# COVID-19 – Immune defense and antiviral strategies for prevention and treatment

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# 1 Summary

The SARS-CoV-2 pandemic is of historic proportions and associated with exceptional morbidity and mortality. To effectively contain and minimize an outbreak of a novel virus, it is important to study intervention strategies from different angles. Within the scope of the present work, we conducted studies investigating COVID-19 on the level of prevention, therapy, and immunity.

SARS-CoV-2 can be transmitted through aerosols containing viral particles and through virus-contaminated surfaces. We tested two commercially available UVC-LED disinfection boxes for their ability to inactivate high viral loads of SARS-CoV-2 on materials representative of personal item surfaces. The UVC-LED boxes effectively inactivated SARS-CoV-2 on glass, metal, and plastic surfaces after 3 minutes of irradiation. Our results showed that UVC-LED boxes can be an affordable and environmentally friendly option for disinfecting personal items.

Next, we tested the antiviral activity of curcumin from turmeric root and glycyrrhizin from licorice root against SARS-CoV-2. We showed that curcumin and glycyrrhizin effectively neutralized SARS-CoV-2 with a half-maximal effective concentration ($EC_{50}$) of 7.9 µg/mL (21.5 µM) and an $EC_{50}$ of 440 µg/mL (534.7 µM), respectively. Both natural products effectively reduced SARS-CoV-2 RNA levels in cell culture. Furthermore, we discovered glycyrrhizin as an inhibitor of the SARS-CoV-2 main protease ($M^{pro}$). We identified curcumin and glycyrrhizin as promising compounds for complementary treatment of COVID-19 that require further investigation in large-scale randomized controlled trials.

The reduced neutralization activity of sera from vaccinated individuals against newly emerging SARS-CoV-2 variants of concern (VOCs) led to an increase in vaccine breakthrough infections. We showed strongly reduced neutralizing activity of sera from vaccinated people and patients with SARS-CoV-2 breakthrough infection against the Omicron sub-variants BA.1 and BA.5. Furthermore, vaccine breakthrough infections with Delta and BA.1, but not BA.5, boosted immunity against SARS-CoV-2 in a variant specific manner. In addition, we showed that kidney transplant (KTX) patients might be partly protected against SARS-CoV-2 after booster vaccination by IL-2 producing T cells or neutralizing antibodies. After treatment with convalescent plasma, we found an increase of antibodies and IFN- γ secreting T cells against SARS-CoV-2 in KTX and hemodialysis patients. In summary, we showed that vaccine breakthrough infections can enhance vaccination-acquired immunity against SARS-CoV-2 in immunocompetent individuals. Immunocompromised patients might benefit from booster vaccination and treatment with convalescent plasma.

# 2 Zusammenfassung

Die durch SARS-CoV-2 ausgelöste Pandemie führte zu einer hohen Morbidität und Mortalität von historischem Ausmaß. Um den Ausbruch eines neuartigen Virus wirksam einzudämmen ist es wichtig, geeignete präventive und therapeutische Maßnahmen zu treffen. Im Rahmen der vorliegenden Arbeit untersuchten wir verschiedene Ansätze zur Prävention und Therapie von COVID-19 sowie die Immunität gegen SARS-CoV-2.

SARS-CoV-2 kann über virushaltige Aerosole und Virus-kontaminierte Gegenstände übertragen werden. Wir haben zwei handelsübliche UVC-LED-Desinfektionsboxen auf ihre Fähigkeit getestet, hohe SARS-CoV-2 Viruslasten auf Oberflächen von persönlichen Gegenständen zu inaktivieren. Die mit hohen Viruslasten von SARS-CoV-2 kontaminierten Glas-, Metall- und Kunststoffoberflächen konnten bereits nach 3 Minuten UVC Bestrahlung im Inneren der UVC-Boxen wirksam dekontaminiert werden. Unsere Ergebnisse ergaben, dass UVC-LED-Boxen eine kostengünstige und umweltfreundliche Option für die Desinfektion von persönlichen Gegenständen sein können.

Anschließend untersuchten wir die antivirale Aktivität von Curcumin aus der Kurkumawurzel und Glycyrrhizin aus der Süßholzwurzel gegen SARS-CoV-2. Wir konnten zeigen, dass Curcumin und Glycyrrhizin SARS-CoV-2 mit einer halbmaximalen wirksamen Konzentration ($EC_{50}$) von 7,9 µg/mL (21,5 µM) bzw. 440 µg/mL (534,7 µM) wirksam neutralisieren. Beide Naturstoffe reduzierten effektiv die Konzentration von SARS-CoV-2-RNA in Zellkultur. Darüber hinaus haben wir herausgefunden, dass Glycyrrhizin die Hauptprotease von SARS-CoV-2 ($M^{pro}$) hemmt. Die Arbeiten zeigen, dass Curcumin und Glycyrrhizin vielversprechende Wirkstoffe für die ergänzende Behandlung von COVID-19 sein können. Die Ergebnisse stellen die Grundlage für die Weiterentwicklung dieser Substanzen in klinischen Studien dar.

Die verminderte Neutralisierungsaktivität von Seren geimpfter Personen gegenüber neu auftretenden bedenklichen SARS-CoV-2-Varianten führte zu einer Zunahme von Durchbruchsinfektionen. Wir konnten zeigen, dass Seren geimpfter Personen und Patienten mit SARS-CoV-2 Durchbruchsinfektionen eine stark reduzierte neutralisierende Aktivität gegenüber den Omikron-Varianten BA.1 und BA.5 aufweisen. Durchbruchsinfektionen mit Delta und BA.1, aber nicht BA.5, führten außerdem zu einer variantenspezifischen Verstärkung der Immunität gegen SARS-CoV-2. Darüber hinaus konnten wir zeigen, dass nierentransplantierte (KTX) Patienten nach einer Auffrischungsimpfung durch IL-2-produzierende T-Zellen oder neutralisierende Antikörper teilweise gegen SARS-CoV-2 geschützt sein könnten. Nach der Behandlung mit Rekonvaleszenzplasma fanden wir bei KTX- und Hämodialyse-Patienten einen Anstieg der Antikörper und IFN-γ-sezernierenden T-Zellen gegen SARS-CoV-2. Zusammenfassend ergaben unsere Ergebnisse, dass Durchbruchsinfektionen die durch die Impfung erworbene Immunität gegen SARS-CoV-2 bei immunkompetenten Personen verstärken können. Immungeschwächte Patienten könnten von einer Auffrischungsimpfung und einer Behandlung mit Rekonvaleszenzplasma profitieren.

# 3 Introduction

## 3.1 Coronaviruses

Coronaviruses are enveloped single-stranded ribonucleic acid (RNA) viruses belonging to the family *Coronaviridae*. They can be found in a wide range of birds and mammals, including humans, cats, dogs, chickens, pigs, and cows (Wiersinga et al., 2020). It was in the mid-1960s that scientists first identified a human coronavirus from nasal washings of patients with colds (Tyrrell and Bynoe, 1966). Their large genomic size (26.4-31.7 kb) and their specific crown-like appearance, formed by glycoproteins on the virus surface, are typical features of coronaviruses (Mousavizadeh and Ghasemi, 2021).

The United States federal agency Centers for Disease Control and Prevention (CDC) lists 229E, NL63, OC43 and HKU1 as common human coronaviruses. People infected with these human coronaviruses usually develop mild to moderate upper respiratory illnesses, such as the common cold (Centers for Disease Control and Prevention, 2020). However, the outbreak of severe acute respiratory syndrome-coronavirus (SARS-CoV) in 2003 demonstrated that human coronaviruses hold the potential to cause severe illnesses with a high fatality rate in humans. SARS was the first known pandemic caused by a human coronavirus, resulting in more than 8000 cases in different countries with a case fatality rate of about 10 % (Cheng et al., 2007). It was suggested that SARS-CoV is of zoonotic origin, since the first cases had contact with wild animals. This hypothesis was supported by the discovery of a closely related bat CoV in Chinese horseshoe bats (Zhong et al., 2003, Lau et al., 2005).

Almost a decade later in 2012, the middle east respiratory syndrome-coronavirus (MERS-CoV) was first identified in Saudi Arabia (Zaki et al., 2012). Since then, over 2600 cases with more than 900 associated deaths have been reported in 27 countries as of February 2023, with most cases occurring in the Eastern Mediterranean Region (World Health Organization, 2023a). MERS-CoV is a zoonotic pathogen and accumulating virological studies suggested that dromedary camels are the reservoir (Haagmans et al., 2014, Adney et al., 2014). The exact origin of the virus remains unknown, but it was suspected that MERS-CoV originated from bats (Cui et al., 2019).

Scientists have warned that “the presence of a large reservoir of SARS-CoV-like viruses in horseshoe bats, together with the culture of eating exotic mammals in southern China, is a time bomb” (Cheng et al., 2007). Cheng et al. (2007) stated that there is a possibility of a reemergence of SARS and that we should be prepared for this eventuality. More than a decade later, in December 2019, an outbreak of novel severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) started in Wuhan, China (Zhou et al., 2020). SARS-CoV-2 rapidly spread over the whole world, resulting in the global coronavirus disease 2019 (COVID-19) pandemic.

As of April 2023, more than 700 million people were infected with SARS-CoV-2 worldwide and almost seven million people have died as a result of COVID-19 (World Health Organization, 2023d).

In contrast to SARS-CoV and MERS-CoV, SARS-CoV-2 is highly transmissible from human to human. Although interpersonal transmission has been reported for SARS-CoV and MERS-CoV, it is much less pronounced compared to SARS-CoV-2 and occurred mainly in hospital settings (Zhou et al., 2021a). The origin of SARS-CoV-2 is still a matter of debate. There are two main hypotheses on the origin of SARS-CoV-2: A zoonotic transmission via bats or an as yet unknown intermediate host or a laboratory leakage in Wuhan (Domingo, 2022).

In April 2023, the last remaining measures, such as wearing an filtering facepiece 2 (FFP2) mask when visiting a hospital or nursing home, were abolished in Germany (Die Bundesregierung, 2023). Due to the decrease of COVID-19 related deaths and hospital admissions, the WHO declared in May 2023 that COVID-19 is an established and persistent health problem and no longer a public health emergency of international concern (PHEIC) (World Health Organization, 2023b). However, COVID-19 continues to be a global threat, in part because of the emergence of highly transmissible VOCs, insufficient vaccine coverage in low- and middle-income countries and the still high number of reported deaths (The Lancet, 2023).

## 3.2 Entry and replication of SARS-CoV-2

SARS-CoV-2, together with SARS-CoV and MERS-CoV, is a betacoronavirus belonging to the subfamily *Orthocoronavirinae* of the family *Coronaviridae* (Lu et al., 2020). The virus shares about 79 % sequence identity to SARS-CoV and about 50 % to MERS-CoV (Lu et al., 2020). The single-stranded, positive-sensed RNA genome of SARS-CoV-2 has a length of around 30 kb nucleotides (Wu et al., 2020, Chan et al., 2020). Similar to MERS-CoV and SARS-CoV, the genome of SARS-CoV-2 consists of the open reading frame (ORF) 1a/b encoding 16 nonstructural proteins that are important for replication (Chan et al., 2020). The genome also encodes accessory proteins and four major structural proteins including spike (S), envelope (E), membrane (M) and nucleocapsid (N) protein (Chan et al., 2020) (Figure 1 A and B). The S protein can be subdivided into two subunits, S1 and S2 (Chan et al., 2020). The S1 subunit contains an N-terminal domain (NTD) and receptor-binding domain (RBD) that binds to the host-cell receptor angiotensin-converting enzyme 2 (ACE2) (Letko et al., 2020, Xia et al., 2020, Chan et al., 2020). Subunit S2 facilitates cell membrane fusion and contains structures including a conserved fusion peptide (FP), heptad repeat (HR) 1 and 2, cytoplasm domain (CP) and transmembrane domain (TM) (Xia et al., 2020, Chan et al., 2020).

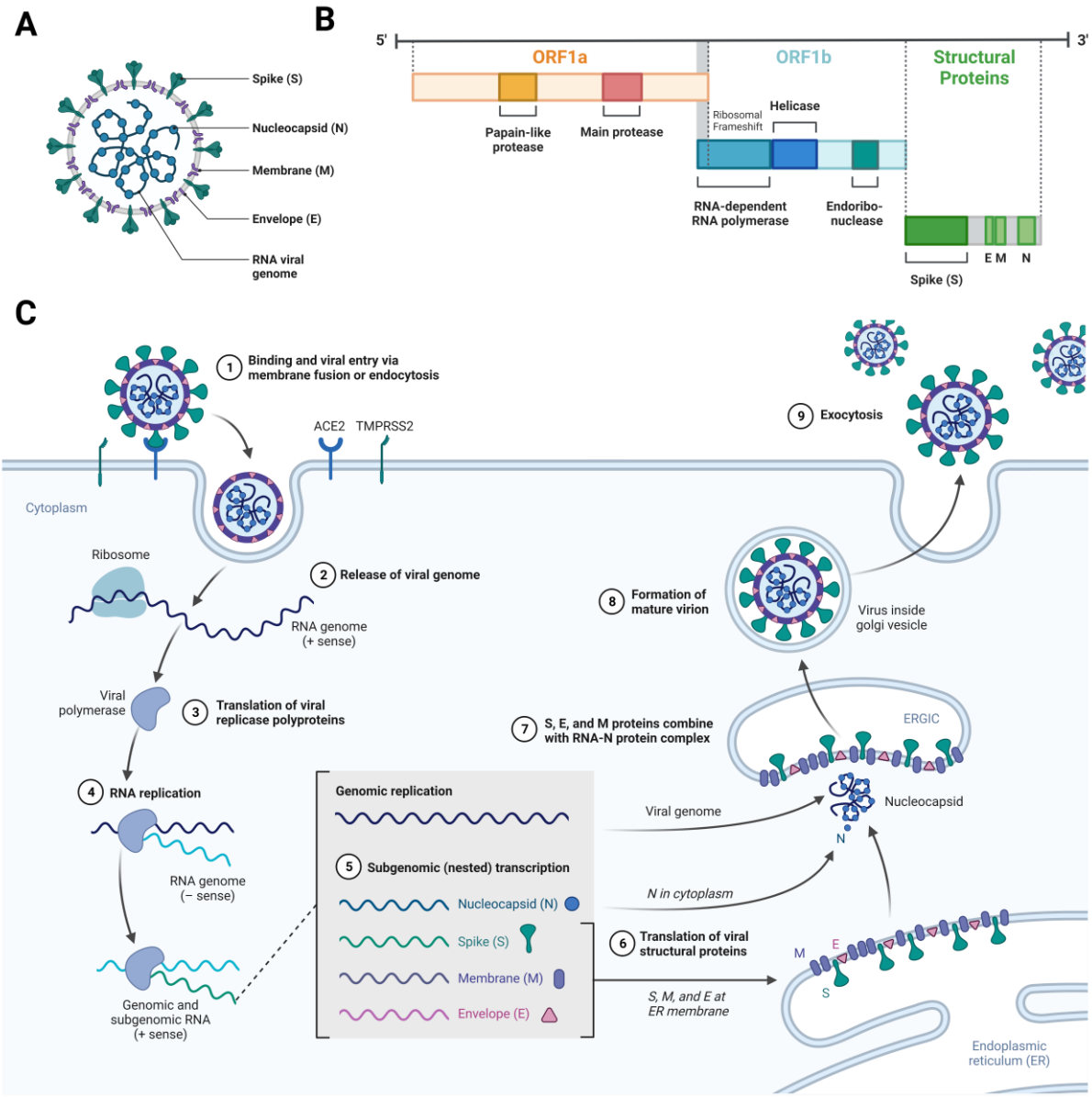


Figure 1. Structure and life cycle of SARS-CoV-2. (A) SARS-CoV-2 has four structural proteins, including the spike (S), envelope (E), membrane (M) and nucleocapsid (N) protein. (B) The SARS-CoV-2 genome consists of the open reading frames 1a (ORF1a) and 1b (ORF1b) encoding nonstructural proteins for replication and a region encoding several accessory and structural proteins. (C) After viral entry via membrane fusion or endocytosis (1), the viral genome is released into the cytoplasm (2) and translated into viral replicase polyproteins (3). During RNA replication (4), the negative-strand RNA is synthesized, which serves as a template for the replication of genomic RNA and subgenomic transcription (5). The subgenomic RNA is translated into the four main structural proteins (6). In the endoplasmic-reticulum-Golgi intermediate compartment (ERGIC) the S, E and M proteins combine with the RNA-N protein complex (7) for assembly mature virions (8). Subsequently, mature virions are released from the cell (9). ACE2, angiotensin-converting enzyme 2; TMPRSS2, transmembrane serine protease 2. (Adapted from “Human Coronavirus Structure” (A), “Genomic Organization of SARS-CoV-2” (B) and “Coronavirus Replication Cycle” (C), by BioRender.com [https://app.biorender.com/biorender-templates] [2023].)

Upon binding of RBD to ACE2, the S protein is activated either at the cell surface by the target-cell protease transmembrane serine protease 2 (TMPRSS2) or in the endosomal compartment following endocytosis by cathepsin L (Hoffmann et al., 2020, Jackson et al., 2022). Activation of the S protein induces cleavage of the S2′ site, exposing the FP (Jackson et al., 2022). S2 then incorporates the FP into the host-cell membrane leading to an interaction between HR1 and HR2 to form a six-helical bundle (6-HB) (Xia et al., 2020). The 6-HB is responsible for bringing the cellular and viral membranes in close contact for fusion (Xia et al., 2020).
Following entry of SARS-CoV-2 into the target-cell, the viral RNA is released into the cytoplasm and translated into viral replicase polyproteins (Harrison et al., 2020, Jiang et al., 2020) (Figure 1 C). The main protease ($M^{pro}$) encoded by the virus cleaves these polyproteins into replicase complex nonstructural proteins (nsps) like RNA-dependent RNA polymerase (RdRp) (Zhao et al., 2022b). Negative-strand RNA is synthesized in virus-induced double-membrane vesicles (DMVs) formed by extrusions of the endoplasmic reticulum (ER) membrane (Harrison et al., 2020, Jiang et al., 2020). This negative-strand RNA serves as a template for subgenomic or genomic positive-strand RNA. The subgenomic RNA is translated into the N protein, which forms helical structures with the genomic RNA in the cytoplasm, and into the S, E and M proteins, which are incorporated into the ER after translation. The S, E and M proteins along with the RNA-N protein complex are then transported to the ER-Golgi intermediate compartment (ERGIC) for assembly of the virion. The newly formed mature virions can then be released from the host cell plasma membrane (Harrison et al., 2020, Jiang et al., 2020).

## 3.3 Transmission of SARS-CoV-2

Unlike SARS-CoV, SARS-CoV-2 can be transmitted by presymptomatic individuals (i.e., those who are infectious before the symptoms appear) and asymptomatic individuals (i.e., those who never develop symptoms). Transmission before the onset of symptoms significantly contributed to the rapid global spread of SARS-CoV-2. It is even assumed that transmission by presymtomatic and asymptomatic individuals contributed to more than half of all transmissions globally (Johansson et al., 2021).
The main mode of transmission of SARS-CoV-2 was considered to be through aerosols (Marr and Tang, 2021, Duval et al., 2022). Presymptomatic, asymptomatic or symptomatic individuals can release respiratory particles that can be directly inhaled by people in close proximity or at a greater distance (Figure 2). Aerosols are densely concentrated in close proximity, and smaller aerosols can travel long distances and survive in the air for hours (Marr and Tang, 2021). Although some studies have demonstrated difficulties in recovering viable SARS-CoV-2 from the air, evidence strongly supports the hypothesis that airborne transmission is the primary mode of transmission of SARS-CoV-2 (Greenhalgh et al., 2021).

Research data indicate an important role for long distance airborne transmission in indoor settings, such as at choirs, work and restaurants, and that inadequate ventilation contributes to transmission (Duval et al., 2022). Superspreading events are even considered as substantial drivers of the COVID-19 pandemic (Greenhalgh et al., 2021). Infectious individuals can also release large respiratory droplets that fall rapidly and usually do not travel more than 1-2 meters (Marr and Tang, 2021). Fomite-mediated transmission via objects contaminated by respiratory droplets or body secretions of infected individuals has also been reported for SARS-CoV-2 (Bak et al., 2021). However, the risk can be regarded as relatively low compared to airborne transmission (Greenhalgh et al., 2021).

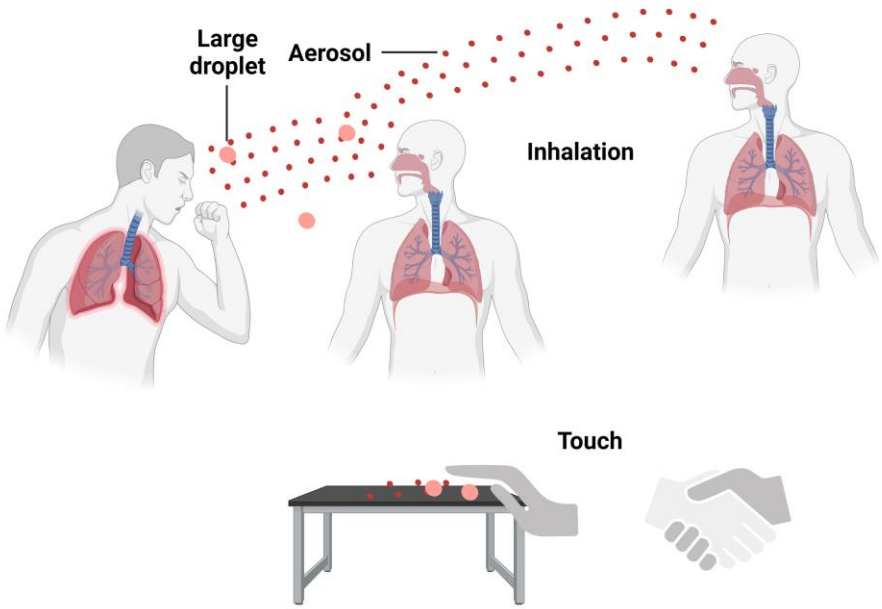


Figure 2. Transmission of SARS-CoV-2. SARS-CoV-2 can be transmitted at close range and at a greater distance by inhalation of virus-containing aerosols. The virus can also be transmitted by touching contaminated skin or surfaces. Created with BioRender.com.

## 3.4 Epidemiology of SARS-CoV-2

Similar to other RNA viruses, SARS-CoV-2 tends to continuously evolve while adapting to its human hosts. During this process, mutations in the genetic code can lead to the emergence of virus variants that may differ from their ancestors. In case of SARS-CoV-2, a rapid increase of variants with various spike protein mutations was observed. Variants that are associated with escape from vaccine-mediated immunity, enhanced disease severity and transmissibility are classified as variants of concern (VOCs) (European Centre for Disease Prevention and Control, 2023). The following SARS-CoV-2 variants were classified as VOCs during the pandemic: Alpha (B.1.1.7) and Beta (B.1.351) in September 2020, Gamma (P.1) and Delta (B.1.617.2) in December 2020 and Omicron (B.1.1.529) in November 2021 (European Centre

for Disease Prevention and Control, 2023) (Figure 3). Meanwhile, Omicron can be divided into several sub-variants, including BA.1, BA.2, BA.4, BA.5, BQ.1 and XBB (Hodcroft, 2021).
The SARS-CoV-2 variants are responsible for several waves of COVID-19 infections. During these infection waves, an increased transmission of the virus took place. In the early phase of the pandemic, a mean basic reproduction number ($R_0$) of 3.28 for SARS-CoV-2 was calculated (Liu et al., 2020b). The $R_0$ indicates the transmissibility of a virus by providing the mean number of new infections caused by an infected individual in a completely naïve population. With the emergence of new variants, the $R_0$ of SARS-CoV-2 increased. Liu and Rocklöv (2021) found a higher transmissibility for Delta compared to the ancestral SARS-CoV-2 strain with a mean $R_0$ of 5.08. For Omicron, the $R_0$ was even estimated at 9.5, which was in line with the rapid spread of the variant around the world (Liu and Rocklöv, 2022).

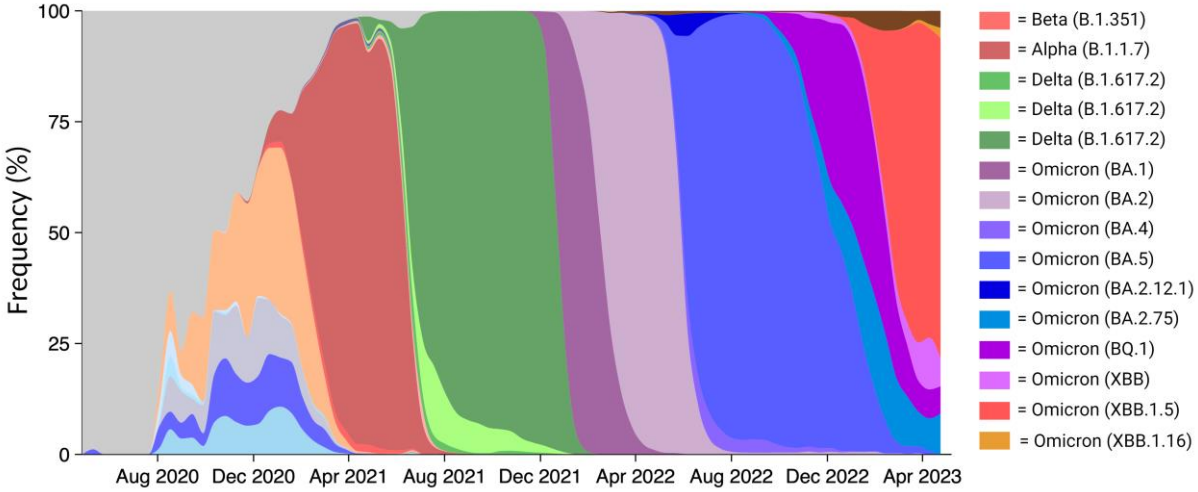


Figure 3. Distribution of variants of SARS-CoV-2 in Germany since the start of the pandemic. Adapted from Emma B. Hodcroft, 2021. "CoVariants: SARS-CoV-2 Mutations and Variants of Interest." (https://covariants.org/).

## 3.5 Pathogenesis of SARS-CoV-2 infection

COVID-19 is characterized by a wide spectrum of clinical manifestations, ranging from asymptomatic infection or mild respiratory illness to acute respiratory distress syndrome (ARDS) and even multiple organ dysfunction syndrome (MODS) (Wang et al., 2020). In the first wave of the pandemic in 2020, fever, cough, fatigue and rhinitis were described as common symptoms, with impaired sense of smell and/or taste as a characteristic feature of COVID-19 (Guan et al., 2020, Schilling et al., 2020, Wang et al., 2020).
COVID-19 most commonly affects the respiratory system, with symptoms including shortness of breath and dry cough (Hernandez Acosta et al., 2022). The surface of lung alveolar epithelial cells shows a high expression ACE2, which is used by SARS-CoV-2 for cellular entry. Besides of epithelial cells in the lung, ACE2 is also expressed in human tissues such as the heart, kidney, arterial and venous endothelial cells, and absorptive enterocytes in the intestine

(Hamming et al., 2004, Ziegler et al., 2020). The wide expression of ACE2 in the human body seems to contribute to extrapulmonary manifestations of COVID-19, leading to acute kidney injury, cardiac injury, gastrointestinal symptoms, neurological symptoms and vascular damage (Gupta et al., 2020).

About one-third of SARS-CoV-2 infections remain asymptomatic, with a higher rate of asymptomatic infections in children (Sah et al., 2021). Among the people that developed a symptomatic infection during the first months of the pandemic, about 81 % percent developed mild COVID-19, 14 % percent moderate and about 5 % severe disease (Wu and McGoogan, 2020). According to Wu and McGoogan (2020), the overall case-fatality rate was 2.3 %. The World Health Organization (WHO) classified COVID-19 into three different levels depending on the severity of the disease: Non-severe/mild COVID-19, severe COVID-19 and critical COVID-19 (World Health Organization, 2023c). Patients with severe COVID-19 either have an oxygen saturation < 90 % on room air, signs of pneumonia or signs of severe respiratory distress. In critical COVID-19, patients suffer from conditions such as ARDS or sepsis that normally demand life-sustaining interventions (World Health Organization, 2023c).

The clinical course of disease can be divided into three phases: Initial phase accompanied with viral replication and high viral loads in the respiratory tract, a second phase characterized by pulmonary vascular disease, and a third hyperinflammatory phase (Siddiqi and Mehra, 2020) (Figure 4). During the first days of infection, most people develop mild or non-specific symptoms (phase I of infection) (Siddiqi and Mehra, 2020). The first phase is characterized by a high viral load in the nasopharynx of patients. For most people, symptoms subside within an average of seven days. Patients that do not recover from the initial cold-like disease may develop pneumonia, and the virus propagates in the lungs (phase II of infection). As a result of the activation of the innate and adaptive immune system, patients may have slightly increased markers of systemic inflammation. Hospitalization is necessary for most of the patients at this stage. From this stage on, the disease may further progress in some patients leading to the development of an extrapulmonary systemic hyperinflammatory syndrome that is associated with a poor prospects of recovery (phase III of infection) (Siddiqi and Mehra, 2020). The hyperinflammation syndrome results from a storm of proinflammatory cytokines, including interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-10 (IL-10), interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α) (Liu et al., 2020a, Silva et al., 2023).

Patients that have survived the acute phase of COVID-19 may develop long-term sequelae of the disease. This post COVID-19 condition is characterized by symptoms or abnormal clinical parameters that last for at least two months and usually occur three months after the onset of COVID-19 (World Health Organization, 2021). The most commonly reported post COVID symptoms are chronic fatigue, hair loss, shortness of breath, attention disorder and headache (Lopez-Leon et al., 2021). Severe disease progression has been reported as a risk factor for

the development of post COVID-19, as well as other factors such as obesity, female sex, smoking, immunosuppression and a wide range of comorbidities (Tsampasian et al., 2023, Subramanian et al., 2022).

During the course of the pandemic, it became clear that the incubation period and symptom profile of COVID-19 changed with the different SARS-CoV-2 variants. A recent meta-analysis identified a decreasing mean incubation period for SARS-CoV-2 variants, with 5 days for Alpha, 4.5 days for Beta, 4.41 days for Delta and 3.42 days for Omicron (Wu et al., 2022). Whitaker et al. (2022) indicated that the loss of sense of smell or taste was less pronounced in patients infected with Omicron than in those infected with former variants. However, more cold- and flu-like symptoms, such as runny nose and sore throat, were reported for Omicron (Whitaker et al., 2022). Recent studies reported a reduced number of hospitalizations and case fatality rate for Omicron sub-lineages BA.1 and BA.2 compared to Delta (Sievers et al., 2022, Wolter et al., 2022a). However, another study reported an increased symptom severity and symptom burden for BA.2 compared to BA.1 (Whitaker et al., 2022). Of note, patients infected with Omicron BA.1, BA.4 or BA.5 showed a similar risk for hospitalization and severe disease outcome (Wolter et al., 2022b).

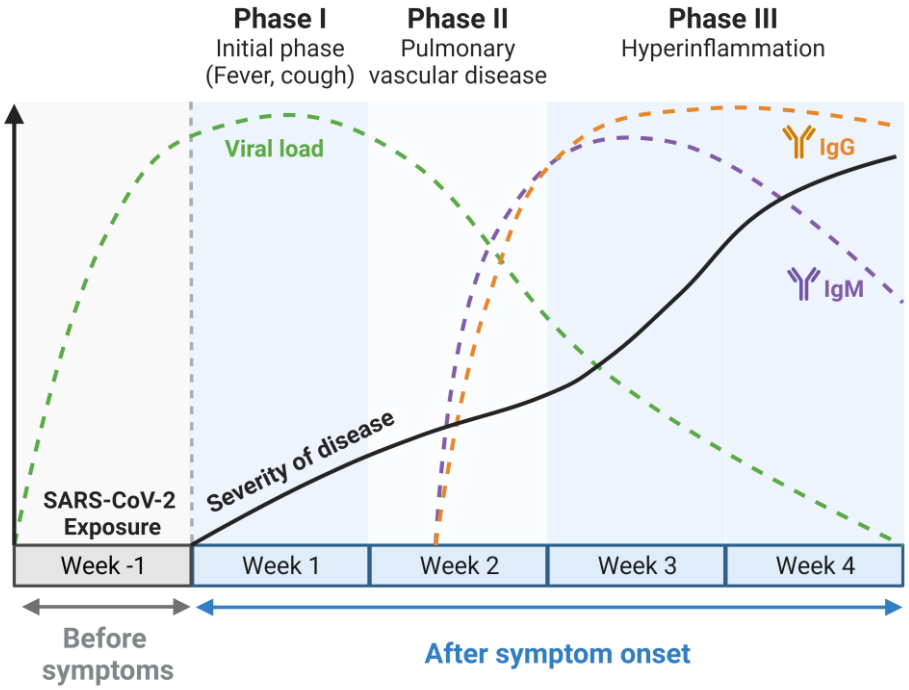


Figure 4. Phases of COVID-19 depending on severity of the disease. Phase I is characterized by mild and non-specific symptoms and a high viral load. In phase II, patients who did not recover from infection may develop pulmonary vascular disease. Phase III is characterized by a hyperinflammatory response that can lead to acute respiratory distress syndrome (ARDS) and organ failure. SARS-CoV-2 specific IgG and IgM antibodies are detectable at week 2 after the onset of symptoms. Adapted from Bormann et al. (2021a) and Siddiqi and Mehra (2020) with BioRender.com.

## 3.6 Risk groups for severe COVID-19

Healthy individuals of all ages can develop severe COVID-19. However, the risk for severe disease progression increases with age and is enhanced in people with comorbidities. The most commonly reported risk factor for severe COVID-19 is older age (especially over 60 years) (Karla Romero et al., 2021, Wolff et al., 2021). Other risk factors are male sex, obesity, hypertension, diabetes, cardiovascular disease, chronic respiratory tract disease and chronic kidney disease (Wolff et al., 2021, Booth et al., 2021, Zhang et al., 2023). Given that COVID-19 can affect organs such as liver, kidney and the heart, pre-existing organ-based comorbidities could further promote organ damage from COVID-19 (Wolff et al., 2021).
Immunocompromised individuals, such as solid organ recipients and cancer patients, have also been associated with an increased risk for severe COVID-19 and a higher mortality rate (Zhang et al., 2023, Gao et al., 2020, Caillard et al., 2021). Caillard et al. (2021) found a significantly higher COVID-19-related mortality in kidney transplant (KTX) patients compared to nontransplant patients but showed no significant difference in severe disease progression between the groups. As these vulnerable groups are at higher risk of mortality and severe disease progression, special attention was paid to their protection during the COVID-19 pandemic.

## 3.7 Immune defense against SARS-CoV-2 infection

Upon infection with SARS-CoV-2, the immune system is activated to eliminate the virus from the body. The innate immune system is the body's first line of defense against invading pathogens such as SARS-CoV-2. It is present since birth and characterized by a fast and non-specific response against pathogens. During this first line defense, pattern recognition receptors (PRRs) on innate immune cells such as dendritic cells (DCs), monocytes, macrophages, neutrophils and natural killer (NK) cells recognize pathogen-associated molecular patterns (PAMPs) as well as damage-associated molecular patterns (DAMPs), which initiates an inflammatory response (Kanneganti, 2020) (Figure 5). Multiple PRRs have been shown to initiate an immune response when recognizing SARS-CoV-2, particularly nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), Toll-like receptors (TLRs) and inflammasomes (Diamond and Kanneganti, 2022). The PRR signaling leads to the secretion of interferons (IFNs) and other pro-inflammatory cytokines that help to stop the viral replication and spread, and to initiate the adaptive immune response by activating antigen-presenting cells (Diamond and Kanneganti, 2022).

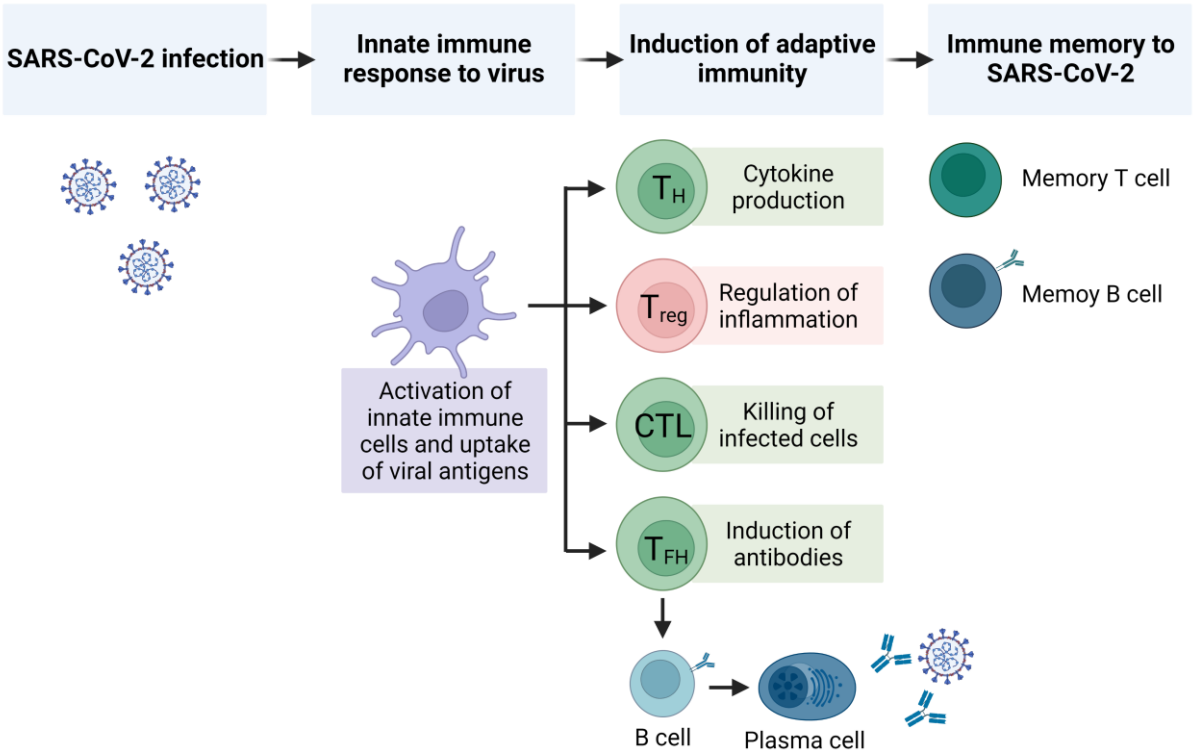


Figure 5. Immune defense against SARS-CoV-2. SARS-CoV-2 infection leads to the activation of innate immune cells, including dendritic cells. These cells help to initiate the adaptive immune response by activating virus-specific T cells and B cells. Memory T and B cells can be detected in the blood for more than a year after convalescence. CTL, cytotoxic T cell; $T_{FH}$, T follicular helper cell; $T_H$, T helper cell; $T_{reg}$, regulatory T cell. Adapted from Cox and Brokstad (2020) with BioRender.com.

Convalescent COVID-19 patients have been shown to exhibit SARS-CoV-2-specific humoral and cellular immunity, which are part of the adaptive immune response (Ni et al., 2020). The adaptive immune response is slower than the innate immune response. Sufficient numbers of immune cells are produced between 6 to 10 days after priming (Sette and Crotty, 2021) (Figure 5). The three main cell types of the adaptive immune system are B cells that produce antibodies, $CD4^+$ T cells that have effector and helper functions and $CD8^+$ T cells that kill virus-infected cells (Sette and Crotty, 2021). A coordinated antigen-specific adaptive immune response between T and B cells has been associated with lower COVID-19 severity (Rydyznski Moderbacher et al., 2020). Studies suggest that $CD4^+$ T cells play the most prominent role in preventing severe disease compared to B cells and $CD8^+$ T cells (Rydyznski Moderbacher et al., 2020, Silva et al., 2022).

B cells produce virus-specific antibodies, including neutralizing antibodies (nAbs), in response to SARS-CoV-2 infection. SARS-CoV-2 specific IgM and IgG antibodies can be detected in patient's serum between 7 to 14 days after the appearance of symptoms (Vabret et al., 2020). A special feature of SARS-CoV-2 infection is the almost simultaneous detection of IgM, IgG and IgA at seroconversion, although IgM is commonly the first antibody secreted by the

adaptive immune system (Carrillo et al., 2021) (Figure 4). Frequently detected antibodies in SARS-CoV-2 infected individuals are targeting the viral N and the S proteins (Vabret et al., 2020). Antibodies against the RBD of the S protein can have a potent neutralizing effect and block the binding of the virus to ACE2 receptor (Vabret et al., 2020). Legros et al. (2021) identified a correlation between nAb titers and disease severity showing that patients with severe COVID-19 expressed high nAb titers and patients with a mild course expressed no or low nAb titers. The neutralizing antibody titer in vaccinated or convalescent individuals is a good indicator for immune protection against symptomatic SARS-CoV-2 infection (Khoury et al., 2021). After symptom onset, IgG levels and neutralizing titers gradually wane over time but stabilize after 6 months (Marcotte et al., 2022). Memory B and T cells can be detected even after more than a year in convalescent individuals (Marcotte et al., 2022) (Figure 5).

## 3.8 Prevention and control strategies for COVID-19

In order to contain the COVID-19 pandemic, a number of public health measures were implemented worldwide. The combined use of interventions such as travel restrictions, lockdowns, crowd restrictions, isolation and quarantine of COVID-19 positive individuals and contacts, social distancing and mandatory mask wearing were effective in reducing the transmission of SARS-CoV-2 (Ayouni et al., 2021). In addition to these public health measures, different non-pharmaceutical and pharmaceutical interventions were applied, such as disinfection of surfaces with disinfectants or ultraviolet-C (UVC) irradiation, vaccination campaigns and antiviral therapy.

### 3.8.1 SARS-CoV-2 UVC disinfection

SARS-CoV-2 can remain stable on materials such as metal, glass, and plastic for up to several days (Xu et al., 2023). To reduce fomite transmission of SARS-CoV-2, soaps and alcohols were used to inactivate SARS-CoV-2 on hands, and disinfectants and UVC irradiation were applied to inactivate SARS-CoV-2 on environmental surfaces (Viana Martins et al., 2022). UVC light has various advantages over chemical disinfectants, including being more environmentally friendly as it does not leave toxic residues (Demeersseman et al., 2023). There are different types of UV light categorized according to their wavelengths, including UVA (315-400 nm), UVB (280-315 nm) and UVC (200-280 nm) light (Figure 6). UVC light is the most energetic UV light due to its short wavelength. It is absorbed by ozone in the atmosphere. Using UVC light to disinfect surfaces has been proven to be effective against a variety of bacteria, fungi, and viruses, including SARS-CoV and SARS-CoV-2 (Duan et al., 2003, Heilingloh et al., 2020, Menetrez et al., 2010). High viral titers of SARS-CoV-2 were completely

inactivated with a UVC dosage of about 1 J/cm$^2$ at a wavelength between 250 and 280 nm (Heilingloh et al., 2020). The UVC dosage was calculated by multiplying intensity (mW/cm$^2$) with the exposure time (s) (Demeersseman et al., 2023). Different commercially available UVC-emitting devices equipped with light emitting diodes (LEDs) can be used to inactivate SARS-CoV-2 on personal items with surfaces like glass, metal, and plastic (Trivellin et al., 2021). In the present work, we investigated the effectivity of UVC-LED boxes designed for private use.

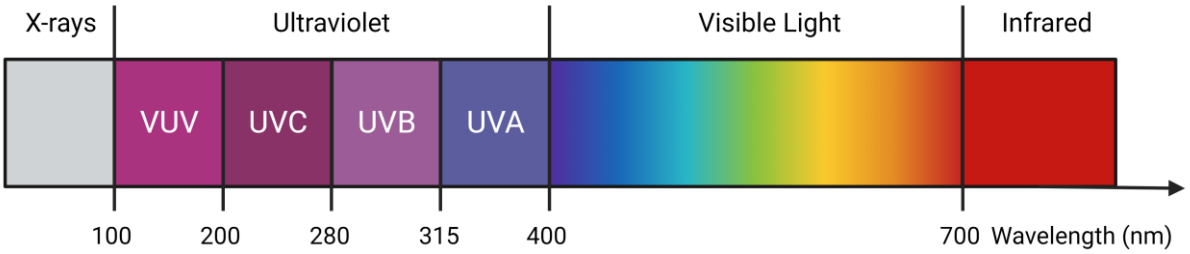


Figure 6. The electromagnetic radiation spectrum. Ultraviolet light is divided into UVA light (315-400 nm), UVB light (280-315 nm), UVC light (200-280 nm) and vacuum ultraviolet (VUV) light (100-200 nm). Adapted from Palma et al. (2022) with the help of BioRender.com.

### 3.8.2 COVID-19 vaccines

The rapid spread and the devastating impact of SARS-CoV-2 in terms of morbidity and mortality alerted governments, companies, and scientists to rapidly develop vaccines against the virus. Besides public health interventions, vaccination campaigns are critical to contain the pandemic by preventing SARS-CoV-2 infection. As a result of an unparalleled effort of scientists worldwide, several effective vaccines targeting the SARS-CoV-2 S protein were developed in less than a year. As of April 2023, more than 13 billion vaccine doses have been administered globally (World Health Organization, 2023d).

The messenger RNA (mRNA) vaccine Comirnaty® from BioNTech/Pfizer was the first vaccine approved by the European Commission (December 2020), followed by Spikevax® from Moderna (January 2021). The mRNA vaccine technology is a promising novel platform for vaccine development. COVID-19 mRNA vaccines contain an mRNA encoding for the SARS-CoV-2 S protein. To efficiently deliver the mRNA into cells, lipid nanoparticles are used as carriers. In Germany, the Standing Committee on Vaccination (STIKO) recommends primary vaccination with Comirnaty® against the original SARS-CoV-2 strain from 6 months of age (Robert Koch Institut, 2023a). Although Spikevax® against the original strain is approved for primary vaccination from 6 months of age, the STIKO advises against vaccination between the ages of 12 and 29 years. This recommendation is based on the occurrence of adverse events such as pericarditis and myocarditis from Spikevax® in this age group (Robert Koch Institut, 2023a).

Shortly after the approval of the COVID-19 mRNA vaccines, the adenoviral vector vaccines Vaxzevria® from AstraZeneca (January 2021) and JCOVDEN® from Janssen (March 2021) were licensed in the European Union. These adenoviral vector vaccines consist of a genetically modified adenovirus that expresses the S protein. As of today, the STIKO only recommends primary vaccination with Vaxzevria® and JCOVDEN® for individuals aged 60 years and older due to rare cases of thrombosis in combination with thrombocytopenia (Robert Koch Institut, 2023a). In this age group, according to the STIKO, the benefit-risk assessment would be in favor of vaccination with these vaccines due to the increased COVID-19 lethality. Since December 2021, Vaxzevria® is no longer available in Germany.

Next to mRNA and adenoviral vector vaccines, immunization against SARS-CoV-2 can also be achieved with protein vaccines. Currently, the protein vaccines Nuvaxovid® from Novavax, VidPrevtyn® Beta from Sanofi Pasteur and recently Bimervax® from HIPRA Human Health S.L.U. are authorized in the European Union (European Medicines Agency, 2023a). Protein vaccines against SARS-CoV-2 contain an adjuvant and a laboratory-produced protein consisting of the whole or parts of the S protein. Nuvaxovid® contains the complete S protein of the original strain, Vidprevtyn® Beta the S protein of the Beta variant (B.1.351) and Bimervax® an RBD fusion heterodimer based on the Alpha (B.1.1.7) and Beta (B.1.351) strains. At present, the STIKO advises against the use of Vidprevtyn® Beta in the 25$^{th}$ update of the COVID-19 vaccination recommendation due to limited data (Robert Koch Institut, 2023a).

The COVID-19 vaccine Valneva is the only inactivated virus vaccine authorized in the European Union (European Medicines Agency, 2023a). Only persons between 18 and 50 years are allowed to receive the vaccine. The vaccine contains adjuvanted, inactivated SARS-CoV-2 virus from the original strain.

A mathematical modelling study assessed the global impact of vaccination during the first year of the pandemic, from December 2020 to December 2021 (Watson et al., 2022). The authors estimated that vaccination prevented a total of 14.4 million deaths from COVID-19 in 185 countries and territories during this period. Efficacy against severe disease remained high after full primary vaccination, even though it declined slightly within 6 months (Feikin et al., 2022). Furthermore, data indicate an increased risk of developing post COVID-19 for unvaccinated individuals compared to vaccinated individuals (Tsampasian et al., 2023).

### 3.8.3 Booster vaccination as a strategy against SARS-CoV-2 variants

Since the primary vaccination of the population was carried out with vaccines based on the original SARS-CoV-2 strain, the high mutation rate of the virus became a threat to the immune protection provided by these vaccines. The Omicron variant harbors a high number of immune-

escape mutations and is associated with an increased transmissibility. Sera of individuals with primary vaccination showed an extensive reduction of neutralizing activity against the Omicron variant, resulting in a marked increase of vaccine breakthrough infections (Tuekprakhon et al., 2022, Edara et al., 2022, Cele et al., 2022, Christensen et al., 2022). To address the substantial immune evasion of emerging VOCs, booster vaccinations were administered to the population. Studies indicated a notably increased protection against severe COVID-19 infections with Omicron and Delta after booster vaccination based on the original strain (Tartof et al., 2022a, Tartof et al., 2022b). However, vaccine-boosted neutralizing antibody titers against Omicron as well as the protective effect of the booster vaccination against severe Omicron infection rapidly decreased over time (Tartof et al., 2022a, Lyke et al., 2022, Collie et al., 2022, Lin et al., 2022).

With the aim of enhancing protection against the Omicron variant, the European Commission authorized mRNA vaccines adapted to this variant from BioNTech/Pfizer and Moderna. These bivalent mRNA vaccines are coding for both the S protein of the ancestral strain as well as the S protein of Omicron BA.1 or BA.4/5 (the S protein of BA.4 and BA.5 is identical). Studies indicated an increased neutralization response against BA.1 and the ancestral strain for both BA.1 bivalent mRNA vaccines compared to the original mRNA vaccines when administered as second booster dose (Chalkias et al., 2022, Barda et al., 2023). In accordance, both BA.4/5 bivalent mRNA vaccines elicited higher neutralization titers against the ancestral strain and BA.4/5 compared to the original vaccine when given as a second booster dose (Zou et al., 2023, Spyros et al., 2022). However, other studies showed that the second booster with the two BA.4/5 bivalent vaccines provided no, or only modestly enhanced neutralizing antibody titers compared to the original vaccines (Wang et al., 2023, Collier et al., 2023). Although neutralizing antibody titers are a good indicator of protection against COVID-19, studies are needed to evaluate the utility of bivalent mRNA vaccines for the prevention of severe COVID-19. Initial studies indicated that the BA.4/5 bivalent boosters were more effective than the monovalent boosters in preventing severe Omicron infections (Lin et al., 2023).

## 3.9 Treatment options for COVID-19

When the COVID-19 pandemic emerged, a rapid development of novel antivirals was a major priority. However, it can take several years before newly developed drugs are approved. In order to accelerate this process, drugs such as remdesivir and dexamethasone, already approved for the treatment of other diseases, were investigated for their effectiveness in the treatment of COVID-19. The phase of the disease is an important factor in selecting the appropriate medication. Antiviral therapy is preferred in an early phase of COVID-19 and anti-inflammatory medication in a later phase (Robert Koch Institut, 2023b) (Figure 7).

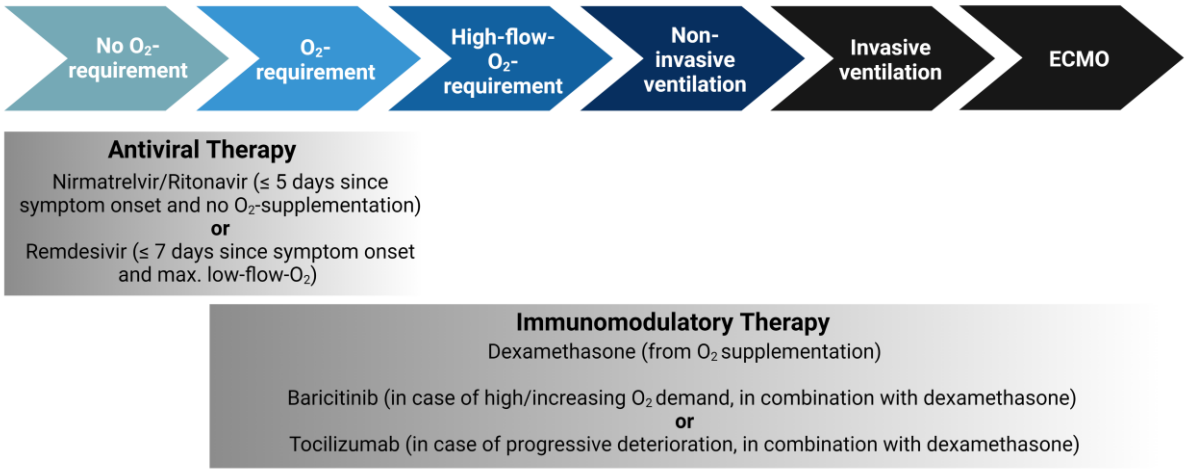


Figure 7. Recommended treatment options for COVID-19 depending on disease severity. ECMO, extra corporeal membrane oxygenation. Adapted from Robert Koch Institut (2023b) with the help of BioRender.com.

### 3.9.1 Antiviral therapy

In the early phase of infection, between 5 and 7 days after symptom onset, antiviral therapy is recommended for patients without COVID-19-related $O_2$ supplementation and at significant risk of a severe course. These are especially immunosuppressed and elderly individuals, regardless of the vaccination status (Robert Koch Institut, 2023b). Different antiviral compounds that inhibit RNA replication are available for antiviral treatment of COVID-19. Treatment of unvaccinated non-hospitalized COVID-19 patients with Paxlovid®, a combination of the antiviral drugs nirmatrelvir and ritonavir, significantly reduced the risk of severe disease progression without apparent safety concerns (Hammond et al., 2022). More recent data confirmed these results for vaccinated non-hospitalized patients during the Omicron BA.1 and BA.2 infection wave (Ganatra et al., 2023). Furthermore, *in vitro* data indicated efficacious neutralization of nirmatrelvir against Omicron sub-variants (Imai et al., 2022). In February 2023, Paxlovid® received full marketing authorization in the European Union for treatment of COVID-19.

In place of Paxlovid®, the antiviral agent remdesivir can also be administered. Remdesivir is a nucleoside analogue that competes with adenosine triphosphate, resulting in inhibition of viral RNA-dependent RNA polymerase. Gilead Sciences' Veklury® (remdesivir) was the first drug gaining conditional marketing authorization in the European Union for the treatment of COVID-19. It was switched to full marketing authorization following confirmational data of its antiviral activity against SARS-CoV-2 variants (Vangeel et al., 2022, Imai et al., 2022, Takashita et al., 2022). A meta-analysis of eight randomized trials found that remdesivir effectively reduced the risk of death in nonventilated patients requiring supplemental oxygen, but not in patients without supplemental oxygen or those requiring mechanical ventilation (Lee et al., 2022).

The use of the nucleoside analogue Lagevrio® (molnupiravir) was recommended by the Robert Koch Institute (RKI) only in patients at high risk for a severe course of COVID-19 when antiviral therapy with Paxlovid® or Veklury® is not possible (Robert Koch Institut, 2023b). However, in February 2023, the European Medicines Agency (EMA) recommended the refusal of marketing authorization for Lagevrio®, which is currently under re-examination following a request by the applicant (European Medicines Agency, 2023b). Randomized controlled trials showed conflicting results for the efficacy of molnupiravir. A meta-analysis summarized trials published until December 2022 that evaluated the efficacy and safety of molnupiravir for COVID-19 patients (Huang et al., 2023). The authors found a significant difference in mortality and hospitalization between molnupiravir and control in non-hospitalized patients but not in hospitalized patients. *In vitro* data showed that molnupiravir is active against SARS-CoV-2 variants including Omicron and its sub-variants (Vangeel et al., 2022, Imai et al., 2022). Molnupiravir is not recommended for use in pregnant women due to evidence of mutagenicity towards mammalian cells (Robert Koch Institut, 2023b, Zhou et al., 2021b).

Next to compounds that inhibit RNA replication, neutralizing monoclonal antibodies (mAbs) can be used for antiviral therapy in the early phase of SARS-CoV-2 infection or for prophylaxis. SARS-CoV-2 mAbs target the RBD of the S protein, which prevents RBD-ACE2 binding and thus cell entry. Several mAbs are authorized in the European Union for the treatment of COVID-19 patients who do not require supplemental oxygen and are at increased risk of severe progression, including a combination of tixagevimab and cilgavimab (Evusheld®), regdanvimab (Regkirona®), a combination of casirivimab and imdevimab (Ronapreve®) and sotrovimab (Xevudy®). However, the emergence of VOCs challenged the neutralizing activity of these mAbs against SARS-CoV-2. MAbs showed strongly impaired neutralizing titers against Omicron sub-variants *in vitro* (Takashita et al., 2022, Imai et al., 2022). Therefore, the RKI advises against monotherapy with mAbs. Early therapy with mAbs can only be justified for specific cases, such as for individuals with a strong immunodeficiency or at high risk for disease progression (Robert Koch Institut, 2023b).

Neutralizing antibodies can also be found in convalescent plasma of patients recovered from COVID-19. Especially in the early phase of a pandemic, when no antiviral treatment was available, convalescent plasma served as a quickly available therapeutic option. However, despite initial promising results, most of the studies demonstrated no beneficial effect of convalescent plasma treatment in hospitalized COVID-19 patients on all-cause mortality, time to death and clinical symptoms (Piechotta et al., 2020). The RKI generally advises against the use of convalescent plasma for the treatment of hospitalized COVID-19 patients (Robert Koch Institut, 2023b). Nevertheless, data of a recent meta-analysis showed a decrease in mortality in immunocompromised COVID-19 patients after transfusion of COVID-19 convalescent plasma (Senefeld et al., 2023). Therefore, the use of convalescent plasma may be considered

for critically ill or immunocompromised patients when an alternative therapeutic option is not available or effective (Robert Koch Institut, 2023b).

### 3.9.2 Immunomodulatory therapy

In contrast to antiviral therapy, immunomodulatory therapy is recommended at a later stage of COVID-19 in patients with supplemental oxygen (Robert Koch Institut, 2023b). Corticosteroids such as dexamethasone have anti-inflammatory and immunosuppressive properties and can be useful to treat hyperinflammation. A recent systematic Cochrane review found that corticosteroids slightly reduce 30 day all-cause mortality in hospitalized COVID-19 patients (Wagner et al., 2022). However, treatment with dexamethasone is not recommended in patients without supplemental oxygen as there is no evidence of a beneficial effect (Horby et al., 2021, Robert Koch Institut, 2023b).

Therapy with dexamethasone can be combined with the Janus kinase inhibitor (JAK-I) baricitinib or the IL-6 inhibitor tocilizumab. A randomized controlled phase 3 trial showed a reduced mortality in hospitalized COVID-19 patients treated with baricitinib added to standard treatment including dexamethasone (Marconi et al., 2021). Tocilizumab in combination with a systemic corticosteroid improved survival in COVID-19 patients who were hypoxic and with systemic inflammation (RECOVERY Collaborative Group, 2021). JAK-I and IL-6 inhibitors should not be administered in parallel (Robert Koch Institut, 2023b).

## 3.10 Natural products

Repurposing drugs already approved for the treatment of other diseases has been an important strategy to find effective candidates for the treatment of COVID-19. However, apart from the antivirals nirmatrelvir/ritonavir and remdesivir and the corticosteroid dexamethasone, there are still limited therapeutic options for the treatment of SARS-CoV-2 infections. In addition, the emergence of highly transmissible VOCs with high immunoevasion potential emphasized the need to investigate effective antiviral agents. Herbal medicines and their ingredients represent a promising source for the identification of antiviral agents for supportive therapy. Especially in low- and middle-income countries, with a shortage of effective therapeutic agents and vaccines, medicinal herbs may be an easily accessible option for treatment of COVID-19.

The bioactive ingredients of medicinal plants exhibit wide range of pharmacological activities, which has encouraged their use in the development of medicines for a variety of diseases (Españo et al., 2021). An increasing number of *in silico*, molecular docking, and *in vitro* studies showed that several natural products such as polyphenols, flavonoids, polyterpenes and

sterols exhibit a high binding affinity for SARS-CoV-2 proteins (Zhao et al., 2022a). Furthermore, some natural products were investigated in clinical trials in COVID-19 patients and showed immunomodulatory and anti-inflammatory effects in addition to their antiviral activity (Zhao et al., 2022a).

Curcumin, the bioactive ingredient of turmeric root, is a natural product that deserves special attention as a potential candidate for the treatment of COVID-19. Turmeric root, also known as *Curcuma longa*, has a long tradition of medical use in Ayurvedic and Chinese medicine for a variety of conditions, such as rheumatoid arthritis, various respiratory conditions, and abdominal pain (Prasad and Aggarwal, 2011). Curcumin was isolated from turmeric root by Vogel and Pelletier in 1815. The compound exhibits anti-inflammatory as well as antiviral properties (Praditya et al., 2019, Abdollahi et al., 2018). Its antiviral potency has been confirmed for a variety of viruses, including Hepatitis C Virus, Zika Virus, Chikungunya Virus and SARS-CoV (Anggakusuma et al., 2014, Mounce et al., 2017, Wen et al., 2007).

Similar to curcumin, glycyrrhizin showed a broad antiviral activity against viruses, including human immunodeficiency virus, herpes simplex virus, and SARS-CoV (Huang et al., 2012, Cinatl et al., 2003, Ito et al., 1987). Glycyrrhizin is the primary active ingredient of the root of licorice, scientifically known as *Glycyrrhiza glabra*. Licorice is a traditional medicinal plant in Chinese medicine where it has been used to treat arthritis as well as lung and gastrointestinal diseases (Pastorino et al., 2018).

*In silico* studies indicated that both curcumin and glycyrrhizin target relevant SARS-CoV-2 proteins such as the S protein and $M^{pro}$ (Srivastava et al., 2022, Jena et al., 2021). In the present work, the role of curcumin and glycyrrhizin as antiviral agents against SARS-CoV-2 were investigated.

## 3.11 Aim and scope of the work

The SARS-CoV-2 pandemic has significantly disrupted the daily lives of people around the world and caused morbidity and mortality on a historic scale. To contain the spread of SARS-CoV-2, a rapid response in form of non-pharmaceutical interventions and the development of vaccines and treatments was essential. In the present work, we aimed to investigate antiviral strategies for the prevention and treatment of SARS-CoV-2 and the immune response against SARS-CoV-2 after vaccination, infection, and under treatment.

SARS-CoV-2 can be transmitted via aerosols or via contaminated surfaces. In order to prevent fomite transmission, surfaces can be disinfected by using UVC irradiation. As personal items could play a role in the transmission of SARS-CoV-2, we aimed to investigate the ability of commercially available UVC-LED boxes in disinfecting surfaces typically found on personal items such as smartphones, coins, or credit cards.

The availability of effective antivirals against SARS-CoV-2 was and is still limited, especially in low- and middle-income countries. Medicinal herbs may be an easily accessible option for the treatment of COVID-19. Testing bioactive constituents of medicinal herbs for their antiviral activity may reveal new effective antiviral compounds for the treatment of COVID-19. In the present work, we aimed to investigate the antiviral activity of the traditional medicinal herbs turmeric root and its bioactive component curcumin as well as licorice root and its component glycyrrhizin against SARS-CoV-2.

Highly transmissible VOCs with the ability to escape from the host's immune response emerged during the pandemic, resulting in an increase of vaccine breakthrough infections. Insights into the immune response following SARS-CoV-2 infection are important for understanding whether infections can successfully induce population-level immunity against SARS-CoV-2. In the present study, we aimed to investigate the humoral and cellular immune response in breakthrough infections during Delta and Omicron waves. Furthermore, we wanted to analyze the humoral and cellular immunity of patients with chronic kidney disease following vaccination and in COVID-19 patients treated with convalescent plasma. As immunocompromised individuals are at high risk of severe COVID-19, special attention needs to be paid to their protection. Insights into the immune responses are essential for assessing the effectiveness of vaccination and treatment strategies for immunocompromised patients.

# 4 Publications

## 4.1 Disinfection of SARS-CoV-2 Contaminated Surfaces of Personal Items with UVC-LED Disinfection Boxes

**Bormann, M.**, M. Alt, L. Schipper, L. van de Sand, M. Otte, T. L. Meister, U. Dittmer, O. Witzke, E. Steinmann, and A. Krawczyk

2021

Contributions:

- Conception: 10 %
- Experimental work: 70 %
- Data analysis: 80 %
- Statistical analysis: 80 %
- Writing the manuscript: 70 %
- Revising the manuscript: 70 %

The presented study was conceptualized by Adalbert Krawczyk, Eike Steinmann, Toni Luise Meister, and Maren Bormann. The experiments were carried out by Maren Bormann, Mira Alt, Leonie Brochhagen (née Schipper), Lukas van de Sand, and Mona Otte. The analysis, evaluation and visualization of the data was carried out by Maren Bormann in collaboration with Adalbert Krawczyk. The manuscript was prepared by Maren Bormann and Adalbert Krawczyk. The manuscript was edited by Maren Bormann, Adalbert Krawczyk, Toni Luise Meister, Eike Steinmann, and Ulf Dittmer. Ulf Dittmer and Oliver Witzke provided resources.

Maren Bormann

Prof. Dr. Adalbert Krawczyk

viruses

MDPI

*Article*

# Disinfection of SARS-CoV-2 Contaminated Surfaces of Personal Items with UVC-LED Disinfection Boxes

**Maren Bormann [1], Mira Alt [1], Leonie Schipper [1], Lukas van de Sand [1], Mona Otte [1], Toni Luise Meister [2], Ulf Dittmer [3], Oliver Witzke [1], Eike Steinmann [2] and Adalbert Krawczyk [1,3,*]**

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**Abstract:** The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is transmitted from person to person by close contact, small aerosol respiratory droplets, and potentially via contact with contaminated surfaces. Herein, we investigated the effectiveness of commercial UVC-LED disinfection boxes in inactivating SARS-CoV-2-contaminated surfaces of personal items. We contaminated glass, metal, and plastic samples representing the surfaces of personal items such as smartphones, coins, or credit cards with SARS-CoV-2 formulated in an organic matrix mimicking human respiratory secretions. For disinfection, the samples were placed at different distances from UVC emitting LEDs inside commercial UVC-LED disinfection boxes and irradiated for different time periods (up to 10 min). High viral loads of SARS-CoV-2 were effectively inactivated on all surfaces after 3 min of irradiation. Even 10 s of UVC-exposure strongly reduced viral loads. Thus, UVC-LED boxes proved to be an effective method for disinfecting SARS-CoV-2-contaminated surfaces that are typically found on personal items.

**Keywords:** SARS-CoV-2; UVC-box; ultraviolet light



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## 1. Introduction

Since the end of 2019, a novel coronavirus called the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been spreading worldwide, thereby causing a major public health issue [1]. Coronavirus disease 2019 (COVID-19) is caused by SARS-CoV-2 and characterized by symptoms ranging from mild respiratory illness to severe life-threatening pneumonia and acute respiratory distress syndrome (ARDS) [2]. Reducing the transmission of the virus by suitable preventive measures is highly important for controlling the pandemic. SARS-CoV-2 is transmitted by direct contact with infected individuals, by virus-containing aerosols and potentially via virus-contaminated surfaces [3]. Recent studies have shown that SARS-CoV-2 can persist on smooth surfaces such as glass, metal, and plastic for up to seven days at room temperature, remaining a potential risk of infection [4].

UV-irradiation is an environmentally friendly method to disinfect surfaces from bacteria, fungi, and viruses such as SARS-CoV-2 [5,6]. Under laboratory conditions, high viral loads of SARS-CoV-2 in cell culture medium could be completely inactivated by UVC-irradiation after 9 min of irradiation with a UVC dose of 1048 mJ/cm$^2$ [7]. For private use, commercial UVC-LED boxes are available for the disinfection of personal items such as smartphones, keys, coins, or credit cards. However, the performance of such devices on the inactivation of SARS-CoV-2 on surfaces has not yet been investigated. Therefore, in the present study, we investigated the ability of two UVC-LED boxes to disinfect surfaces such

as glass, metal, and plastics typically found on personal items from contamination with high viral loads of SARS-CoV-2.

## 2. Materials and Methods

### *2.1. Cells and Viruses*

Vero E6 cells (American Type Culture Collection, ATCC, CRL-1586, Rockville, MD, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (*v*/*v*) fetal calf serum (FCS), penicillin (100 IU/mL), and streptomycin (100 µg/mL) (all Life Technologies Gibco, Darmstadt, Germany). SARS-CoV-2 was isolated from a nasopharyngeal swab of a patient hospitalized due to COVID-19 at the Department of Infectious Diseases of the University Hospital Essen in April 2020 [7]. In brief, the virus was propagated on Vero E6 cells cultured in DMEM supplemented with 10% (*v*/*v*) FCS, penicillin (100 IU/mL), streptomycin (100 µg/mL), and ciprofloxacin (10 µg/mL). After 5 days of incubation, virus suspension was harvested, cleared from cell debris by centrifugation, and stored at −80°. Viral titers were determined by endpoint dilution according to Spearman and Kärber [8] and calculated as $TCID_{50}$ (tissue culture infectious dose, 50%).

### *2.2. Measurement of the Emitted Light Intensity*

Two UVC-LED boxes were investigated for their capability to inactivate SARS-CoV-2 (UVC-LED box 1, Horcol; UVC-LED box 2, expondo GmbH, Berlin, Germany). A radiometrically calibrated spectrometer (STS-UV-L-50-400-SMA, Ocean Optics B.V., Ostfildern, Germany) with a sensitivity range between 190 and 650 nm (1.5 nm resolution) equipped with a CC-3-UV-S corrector (Ocean Optics B.V., Ostfildern, Germany) was used to determine the light intensity emitted by the LEDs inside the UVC-LED boxes. The emitted light intensity was determined between a wavelength of 250 and 280 nm at specific distances from the light source (LED) of the UVC-LED boxes. The corrector was placed at the same locations as the virus carriers (UVC-LED box 1: 1 and 5 cm horizontal distance; UVC-LED box 2: 1 cm vertical distance). For UVC-LED box 2, emitted light could only be measured at a distance of 1 cm. A measurement at 4 cm distance would have required drilling a hole into the bottom of the box, which was not possible without damaging the box. The data were recorded using OceanView 2.0 Software and visualized using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

### *2.3. UVC-LED Decontamination of SARS-CoV-2 Contaminated Surfaces*

SARS-CoV-2 working stocks ($5 \times 10^6$ $TCID_{50}$/mL for UVC-LED box 1; $2 \times 10^6$ $TCID_{50}$/mL for UVC-LED box 2) were diluted in a defined organic matrix mimicking respiratory secretions [9]. In brief, 900 µL of the respective virus stock was added to 100 µL organic matrix consisting of 2.5 mg/mL mucin type I-S, 7.8 mg/mL BSA Fraction V, and 11 mg/mL yeast extract (all Sigma-Aldrich, Darmstadt, Germany). We used glass (cover glass, 1.8 × 1.8 cm, Carl Roth, Karlsruhe, Germany), metal (stainless steel, diameter of about 2 cm), and plastic (polyvinylchloride, diameter of about 2 cm) carriers for viral contamination. Before inoculating the virus suspension to the carriers, the carriers were sterilized for 10 min with a UV-lamp emitting 1940 µW/cm$^2$ UVC at 254 nm (UV-4 S/L, Herolab, Wiesloch, Germany) [7]. After sterilizing, 50 µL of the respective virus suspension were placed on the center of the carriers and allowed to dry for 1 h at room temperature. The carriers were positioned at different distances from the UVC-LEDs inside the UVC-LED boxes (UVC-LED box 1: 1 and 5 cm horizontal distance; UVC-LED box 2: 1 and 4 cm vertical distance). The carriers were irradiated for specific durations (0 s, 10 s, 30 s, 1 min, 3 min, and 10 min). Subsequently, the infectious virus was recovered by vortexing the carriers placed in plastic containers (SARSTEDT, Nümbrecht, Germany) filled with 2 mL DMEM for 1 min. As control, the virus was recovered 10 min after drying without irradiation. The experiments were conducted in triplicates, and the viral loads were determined by endpoint dilution according to Spearman and Kärber. The limit of detection of the assay was at 159 $TCID_{50}$/mL. The means, standard deviations of the viral titers, and 90% effective concentration ($EC_{90}$)

values were calculated with GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). The statistical significances were determined with the t-test. Comparisons were considered significant at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$.

### 3. Results

SARS-CoV-2 can potentially be transmitted via virus-contaminated surfaces of personal items such as smartphones, keys, coins, or credit cards that have been contaminated with the virus. We investigated the performance of two commercially available UVC-LED boxes for virus inactivation on surfaces typically found on personal belongings such as glass, metal, and plastic. We used two different UVC-LED boxes for sterilizing, one with lateral UVC-LEDs (Figure 1A) and one with UVC-LEDs incorporated in the lid of the box (Figure 1B). Additionally, a mirror was installed to the bottom of the chamber. For UVC-LED box 1, the emitted light intensity was determined with 245 $\mu W/cm^2$ at horizontal distance of 1 cm and 65 $\mu W/cm^2$ in the center of the box at horizontal distance of 5 cm from the UVC-LEDs (corresponding to 0.245 and 0.065 $mJ/cm^2$ per second, respectively; Figure 2A). For both distances, the peak wavelength emission was measured at around 254 nm. For UVC-LED box 2, the emitted light intensity was measured with 117 $\mu W/cm^2$ at 1 cm vertical distance from the LEDs, which corresponds to 0.117 $mJ/cm^2$ per second (Figure 2B). The peak wavelength emission was detected at 280 nm.

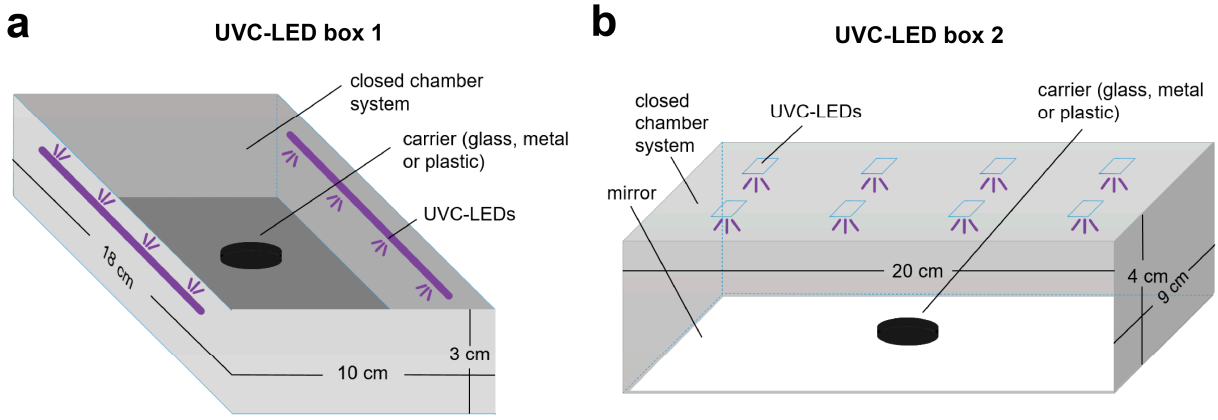


**Figure 1.** Schematic illustrations of the UVC-LED boxes used for UVC-disinfection. (**a**) UVC-LED box 1 (Horcol) was equipped with lateral UVC-LEDs. (**b**) UV-LED box 2 (expondo GmbH, Berlin, Germany) was equipped with UVC-LEDs incorporated in the lid and a mirror installed at the bottom of the chamber.

Next, metal, glass, or plastic samples were overlaid with SARS-CoV-2. The SARS-CoV-2 stocks were diluted with an organic matrix mimicking respiratory secretions, resulting in a final virus concentration of the samples of $4.5 \times 10^6$ $TCID_{50}/mL$ for the experiments in UVC-LED box 1 and $1.8 \times 10^6$ $TCID_{50}/mL$ for the experiments in UVC-LED box 2, respectively. Compared to the viral load immediately after drying, there was no significant reduction of the viral loads after 10 min without irradiation (Figure 3).

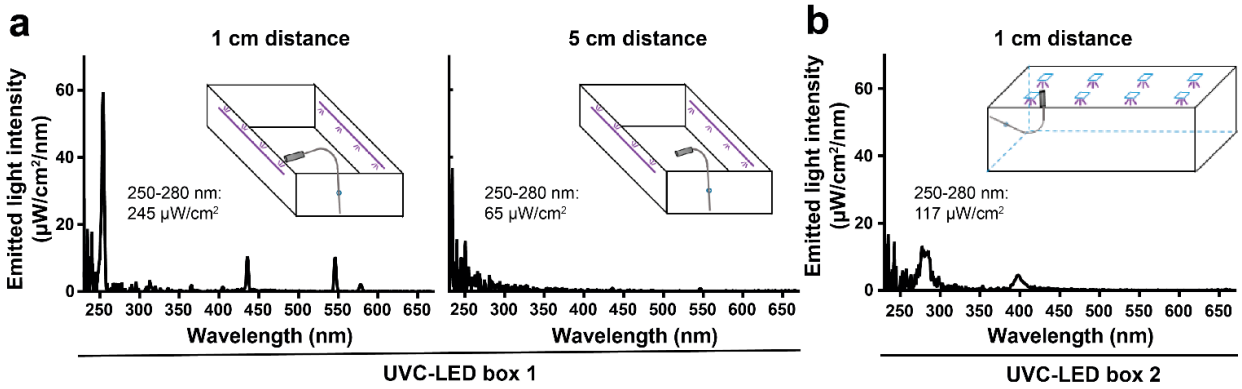


**Figure 2.** Spectrum of the emitted light intensity by LEDs inside of the UVC-LED boxes. The emitted light energy was measured by using a radiometrically calibrated spectrometer (STS-UV-L-50-400-SMA, Ocean Optics B.V., Ostfildern, Germany) with a sensitivity range between 190 and 650 nm (1.5 nm resolution) equipped with a CC-3-UV-S corrector (Ocean Optics B.V., Ostfildern, Germany). (**a**) The spectrum of emitted light intensity of UVC-LED box 1 was measured at 1 and 5 cm horizontal distance from the lateral UVC-LEDs. (**b**) The spectrum of emitted light intensity of UVC-LED box 2 was measured at 1 cm vertical distance from the UVC-LEDs. Emitted light intensity ($\mu W/cm^2$) between 250 and 280 nm is displayed for each spectrum. Schematic illustrations indicate the borehole and the UVC-detector placed inside of the box for the respective measurement.

UVC-LED irradiation conducted with UVC-LED boxes proved to be an appropriate method for the disinfection of SARS-CoV-2-contaminated surfaces. SARS-CoV-2-contaminated glass, metal, or plastic samples were effectively UVC-disinfected inside of both UVC-LED boxes (Figure 3). A significant reduction of viral loads of SARS-CoV-2 on glass, metal, and plastic was achieved even after 10 s of irradiation (UVC-LED box 1: 2.45 $mJ/cm^2$; UVC-LED box 2: 1.17 $mJ/cm^2$) at a distance of 1 cm from the LEDs in both UVC-LED boxes (Figure 3). When using UVC-LED box 1, no virus was detectable after 3 (glass and plastic) or 10 min (metal) of irradiation at a distance of 1 cm (Figure 3A). At a distance of 5 cm from the LEDs (UVC-LED box 1), viral loads were strongly reduced after 3 and 10 min of irradiation (3 min: glass: 88.27 ± 1.49% (0.93 ± 0.05 $\log_{10}$), metal: 88.26 ± 3.32% (0.93 ± 0.09 $\log_{10}$), plastic: 90 ± 1.38% (1 ± 0.05 $\log_{10}$); 10 min: glass: 91.92 ± 1.85% (1.1 ± 0.08 $\log_{10}$), metal: 98.52 ± 0.42% (1.83 ± 0.09 $\log_{10}$), plastic: 92.56 ± 1.7% (1.13 ± 0.8 $\log_{10}$); Figure 3A). When using UVC-LED box 2, SARS-CoV-2 was almost completely inactivated after 3 and 10 min of irradiation at a distance of 1 cm (3 min: glass: 97.49 ± 0.35% (1.6 ± 0.05 $\log_{10}$), metal: 99.21 ± 0.1% (2.1 ± 0.05 $\log_{10}$), plastic: 98.74 ± 0.16% (1.9 ± 0.05 $\log_{10}$); 10 min: glass: 97.85 ± 0.27% (1.67 ± 0.05 $\log_{10}$), metal: 99.42 ± 0.08% (2.23 ± 0.05 $\log_{10}$), plastic: 99.46% (2.27 $\log_{10}$); Figure 3B). At a distance of 4 cm, no SARS-CoV-2 was detectable on glass, metal, and plastic after 10 min of irradiation (Figure 3B).

Taken together, our data demonstrate that UVC-LED sterilization boxes can effectively inactivate SARS-CoV-2 on surfaces such as glass, metal, or plastic.

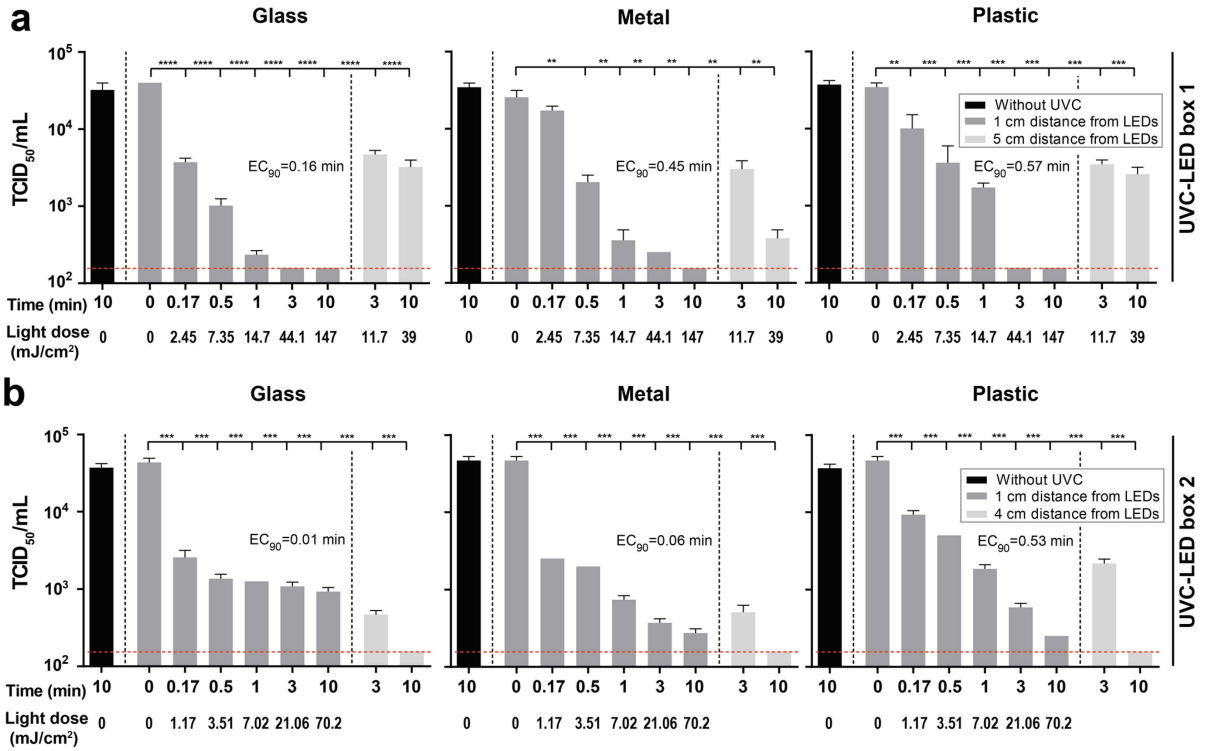


**Figure 3.** Disinfection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-contaminated objects by two distinct UVC-LED boxes. Glass, metal, and plastic samples were contaminated with SARS-CoV-2 at a final concentration of $4.5 \times 10^6$ $TCID_{50}$/mL for UVC-LED box 1 and $1.8 \times 10^6$ $TCID_{50}$/mL for UVC-LED box 2 in a cell culture medium mixed with defined organic matrix, thereby mimicking the viral contamination on the surfaces of personal belongings such as smartphones, keys, coins, or credit cards. The samples were exposed to LED-UVC light for 0 s, 10 s, 30 s, 1 min, 3 min, and 10 min at different distances from UVC-LEDs inside the UVC-LED boxes. Experiments were conducted in triplicates. (**a**) In UVC-LED box 1, samples were irradiated at a distance of 1 and 5 cm from the UVC-LEDs. (**b**) In UVC-LED box 2, samples were irradiated at 1 and 4 cm from the UVC-LEDs. Light doses at 4 cm distance cannot be shown, as an irradiance measurement of the LEDs at that distance would have required drilling a hole into the bottom of the box, which was not possible without damaging the box. The limit of detection of the assay was at 159 $TCID_{50}$/mL (indicated by the red dotted line). Data are displayed as mean ± SD. ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$. $TCID_{50}$ = tissue culture infectious dose, 50%; $EC_{90}$ = 90% effective concentration.

## 4. Discussion

SARS-CoV-2 is transmitted through direct contact with infected individuals, by virus-containing aerosols and potentially via contact with virus-contaminated surfaces [3]. In the present study, we investigated the performance of UVC-LED sterilization boxes on the inactivation of SARS-COV-2 on surfaces such as glass, metal, and plastics that are typically found on personal items like smart phones, credit cards, or keys. We demonstrated that UVC-LED boxes can effectively inactivate SARS-CoV-2 on glass, metal, and plastic. Independent of the used UVC-LED box and the materials, SARS-CoV-2 could be almost completely inactivated after 3 min exposure (UVC-LED box 1: 1 cm: 44.1 mJ/cm$^2$, 5 cm: 11.7 mJ/cm$^2$; UVC-LED box 2: 1 cm: 21.06 mJ/cm$^2$), which represents the standard exposure time provided by the UVC-LED boxes. Even 10 s of exposure strongly reduced viral loads on the contaminated surfaces.

The findings are in line with other studies that report susceptibility of coronaviruses, including SARS-CoV-1 and SARS-CoV-2 to UVC irradiation [7,10]. To almost completely inactivate high viral loads of SARS-CoV-1, a UVC-dose of 1446 mJ/cm$^2$ was necessary [10].

High viral loads of SARS-CoV-2 could be completely inactivated by a UVC dose of 1048 mJ/cm$^2$ [7]. The results of the present study show that the distance of the inoculated materials from the LEDs and thus the emitted light intensity is a decisive factor for achieving the complete inactivation of SARS-CoV-2. Accordingly, it has recently been reported that distance and angle of UVC light source in closed box systems are important factors for irradiance over respirator surfaces [11]. UVC-LED box 1 and 2 showed a similar reduction of viral load for the materials glass and plastic. At a distance of 5 cm from the LED of UVC-LED box 1, although the viral load was reduced by 91.92 ± 1.85% (1.1 ± 0.08 $\log_{10}$) on glass, 98.52 ± 0.42% (1.83 ± 0.09 $\log_{10}$) on metal and 92.56 ± 1.7% (1.13 ± 0.8 $\log_{10}$) on plastic, the emitted light intensity (65 µW/cm$^2$) was not sufficient to completely inactivate the virus after 10 min exposure. The mirror at the bottom of UVC-LED box 2 may have contributed to facilitating the inactivation of the virus. Van Doremalen et al. [4] indicated that a SARS-CoV-2 stock with a viral concentration of $10^5$ $TCID_{50}$/mL corresponds to cycle threshold values between 20 and 22, which is similar to the thresholds of samples from the upper and lower respiratory tracts of infected individuals. In this study, SARS-CoV-2 stocks with even higher viral concentrations of about $10^6$ $TCID_{50}$/mL were used for the inoculation of the materials.

Overall, both UVC-LED boxes were highly effective in inactivating the high-titer viral stocks of SARS-CoV-2. However, in this study, we did not test the stability of the emission of the UVC-LED boxes. Therefore, no statement can be made as to whether the emission of the devices is stable during prolonged use over weeks or months.

The encouraging results of the study make the UVC-LED boxes an affordable option for the public to disinfect a variety of items, including phones, watches, headphones, masks, and makeup utensils, as long as the item size fits the device. Because SARS-CoV-2 can also be detected on different surfaces in hospital environment [12,13], UVC-LED boxes might also be an effective tool for environmental decontamination in hospitals.

**Author Contributions:** Conceptualization, A.K.; methodology, A.K., M.B., T.L.M. and E.S.; investigation, M.B., L.S., M.O. and L.v.d.S.; resources, U.D. and O.W.; data analysis, M.B. and M.A.; writing—original draft preparation, M.B. and A.K.; writing—review and editing, M.B., A.K., T.L.M., E.S. and U.D.; visualization, M.B. and M.A.; supervision, A.K. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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## 4.2 Turmeric Root and Its Bioactive Ingredient Curcumin Effectively Neutralize SARS-CoV-2 In Vitro

**Bormann, M.**, M. Alt, L. Schipper, L. van de Sand, V. T. K. Le-Trilling, L. Rink, N. Heinen, R. J. Madel, M. Otte, K. Wuensch, C. S. Heilingloh, T. Mueller, U. Dittmer, C. Elsner, S. Pfaender, M. Trilling, O. Witzke, and A. Krawczyk

2021

Contributions:

- Conception: 40 %
- Experimental work: 60%
- Data analysis: 80 %
- Statistical analysis: 80 %
- Writing the manuscript: 70 %
- Revising the manuscript: 70 %

The study was conceptualized by Adalbert Krawczyk and Maren Bormann. Experiments were performed by Maren Bormann, Mira Alt, Leonie Brochhagen (née Schipper), Lukas van de Sand, Khanh Le-Trilling, Lydia Rink, Natalie Heinen, Mona Otte, Korbinian Wünsch and Carina Elsner. The analysis, evaluation and visualization of the data was carried out by Maren Bormann in collaboration with Adalbert Krawczyk. The manuscript was prepared by Maren Bormann and Adalbert Krawczyk. The manuscript was edited by Maren Bormann, Adalbert Krawczyk, Mira Alt, Rabea Julia Madel, Lukas van de Sand, Toni Luise Meister, Mirko Trilling, Stephanie Pfaender, and Christiane Silke Heilingloh. Ulf Dittmer and Oliver Witzke provided resources.

Maren Bormann

Prof. Dr. Adalbert Krawczyk

viruses

MDPI

*Article*

# Turmeric Root and Its Bioactive Ingredient Curcumin Effectively Neutralize SARS-CoV-2 In Vitro

**Maren Bormann [1], Mira Alt [1], Leonie Schipper [1], Lukas van de Sand [1], Vu Thuy Khanh Le-Trilling [2], Lydia Rink [2], Natalie Heinen [3], Rabea Julia Madel [1], Mona Otte [1], Korbinian Wuensch [1], Christiane Silke Heilingloh [1], Thorsten Mueller [4,5], Ulf Dittmer [2], Carina Elsner [2], Stephanie Pfaender [3], Mirko Trilling [2], Oliver Witzke [1] and Adalbert Krawczyk [1,2,*]**

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**Abstract:** Severe Acute Respiratory Syndrome Coronavirus Type 2 (SARS-CoV-2) is the causative agent of the coronavirus disease 2019 (COVID-19). The availability of effective and well-tolerated antiviral drugs for the treatment of COVID-19 patients is still very limited. Traditional herbal medicines elicit antiviral activity against various viruses and might therefore represent a promising option for the complementary treatment of COVID-19 patients. The application of turmeric root in herbal medicine has a very long history. Its bioactive ingredient curcumin shows a broad-spectrum antimicrobial activity. In the present study, we investigated the antiviral activity of aqueous turmeric root extract, the dissolved content of a curcumin-containing nutritional supplement capsule, and pure curcumin against SARS-CoV-2. Turmeric root extract, dissolved turmeric capsule content, and pure curcumin effectively neutralized SARS-CoV-2 at subtoxic concentrations in Vero E6 and human Calu-3 cells. Furthermore, curcumin treatment significantly reduced SARS-CoV-2 RNA levels in cell culture supernatants. Our data uncover curcumin as a promising compound for complementary COVID-19 treatment. Curcumin concentrations contained in turmeric root or capsules used as nutritional supplements completely neutralized SARS-CoV-2 in vitro. Our data argue in favor of appropriate and carefully monitored clinical studies that vigorously test the effectiveness of complementary treatment of COVID-19 patients with curcumin-containing products.

**Keywords:** SARS-CoV-2; COVID-19; herbal medicine; antiviral; *Curcuma longa*; turmeric root; curcumin

## 1. Introduction

In early 2020, Severe Acute Respiratory Syndrome Coronavirus Type 2 (SARS-CoV-2) was identified as the causative agent of the coronavirus disease 2019 (COVID-19) that is currently causing a global pandemic [1]. The major transmission routes of SARS-CoV-2 are droplet and airborne transmission [2]. SARS-CoV-2 infections can be asymptomatic

or cause a respiratory and other severe diseases. Symptoms range from mild, cold-like symptoms including fever and cough to severe, life-threatening disease [3].

The current therapeutic treatment options for COVID-19 comprise antiviral therapeutics such as remdesivir as well as immunomodulatory therapeutics such as dexamethasone to downregulate hyper-inflammatory immune responses [4,5]. However, the availability of potent antiviral compounds is limited, particularly in developing areas, and the development of novel antiviral compounds is time- and cost-intensive and may take years before approval. Traditional herbal medicines represent promising options for complementary treatment of COVID-19 diseases. To date, numerous plants and their ingredients exhibit potent antimicrobial and antiviral effects [6–8]. Notably, curcumin showed antimicrobial activity toward bacteria, malaria, fungi, and viruses [9].

Turmeric root, also known as *Curcuma longa*, is broadly used as a spice widely cultivated in Southeast Asia. The rhizome of *Curcuma longa* contains several structurally related curcuminoids. Sixty to 75% of the curcuminoid content consists of curcumin, also known as diferuloylmethane. The remaining fraction is a combination of demethoxycurcumin (20–25%) and bisdemethoxycurcumin (5–15%) [10,11]. Turmeric root has been used for thousands of years as medicine for the complementary treatment of a wide variety of diseases. As early as 1815, the bioactive ingredient curcumin was first isolated from turmeric root by Vogel and Pelletier. Curcumin reveals a broad spectrum of bioactivities such as antioxidant, anti-inflammatory, antibacterial, antiviral, antitumor, and hepatoprotective activities [12–15].

We and others have demonstrated the antiviral activities of curcumin against various viruses, including Dengue Virus, Human Immunodeficiency Virus (HIV), Kaposi Sarcoma-associated Herpesvirus, Enterovirus, Zika Virus, Chikungunya Virus, Vesicular Stomatitis Virus, the Human Respiratory Syncytial Virus, Viral Hemorrhagic Septicemia Virus, Influenza A Virus, Herpes Simplex Type 2, Norovirus, and Hepatitis C Virus [16,17]. Furthermore, curcumin is known for its pharmacological abilities especially as an anti-inflammatory and antiviral agent [18,19]. Moreover, curcumin was discussed as a potential candidate in the therapeutic regimen of COVID-19 [20]. However, its antiviral activity against SARS-CoV-2 has, at least to our knowledge, not been thoroughly proven so far. There is clearly a medical need to determine if curcumin may have a direct antiviral activity and thus may be suitable for complementary treatment of COVID-19.

In the present study, we investigated the neutralizing activity of aqueous turmeric root extract, curcumin-containing nutritional supplement capsules, and pure curcumin against SARS-CoV-2.

## 2. Materials and Methods

### *2.1. Aqueous Turmeric Root Extract*

Turmeric root was comminuted through a grater and subsequently centrifuged (10 min, 3985 RCF (relative centrifugal force)) to remove solid components. The supernatant was further purified by ultracentrifugation at 50,624 RCF and 4 °C for two hours.

### *2.2. Curcumin-Containing Nutritional Supplement Capsule*

One curcumin-containing nutritional supplement capsule (Nature Love, Tauron Ventures GmbH, Monheim am Rhein, Germany) was dissolved in 10 mL of dimethyl sulfoxide (DMSO) (Roth, Karlsruhe, Germany). The solution was subsequently diluted 1:10 ($v/v$) in Dulbecco's modified Eagle's medium (DMEM), containing 10% ($v/v$) fetal calf serum (FCS), penicillin (100 IU/mL), and streptomycin (100 µg/mL) (all Life Technologies Gibco, Darmstadt, Germany) and stored at 37 °C. One capsule contained 640 mg turmeric powder, 105 mg turmeric extract (containing 99.9 mg curcumin), and 5 mg black pepper (containing 4.7 mg piperine).

### 2.3. Curcumin

For cell culture experiments, 200 mg of curcumin (diferuloylmethane) (Sigma Aldrich, Darmstadt, Germany) was dissolved in 10 mL DMSO and subsequently diluted 1:10 (*v*/*v*) in DMEM, containing 10% (*v*/*v*) FCS, penicillin (100 IU/mL), and streptomycin (100 µg/mL) and stored at 37 °C.

### 2.4. Cells and Virus

Vero E6 cells (African green monkey kidney cells, American Type Culture Collection, ATCC, Manassas, Virginia, USA; ATCC® CRL-1586™) were cultivated in DMEM, containing 10% (*v*/*v*) FCS, penicillin (100 IU/mL), and streptomycin (100 µg/mL) at 5% $CO_2$ and 37 °C. Calu-3 cells (human lung cancer cell line, American Type Culture Collection, ATCC, Manassas, Virginia, USA; ATCC® HTB-55™) were cultivated in Eagle's minimum essential medium (EMEM; ATCC, Manassas, Virginia), containing 10% (*v*/*v*) FCS, penicillin (100 IU/mL), and streptomycin (100 µg/mL) at 5% $CO_2$ and 37 °C. The clinical SARS-CoV-2 isolate was derived from a nasopharyngeal swab of a patient with COVID-19 hospitalized in April 2020 at the University Hospital in Essen as previously described [21]. The virus was propagated on Vero E6 cells maintained in DMEM supplemented with 10% (*v*/*v*) FCS, penicillin (100 IU/mL), streptomycin (100 µg/mL), ciprofloxacin (10 µg/mL), and amphotericin B (2.5 µg/mL). After five days of incubation, the supernatant was harvested, cleared from cell debris by centrifugation, and stored at −80 °C. Viral titers were determined by a standard endpoint dilution assay and calculated as 50% tissue culture infective dose ($TCID_{50}$)/mL as previously described [22].

### 2.5. Neutralization Assay on Vero E6 Cells

The neutralization capacity of aqueous turmeric root extract, curcumin-containing nutritional supplement capsules, and pure curcumin were determined in cell culture by endpoint dilution as described previously [23]. For this purpose, serial twofold dilutions of turmeric root extract (1:8–1:1024), nutritional supplement capsules (468.8–3.7 µg/mL), and curcumin (125–1 µg/mL) were pre-incubated with 100 $TCID_{50}$ of SARS-CoV-2 for one hour at 37 °C and subsequently incubated on confluent Vero E6 cells grown on 96-well microtiter plates. Untreated Vero E6 cells served as negative control and cells infected with 100 $TCID_{50}$ of SARS-CoV-2 in the absence of antiviral compounds served as positive control. After 48 h, cells were stained with 0.5% crystal violet (*w*/*v*) (Roth, Karlsruhe, Germany), solved in 20% (*v*/*v*) methanol (Merck, Darmstadt, Germany), and analyzed for cytopathic effects (CPE) by transmitted light microscopy (Carl Zeiss AG, Oberkochen, Germany). The concentration required for reducing virus-induced CPE by 100% was determined as the complete neutralization titer. To determine the half-maximal effective concentration ($EC_{50}$) sufficient to neutralize the virus, for each dilution, the percentage of cell cultures showing CPEs was determined. $EC_{50}$ values were calculated by nonlinear regression, and the means were calculated using GraphPad Prism 8.0.1. The experiment was performed three times independently.

### 2.6. Neutralization Assay via icELISA on Human Calu-3 Cells

The neutralization efficacy of curcumin against SARS-CoV-2 on human Calu-3 cells was assessed by an in-cell ELISA (icELISA)-based neutralization test (icNT). The icNT was performed as described previously [24]. In brief, 5000 plaque-forming units (PFU) of SARS-CoV-2 were incubated with different dilutions of turmeric root extract (1:16–1:128), nutritional supplement capsule content (468.8–58.6 µg/mL), or curcumin (125–15.6 µg/mL) for 1 h prior to inoculation of Calu-3 cells seeded on 96-well plates (≈$5 \times 10^4$ cells per well). At 24 h post infection, Calu-3 cells were fixed with 4% (*w*/*v*) paraformaldehyde/PBS, permeabilized with 1% (*v*/*v*) Triton-X-100/PBS, and blocked with 3% (*v*/*v*) FCS/PBS. An SARS-CoV-2 N-specific primary antibody was added and incubated overnight at 4 °C. The cells were washed three times with 0.05% (*v*/*v*) Tween-20/PBS followed by incubation with a peroxidase-labeled secondary antibody for 1 h. After four washing steps, the enzyme

reaction was visualized by adding tetramethylbenzidine (TMB) substrate and stopped with 0.5 M HCL. The absorbance was measured with a microplate multireader at OD 450 (Mithras2 LB 943; Berthold Technologies). Means and standard errors of the mean were calculated and significance was assessed by a one-way analysis of variance (ANOVA) and Dunnett's multiple comparison test using GraphPad Prism 8.0.1.

### *2.7. Cell Viability Assay*

A potential cytotoxicity of various concentrations of aqueous turmeric root extract, nutritional supplement capsules, and curcumin toward Vero E6 cells was determined using the Orangu cell-counting solution (Cell guidance systems, Cambridge, UK) as described before [7]. Orangu™ is a colorimetric assay used in cytotoxicity assays for the calculation of viable cell numbers. In this assay, WST-8 tetrazolium salt is reduced by cellular dehydrogenase activities to an orange formazan product. The quantity of living cells is directly proportional to the amount of chemically converted orange-colored formazan dye [25]. Orangu assay was conducted according to the manufacturer's instructions. Identical twofold dilutions of aqueous turmeric root extract, nutritional supplement capsules, and curcumin as used in the neutralization assays and icNTs were utilized to overlay 96-well microtiter plates, which contained Vero E6 or Calu-3 cells. The further incubation took place at 37 °C and 5% $CO_2$ according to the duration of the corresponding neutralization test for 48 h (Vero E6 cells) or 24 h (Calu-3 cells). After incubation, 10 µL of Orangu cell counting solution was added to the wells and incubated for 120 min (37 °C, 5% $CO_2$). Cell viability was measured at an absorbance of 450 nm using Mithras LB 940 (Berthold Technologies, Oak Ridge, TN, USA). The experiment was performed three times independently. The means and standard error of the mean were calculated using GraphPad Prism 8.0.1 (Graph Pad Software, San Diego, CA, USA).

### *2.8. Quantitative SARS-CoV-2 RT-PCR*

We assessed the effect of aqueous turmeric root extract, nutritional supplement capsules, and curcumin on SARS-CoV-2 RNA levels by qRT-PCR. In brief, we co-incubated 100 $TCID_{50}$ SARS-CoV-2 with serial dilutions of curcumin (125–1 µg/mL) at 37 °C for one hour. Virus–curcumin suspensions were added to confluent Vero E6 cells grown in 96-well plates. Untreated Vero E6 cells and cells treated with 100 $TCID_{50}$ SARS-CoV-2 served as controls. Subsequently, supernatants were harvested, and the viral RNA was purified using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). The genomic SARS-CoV-2 RNA was quantified by RT-qPCR, using primers targeting the viral M or N gene [26]. Plasmid dilution series of 1:10 were used as reference to assess the M and N gene copy numbers (details and sequence information available upon request). The experiment was performed three times independently. $EC_{50}$ were calculated by nonlinear regression, and the means were calculated using GraphPad Prism 8.0.1.

## 3. Results

### *3.1. In Vitro Neutralization of SARS-CoV-2*

The turmeric root ingredient curcumin plays an important role in traditional medicine because of its anti-inflammatory and antimicrobial activity. Here, we investigated the antiviral effect of an aqueous turmeric root extract, curcumin-containing nutritional supplement capsules, and pure curcumin against SARS-CoV-2.

Various concentrations of turmeric root extract (1:8–1:1024 dilution), curcumin-containing nutritional supplement capsules (468.8–3.7 µg/mL), and pure curcumin (125–1 µg/mL) dissolved in cell-culture medium were incubated with 100 $TCID_{50}$ of SARS-CoV-2 for 1 h. Subsequently, mixtures were incubated on confluent Vero E6 cells. Two days after infection, cell cultures were fixed and stained with crystal violet and microscopically inspected for a CPE. The concentration required for complete virus neutralization (Figure 1) was determined, and $EC_{50}$ values were calculated (Figure 2).

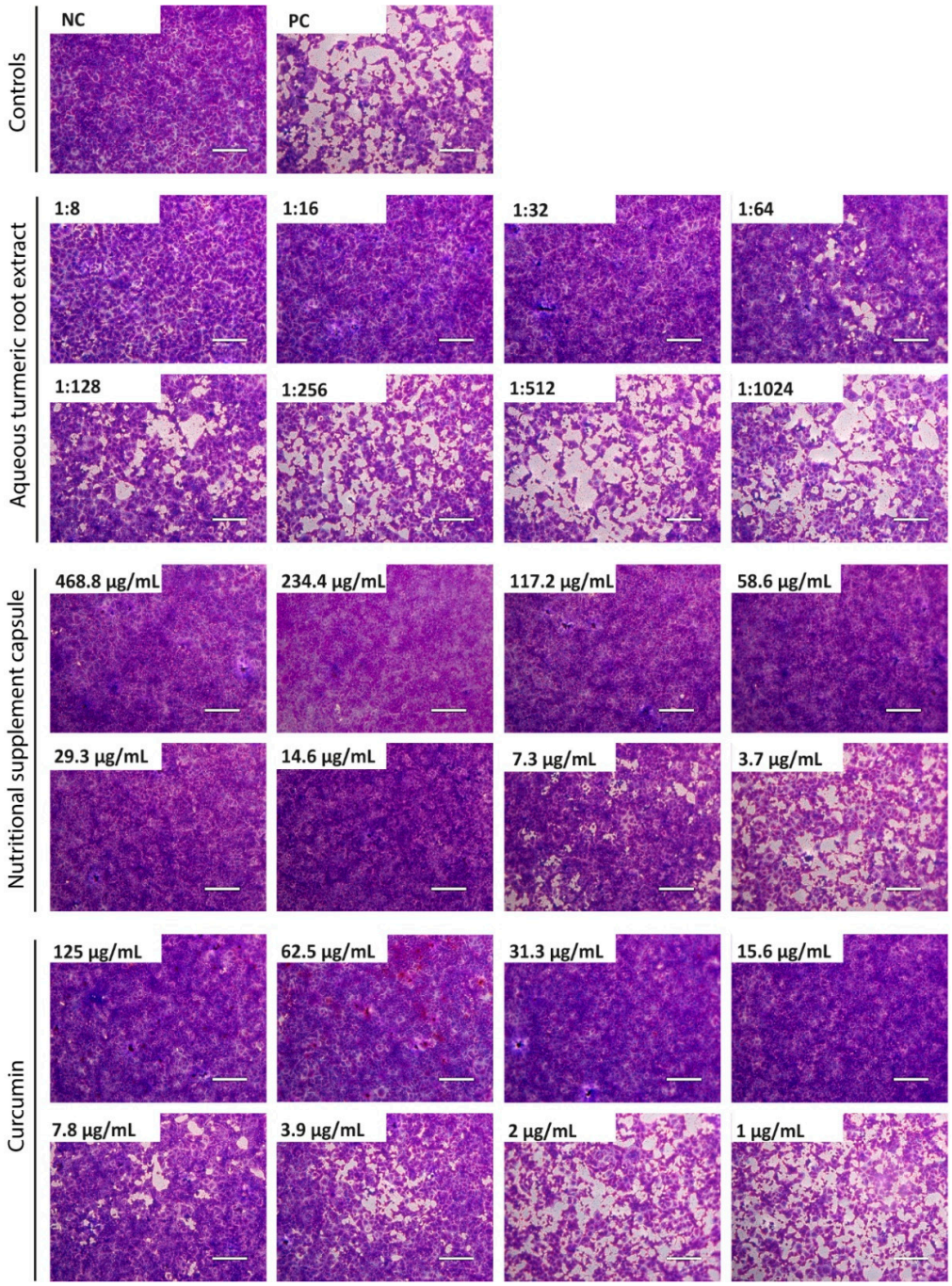


**Figure 1.** Neutralization of SARS-CoV-2 by aqueous turmeric root extract, curcumin-containing nutritional supplement capsules, and curcumin. Decreasing concentrations of aqueous turmeric root extract (1:8–1:1024 dilution), nutritional supplement capsules (468.8–3.7 µg/mL), and curcumin (125–1 µg/mL) were pre-incubated with 100 $TCID_{50}$ of SARS-CoV-2 for one hour and subsequently added to confluent Vero E6 cells. After 48 h, cells were stained with crystal violet and analyzed for cytopathic effects using transmitted light microscopy. The experiment was performed three times independently. Representative images are displayed. NC = negative control (medium); PC = positive control (100 $TCID_{50}$ SARS-CoV-2); scale bar = 200 µm.

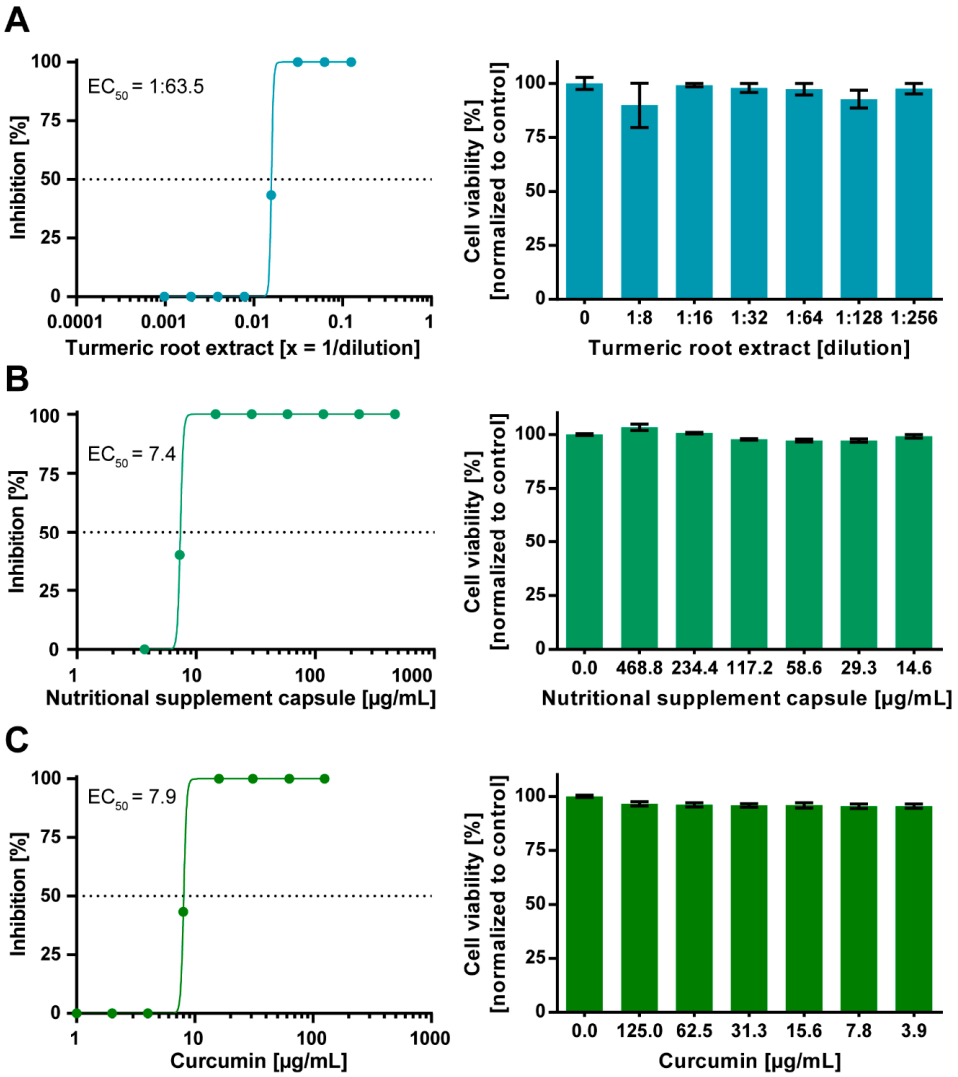


**Figure 2.** Dose-dependent antiviral activity of aqueous turmeric root extract, curcumin-containing nutritional supplement capsules, and curcumin against SARS-CoV-2. Decreasing concentrations of aqueous turmeric root extract (1:8–1:1024 dilution) (**A**), nutritional supplement capsules (468.8–3.7 µg/mL) (**B**), and curcumin (125–1 µg/mL) (**C**) were pre-incubated with 100 $TCID_{50}$ of SARS-CoV-2 for one hour. Subsequently, each dilution of virus–herb suspensions was incubated on confluent Vero E6 cells grown on a 96-well plate. After 48 h, cells were stained with crystal violet and analyzed regarding cytopathic effects. The half-maximal effective concentration ($EC_{50}$) was calculated by nonlinear regression using GraphPad Prism. The cytotoxic effect of various concentrations of aqueous turmeric root extract, nutritional supplement capsules, and curcumin toward Vero E6 cells was determined by Orangu Cell Counting Solution (Cell guidance systems) after 48 h. Cell viability was normalized to untreated control cells. The experiment was performed three times independently. Error bars represent the standard error of the mean (SEM).

Complete neutralization of 100 $TCID_{50}$ SARS-CoV-2 was achieved by aqueous turmeric root extract at a dilution of 1:32 (Figure 1). In order to calculate the $EC_{50}$ of turmeric root extract, we quantitatively analyzed the neutralization assay. Dose–response assessment showed that the $EC_{50}$ of turmeric root extract was achieved at a dilution of 1:63.5 (Figure 2A). No cytotoxic effect for turmeric root extract was observed at the indicated dilutions (Figure 2A). The dissolved nutritional supplement capsules completely neutralized SARS-CoV-2 at a concentration of 14.6 µg/mL (Figure 1). The $EC_{50}$ was determined at a concentration of 7.4 µg/mL (Figure 2B). No cytotoxic effect for the nutritional sup-

plement capsule was observed (Figure 2B). Curcumin is the main bioactive component of turmeric root and was already shown to have an elicit antiviral activity against various viruses [11,16]. Curcumin achieved the complete neutralization of SARS-CoV-2 until a subtoxic concentration of 15.6 µg/mL (Figure 1) with an $EC_{50}$ of 7.9 µg/mL (Figure 2C). Effects related to cytotoxicity were excluded, since none of the applied concentrations exhibited cytotoxicity (Figure 2C).

To assess the antiviral effect of turmeric root extract, dissolved nutritional supplement capsules, and curcumin against SARS-CoV-2 on human cells, we conducted an icNT on Calu-3 cells. Different compound dilutions were incubated with 5000 PFU of SARS-CoV-2 for 1 h prior to inoculation of Calu-3 cells. After 24 h, the extent of the infection was quantified after staining of the SARS-CoV-2 N antigen by icELISA. All tested mixtures potently neutralized SARS-CoV-2 at subtoxic concentrations (Figure 3A). Low concentrations of 58.6 µg/mL of the dissolved supplement capsules, 15.6 µg/mL of curcumin, and a high dilution of 1:128 of turmeric root extract were sufficient to significantly neutralize SARS-CoV-2. No cytotoxic effect could be observed at the indicated concentrations (Figure 3B).

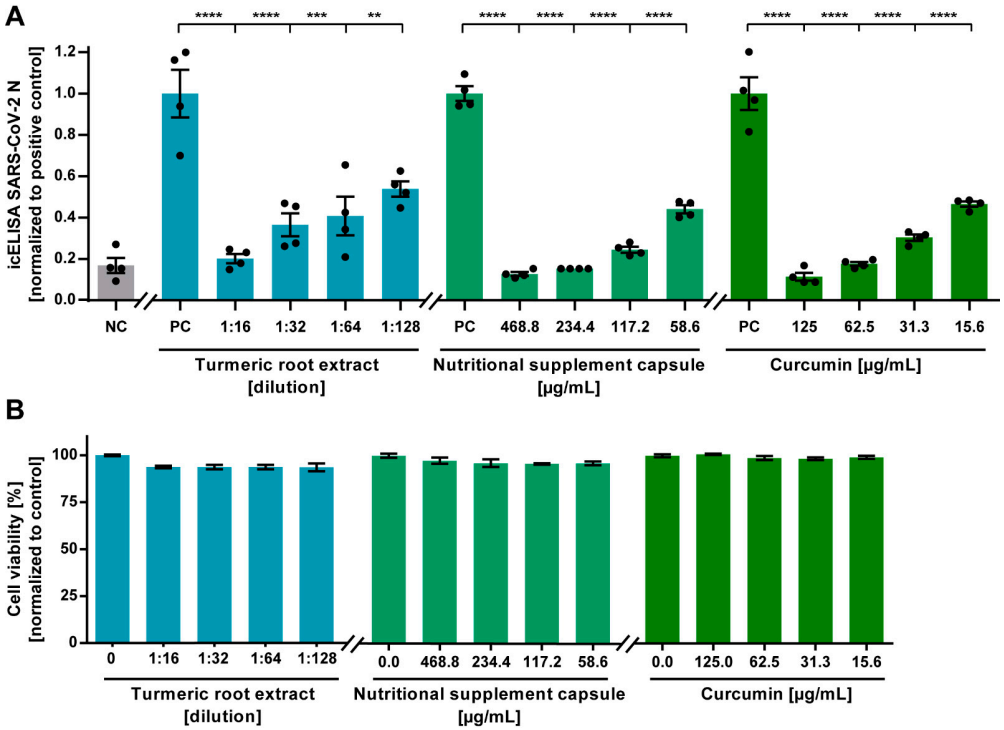


**Figure 3.** Neutralization of SARS-CoV-2 by aqueous turmeric root extract, curcumin-containing nutritional supplement capsules, and curcumin on a human cell line assessed by an in-cell ELISA (icELISA)-based neutralization test (icNT). (**A**) Decreasing concentrations of aqueous turmeric root extract (1:16–1:128 dilution), nutritional supplement capsule content (468.8–58.6 µg/mL), or curcumin (125–15.6 µg/mL) were pre-incubated with 5000 plaque-forming units (PFU) of SARS-CoV-2 for one hour. Subsequently, mixtures were added to human Calu-3 cells and incubated for 24 h. After incubation with a SARS-CoV-2 N-specific primary antibody and peroxidase-labelled secondary antibody, the enzyme reaction was visualized by adding tetramethylbenzidine. Absorbance was measured with a microplate multireader at OD450. Statistical analysis was performed with one-way analysis of variance (ANOVA) and Dunnett's multiple comparison test using GraphPad Prism. ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$; error bars represent the standard error of the mean (SEM). NC = negative control (medium); PC = positive control (5000 PFU SARS-CoV-2). (**B**) The cytotoxic effect of various concentrations of aqueous turmeric root extract, nutritional supplement capsule, and curcumin toward Calu-3 cells was determined by Orangu™ Cell Counting Solution (Cell guidance systems) after 24 h. The cell viability was normalized to untreated control cells. Error bars represent the SEM.

In conclusion, we demonstrated, to our knowledge, for the first time a potent antiviral activity of turmeric root and its bioactive ingredient curcumin against SARS-CoV-2 on Vero-E6 and Calu-3 cells.

### *3.2. Effect of Curcumin on SARS-CoV-2 RNA*

Curcumin was described as the main active ingredient of turmeric root. Therefore, we further tested whether curcumin may have an effect on RNA levels of SARS-CoV-2 in cell culture. For this purpose, serial dilutions of curcumin were co-incubated with 100 $TCID_{50}$ SARS-CoV-2 for 1 h prior to inoculation of Vero E6 cells. Two days post infection, supernatants were harvested, and SARS-CoV-2 RNA was quantified by RT-qPCR.

The RT-qPCR results correlated very well with the aforementioned experiments assessing the neutralization efficacy by CPE inspection. Curcumin significantly reduced SARS-CoV-2 RNA levels in cell culture supernatants with an $EC_{50}$ of ≈14 µg/mL (Figure 4).

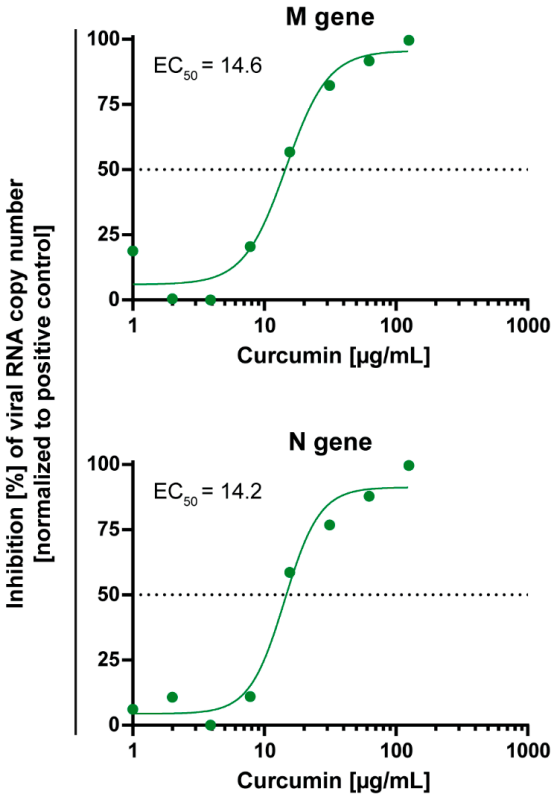


**Figure 4.** Dose-dependent activity of curcumin on SARS-CoV-2 RNA genome copy numbers. Decreasing concentrations of curcumin (125–1 µg/mL) were pre-incubated with 100 $TCID_{50}$ of SARS-CoV-2 for one hour and subsequently added to confluent Vero E6 cells. After 48 h, cell culture supernatants were harvested, and the genomic SARS-CoV-2 RNA was quantified via RT-qPCR, using primer targeting the viral M or N gene. Cells infected with 100 $TCID_{50}$ of SARS-CoV-2 served as positive control. The experiment was performed three times independently. The half-maximal effective concentration ($EC_{50}$) was calculated by nonlinear regression using GraphPad Prism.

## 4. Discussion

Herbal medicines with antiviral activity are promising candidates for complementary treatment of viral infections such as SARS-CoV-2 infections since they are cost-effective and broadly available around the world. In the present study, we showed that turmeric root and its bioactive ingredient curcumin have a strong antiviral effect against SARS-CoV-2

in cell culture at low subtoxic concentrations. These findings highlight curcumin as an antiviral compound against SARS-CoV-2.

Using natural products or repurposing drugs to develop antiviral agents can be an alternative strategy to the time-consuming process of developing or designing new compounds. Turmeric root has a long history as a medicine for a variety of uses around the world, including as an antiseptic, anti-inflammatory agent with antimicrobial activity [27]. Aqueous turmeric root extract, dissolved nutritional supplement capsules, as well as curcumin potently neutralized SARS-CoV-2 in Vero E6 and Calu-3 cell culture models. Furthermore, curcumin significantly reduced SARS-CoV-2 RNA levels in cell culture supernatants. A possible mechanism of action may be the inhibition of viral entry by curcumin. Former in silico studies indicated that curcumin may interfere with the binding of the spike (S) glycoprotein of SARS-CoV-2 to angiotensin-converting enzyme 2 (ACE-2) receptor [28,29]. The ACE-2 receptor is located on the surface of several cell types in humans, including secretory goblet cells in the nasal mucosa, absorptive enterocytes in the intestine, as well as type II pneumocytes in the lung [30]. In silico, the keto and enol forms of curcumin established potent hydrogen bonding with the ACE-2 receptor [28]. Recently, another in silico study predicted that curcumin strongly binds to the receptor-binding domain (RBD) of the S-protein, the ACE-2 receptor, and the complex between the RBD and ACE-2 [29].

Curcumin acts as an antiviral agent against a variety of viruses, including HIV, HCV, Influenza A, and Severe Acute Respiratory Syndrome Coronavirus 1 (SARS-CoV-1) [16,17,31]. To inhibit the integrase of HIV-1, an $EC_{50}$ of 40 μM curcumin is necessary compared to the effective dose of 10 μM against influenza A virus [16]. Furthermore, curcumin inhibits SARS-CoV-1 with an $EC_{50}$ of >10 μM [31]. We showed that curcumin also inhibits SARS-CoV-2 CPEs with an $EC_{50}$ of 7.8 μg/mL (21.2 μM) of infected Vero E6 cells, which is a standard in vitro model in SARS-CoV-2 research. Furthermore, curcumin treatment significantly reduced SARS-CoV-2 RNA levels in cell culture supernatants of Vero E6 cells with an $EC_{50}$ of ≈14 μg/mL (≈38 μM).

Recently, it was shown that the antiviral effect of potential antiviral compounds against SARS-CoV-2 can be cell-line-dependent. Notably, chloroquine blocked SARS-CoV-2 infection in Vero E6 cells, but it failed to neutralize SARS-CoV-2 infection in human Calu-3 cells [32]. This finding highlights the importance of using human cell line models such as the Calu-3 cells in addition to the commonly used Vero E6 cell line. We demonstrated that curcumin efficiently inhibited SARS-CoV-2 infection in both cell lines, Vero E6, and human Calu-3 lung cells, thereby indicating a genuine antiviral effect of curcumin against SARS-CoV-2.

In addition to the antiviral activity, curcumin also exhibits anti-inflammatory effects. Randomized controlled trials indicated a significant downregulation of the human tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6) through curcumin supplementation [33,34]. A meta-analysis showed that 8–12 weeks of treatment with 1 g curcumin per day can reduce symptoms of rheumatoid arthritis such as pain and symptoms related with inflammation [35]. However, no definitive conclusion can be drawn due to the small number of randomized controlled trials included in the analysis and small sample sizes. Furthermore, an add-on therapy with curcumin capsules improved airway obstruction in bronchial asthma patients [36]. Due to the antiviral as well as anti-inflammatory effect of curcumin, the compound might have a positive effect on COVID-19 progression. A clinical trial registered in Iran currently investigates the effect of curcumin–piperine co-supplementation on clinical symptoms, duration, severity, and inflammatory factors in patients with COVID-19 (IRCT20121216011763N46). Moreover, another clinical trial from Iran is studying the effect of curcumin-containing nanocarriers on symptoms of COVID-19 and inflammatory markers (IRCT20200611047735N1).

Turmeric root is generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA) [37]. Furthermore, the FDA concluded that when used as a flavoring agent or ingredient of specific foods, levels of up to 20 mg curcumin per serving are safe [38].

The European Food Safety Authority (EFSA) panel concluded that evidence supports an acceptable daily intake (ADI) of 3 mg/kg bodyweight per day for curcumin [39]. A clinical trial indicated that curcumin is not toxic for humans when administered at doses ranging from 1 to 8 g/day for up to 3 months [40]. In addition, other human trials using 1–2 g/day also reported curcumin as safe [41]. When administered at high doses of up to 12 g/day, only mild side effects such as diarrhea, headache, rash, and yellow stool were reported [42].

The clinical use of curcumin is hindered by its poor bioavailability. Only 1% of curcumin is absorbed by the body, and after a half-life of approximately 8 h, it degrades into several ineffective products [43–46]. Methods, involving the use of nanoparticles, liposomes, micelles, and adjuvants should be used to enhance the bioavailability of curcumin [47,48]. For instance, the bioavailability of curcumin can be increased by 2000% when using piperine as an adjuvant [47]. Further studies are required to determine the dose needed to reach adequate serum and lung tissue concentrations sufficient for virus neutralization.

Taken together, we demonstrated that curcumin potently neutralizes SARS-CoV-2 in vitro at low subtoxic concentrations. The good safety profile of curcumin and its immunomodulatory as well as the antiviral effect make curcumin a promising candidate for complementary treatment of COVID-19. Clinical studies evaluating the benefit of curcumin treatment in COVID-19 patients are pending.

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**Conflicts of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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## 4.3 Immune responses in COVID-19 patients during breakthrough infection with SARS-CoV-2 variants Delta, Omicron-BA.1 and Omicron-BA.5

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2023

Contributions:

- Experimental work: 60%
- Data analysis: 80 %
- Statistical analysis: 80 %
- Writing the manuscript: 70 %
- Revising the manuscript: 70 %

Adalbert Krawczyk, Andreas Schönfeld, and Hana Rohn conceptualized the study. The patient samples were collected and processed by Hana Rohn, Maren Bormann, Leonie Brochhagen, Mira Alt, and Mona Otte. Experiments were performed by Maren Bormann, Leonie Brochhagen, Mira Alt, Mona Otte, Laura Thümmler, Lukas van de Sand, Ivana Kraiselburd, Alexander Thomas, and Peer Braß. The analysis, evaluation and visualization of the data was conducted by Maren Bormann in collaboration with Adalbert Krawczyk. The manuscript was prepared and revised by Maren Bormann and Adalbert Krawczyk. Ulf Dittmer, Oliver Witzke, Sebastian Dolff, Monika Lindemann, and Folker Meyer provided resources.

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# Immune responses in COVID-19 patients during breakthrough infection with SARS-CoV-2 variants Delta, Omicron-BA.1 and Omicron-BA.5

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**Background:** Breakthrough infections with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants are increasingly observed in vaccinated individuals. Immune responses towards SARS-CoV-2 variants, particularly Omicron-BA.5, are poorly understood. We investigated the humoral and cellular immune responses of hospitalized COVID-19 patients during Delta and Omicron infection waves.

**Methods:** The corresponding SARS-CoV-2 variant of the respective patients were identified by whole genome sequencing. Humoral immune responses were analyzed by ELISA and a cell culture-based neutralization assay against SARS-CoV-2 D614G isolate (wildtype), Alpha, Delta (AY.43) and Omicron (BA.1 and BA.5). Cellular immunity was evaluated with an IFN-γ ELISpot assay.

**Results:** On a cellular level, patients showed a minor IFN-γ response after stimulating PBMCs with mutated regions of SARS-CoV-2 variants. Neutralizing antibody titers against Omicron-BA.1 and especially BA.5 were strongly reduced. Double-vaccinated patients with Delta breakthrough infection showed a significantly increased neutralizing antibody response against Delta compared to double-vaccinated uninfected controls (median complete neutralization titer ($NT_{100}$) 640 versus 80, $p<0.05$). Omicron-BA.1 infection increased neutralization titers against BA.1 in double-vaccinated patients (median $NT_{100}$ of 160 in patients versus 20 in controls, $p=0.07$) and patients that received booster vaccination (median $NT_{100}$ of 50 in patients versus 20 in controls, $p=0.68$). For boosted

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patients with BA.5 breakthrough infection, we found no enhancing effect on humoral immunity against SARS-CoV-2 variants.

**Conclusion:** Neutralizing antibody titers against Omicron-BA.1 and especially BA.5 were strongly reduced in SARS-CoV-2 breakthrough infections. Delta and Omicron-BA.1 but not Omicron-BA.5 infections boosted the humoral immunity in double-vaccinated patients and patients with booster vaccination. Despite BA.5 breakthrough infection, those patients may still be vulnerable for reinfections with BA.5 or other newly emerging variants of concern.

KEYWORDS

SARS-CoV-2, breakthrough infections, Omicron, Delta, COVID-19

# 1 Introduction

Since the beginning of the coronavirus disease 2019 (COVID-19) pandemic, more than 700 million people worldwide have been infected with severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) and about seven million people have died as a result of COVID-19 (1). In an attempt to reduce the number of SARS-CoV-2 infections and severe COVID-19 cases, SARS-CoV-2 vaccines have been effectively deployed. The mRNA vaccines Comirnaty (BioNTech/Pfizer) and Spikevax (Moderna) have been administered most frequently in Germany, followed by Vaxzevria (AstraZeneca), Janssen (Johnson & Johnson) and Nuvaxovid (Novavax) (2). In particular, SARS-CoV-2 mRNA vaccines effectively protect against SARS-CoV-2 infection and severe COVID-19 (3, 4).

Throughout the COVID-19 pandemic, highly transmissible variants of concern (VOCs) have emerged, harboring multiple immune-escape mutations towards the available vaccines (5). By the end of 2021, the Omicron (B.1.1.529) variant displaced the Delta (B.1.617.2) variant as the leading VOC in Germany (6). Monoclonal antibodies as well as sera from vaccinated individuals are less effective in neutralizing Delta and Omicron compared to the D614G ancestral strain, with Omicron exhibiting the strongest immune evasiveness (7, 8). Despite the reduced neutralization capacity of vaccine-induced antibodies against these SARS-CoV-2 variants and the resulting increase of breakthrough infections among vaccinated individuals, most of the individuals with SARS-CoV-2 breakthrough infections were still protected against a lethal disease course (9–11). However, the humoral and cellular immune responses towards Omicron sub-variants BA.1 and in particular BA.5 are poorly understood.

In the present study, we assessed the humoral and cellular immune response in a group of patients hospitalized with SARS-CoV-2 breakthrough infection during Delta and Omicron infection waves. Our study sheds light on the extent of immune recall during breakthrough infection with Delta and Omicron-BA.1 and BA.5 in hospitalized patients and whether these infections provide a variant-specific immune boost or even cross-protective immunity.

# 2 Materials and methods

## 2.1 Study population

The study population consisted of 52 patients with a PCR-confirmed SARS-CoV-2 breakthrough infection hospitalized at the University Hospital Essen and a control group of 28 people without verified SARS-CoV-2 infection (Table 1). In total, 25 patients were infected with Delta, 15 with Omicron-BA.1 and 12 with Omicron-BA.5. The majority of Delta-infected patients were double vaccinated at the time of sample collection (88%). Patients with Omicron-BA.1 infection were predominantly double (53.3%) and triple (40%) vaccinated. All patients with Omicron-BA.5 infection was boosted, either with one booster dose (83.3%) or two booster doses (16.7%). Of the control group, 16 individuals were double vaccinated (57.1%), 10 were triple vaccinated (35.7%) and two were quadruple vaccinated (7.1%). Based on the definition of disease severity of COVID-19 by the World Health Organization (WHO), 42.2% of the patients had a non-severe course of COVID-19, 51.9% a severe course and 5.8% a non-severe course (12). Patient samples were collected from August 2021 to July 2022. Nasopharyngeal swabs and blood samples were collected to characterize the corresponding SARS-CoV-2 strain and the humoral and cellular immunity. Breakthrough infections were classified as Delta or Omicron based on sequencing information as well as information about infection waves from healthcare workers and patients at the University Hospital Essen (13).

The study was approved by the local ethics committee and was performed in accordance with the ethical standards noted in the 1964 Declaration of Helsinki and its later amendments or comparable ethics standards (approval no. 20-9665-BO). Informed consent was obtained from all participants in the study.

## 2.2 Cells and viruses

A549-AT cells were cultivated in minimum essential media (MEM), supplemented with 10% (v/v) fetal calf serum (FCS),

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TABLE 1 Overview of study cohort. Data indicate median (interquartile range) or absolute numbers (percentage).

| Characteristics | Patients with Delta breakthrough infection (N=25) | Patients with Omicron-BA.1 breakthrough infection (N=15) | Patients with Omicron-BA.5 breakthrough infection (N=12) | Uninfected controls (N=28) | p |
|---|---|---|---|---|---|
| Sex: | | | | | |
| Men (%) | 17 (68) | 10 (66.7) | 4 (33.3) | 14 (50) | n.s. |
| Women (%) | 8 (32) | 5 (33.3) | 8 (66.7) | 14 (50) | |
| Age: | | | | | |
| Total | 73 (51-82) | 60 (55-77) | 69 (62-79) | 53 (49-63) | C-BA.5 and C-D: p<0.01 |
| 2 doses of vaccine | 71 (49-83) | 58 (44-63) | N/A | 52 (48-64) | C-D: p=0.0457 |
| Booster vaccination | 71 (60-81) | 74 (55-82) | 69 (62-79) | 54 (50-61) | C-BA.1 and C-BA.5: p<0.01 |
| Vaccine: | | | | | |
| Comirnaty® (BioNTech/Pfizer) (%) | 23 (92) | 8 (53.3) | 6 (50) | 15 (53.6) | D-BA.1, D-BA.5 and C-D: p<0.01 |
| Spikevax® (Moderna) (%) | 1 (4) | 2 (13.3) | 0 | 12 (42.9) | C-BA.5 and C-D: p<0.01 |
| Janssen® (Johnson & Johnson) (%) | 1 (4) | 0 | 0 | 0 | n.s. |
| Combination (%) | 0 | 5 (33.3) | 5 (41.7) | 1 (3.6) | D-BA-1, D-BA-5 and C-BA.5: p<0.01; C-BA.1: p<0.05 |
| Unknown (%) | 0 | 0 | 1 (8.3) | 0 | n.s. |
| Vaccine doses: | | | | | |
| 1 (%) | 1 (4) | 1 (6.7) | 0 | 0 | n.s. |
| 2 (%) | 22 (88) | 8 (53.3) | 0 | 16 (57.1) | D-BA.1 and C-D: p<0.05; D-BA.5: p<0.0001; BA.1-BA.5: 0.01; C-BA.5: p<0.001 |
| 3 (%) | 2 (8) | 6 (40) | 10 (83.3) | 10 (35.7) | D-BA.1, BA.1-BA.5, C-D and C-BA.5: p<0.05; D-BA.5: p<0.0001 |
| 4 (%) | 0 | 0 | 2 (16.7) | 2 (7.1) | n.s. |
| Days since vaccination: | | | | | |
| Total | 149.5 (97-184.3) | 134.5 (66.25-192.5) | 184.5 (133-222.5) | 186 (45.75-199.5) | n.s. |
| Since 2nd vaccination | 160 (113-188) | 176 (90.5-229.3) | N/A | 54 (29-186) | D-C: p=0.0414 |
| Since booster | 96.5 (91-102) | 69 (46-140) | 184.5 (133-222.5) | 199 (192.3-208) | D-BA.5, BA.1-BA.5 and D-C: p<0.05; C-BA.1: p<0.01 |
| Unknown | 7 | 1 | 4 | 0 | |

Data indicate median (interquartile range) or absolute numbers (percentage).
Differences between groups for the categorical variables were analyzed by Fisher's exact test and for the continuous variables by two-tailed Mann-Whitney U test. N/A, not applicable; D, Delta; C, uninfected control; n.s., not significant.

penicillin (100 IU/mL) and streptomycin (100 µg/mL) at 37 °C in an atmosphere of 5% $CO_2$ (all Life Technologies Gibco, Darmstadt, Germany) (14). These cells overexpress both the carboxypeptidase angiotensin I converting enzyme 2 (ACE2) receptor and the cellular transmembrane protease serine 2 (TMPRSS2), allowing for high SARS-CoV-2 susceptibility and formation of cytopathic effects (CPEs).

Nasopharyngeal swabs from COVID-19 patients were used to isolate variants of SARS-CoV-2 (15, 16). In brief, the swab medium was incubated on A549-AT cells for several days until a profound CPE became apparent. Subsequently, supernatant was harvested, cleared from cell debris by centrifugation and stored at -80°. Viral titers were determined using A549-AT cells by a standard end-point

dilution assay and calculated as 50% tissue culture infective dose ($TCID_{50}$)/mL as previously described (17).

## 2.3 Sequencing and phylogenetic characterization

SARS-CoV-2 RNA of cell culture supernatants and nasopharyngeal swabs was purified using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). SARS-CoV-2 whole genome libraries were obtained with the EasySeq™ SARS-CoV-2 Whole Genome NGS Sequencing kit (Nimagen, Nijmegen, Netherlands) after cDNA generation from 5.5 µl of viral RNA with the LunaScript RT SuperMix Kit (NEB). Pooled and normalized libraries were sequenced on an Illumina MiSeq instrument employing the V2 chemistry (300 cycles).

Data analysis was conducted by the opensource pipeline UnCoVar (18). Briefly, UnCoVar performs a series of QC steps, initially attempts *de-novo* assembly with reference guided scaffolding to achieve full genome reconstruction. Alternatively, the genome of recalcitrant samples is generated via incorporation of observed mutations to the Wuhan reference genome using variants called with Freebayes (19), Delly (20) and Varlociraptor (21). The workflow subsequently uses Pangolin (22) for genome lineage calling and Kallisto (23) for read based matching to 24 (25).

After obtaining whole genome sequences, sub-sequences were extracted according to the observed genomic features of the Wuhan reference genomes. For the selected features, e.g., the spike (S) protein coding region, as well as for the whole genome, sequences were aligned [mafft] and phylogenetic trees were calculated [iqtree] to obtain the evolutionary correlations between the samples.

## 2.4 SARS-CoV-2 S and NCP ELISA

IgG antibodies against subunit 1 of the SARS-CoV-2 S protein (S1; Wuhan-Hu-1 isolate) and IgG and IgM antibodies against the nucleocapsid protein (NCP) were measured from patient sera with an enzyme-linked immunosorbent assay (ELISA) (Euroimmun Medizinische Labordiagnostika, Lübeck, Germany). A ratio between the absorbance of the sample and calibrator of <0.8 was regarded as negative, ≥0.8 to <1.1 borderline, and ≥1.1 positive.

## 2.5 Neutralization Assay on A549-AT cells

The neutralization capacity of serum samples against a SARS-CoV-2 clinical isolate from September 2020 with the D614G mutation (wildtype) as well as the variants Alpha (B.1.1.7), Delta (AY.43) and Omicron (BA.1 and BA.5) was analyzed. Additionally, the neutralizing capacity of sera from ten patients was investigated (patient 1, 6, 9, 10, 24, 27, 33, 45, 48, 52) towards their equivalent clinical isolate that caused the SARS-CoV-2 breakthrough infection in comparison to wildtype isolate.

Neutralization assays were conducted as described previously (26). Briefly, two-fold serial dilutions of patient sera (1:20 to 1:2560) were pre-incubated with 100 $TCID_{50}$/50 µL SARS-CoV-2 for one hour at 37 °C. These mixtures were added to A549-AT cells and incubated for three days at 37 °C and 5% $CO_2$. Cell cultures were stained with 0.5% crystal violet (w/v) (Roth, Karlsruhe, Germany), solved in 20% (v/v) methanol (Merck, Darmstadt, Germany) and evaluated for CPEs by transmitted light microscopy. The highest serum dilution at which none of the triplicate cultures displayed CPEs was defined as the complete neutralization titer ($NT_{100}$).

## 2.6 ELISpot Assay for SARS-CoV-2 S and NCP

An IFN-γ enzyme-linked-immuno-spot (ELISpot) assay was conducted to evaluate the cell-mediated immune response to SARS-CoV-2, as described before (27, 28). Plates equipped with polyvinylidene difluoride (PVDF) membranes (MilliporeSigma™ MultiScreen™ HTS, Fisher Scientific, Schwerte, Germany) were activated with ethanol. Subsequently, plates were coated with 60 µL monoclonal antibodies against IFN-γ (10 µg/mL of clone 1-D1K, Mabtech, Nacka, Sweden). After washing and blocking with 150 µL AIM-V® (Thermo Fisher Scientific, Grand Island, NY, USA) for 30 minutes at 37 °C, 250,000 peripheral blood mononuclear cells (PBMCs) in 150 µL of AIM-V® in the presence or absence of PepTivator® proteins (600 pmol/mL of each peptide, all Miltenyi Biotec, Bergisch Gladbach, Germany) were added. The NCP, S protein of Wuhan wildtype and selectively mutated regions of Alpha (B.1.1.7), Delta (AY.1) and Omicron (B.1.1.529) were incubated for 19 hours at 37 °C followed by washing. To detect captured IFN-γ, 50 µL alkaline phosphatase conjugated monoclonal antibody against IFN-γ (clone 7-B6-1, Mabtech) diluted 1:200 in PBS containing 0.5% bovine serum albumin (BSA) was incubated for one hour. Plates were washed again, and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate was added. Spots were quantified using an ELISpot reader (AID Fluorospot, Autoimmun Diagnostika GmbH, Strassberg, Germany). Non-stimulated values were subtracted from stimulated values to obtain the SARS-CoV-2 specific spots. A spot increment of three was considered positive.

## 2.7 Statistical analyses

Statistical analyses and data visualization were conducted using GraphPad Prism 9.4.0 (San Diego, CA, USA) software. For continuous variables, the median and interquartile range were calculated. Significant differences were assessed using Kruskal-Wallis test with post-hoc Dunn's test for multiple comparison, Mann-Whitney U test and Wilcoxon signed-rank test for analyses of more than two independent groups, two unpaired samples and two paired samples, respectively. Categorical variables were analyzed by Fisher's exact test. Correlation coefficients were calculated using Spearman's rank analysis. *P*-values <0.05 were considered significant.

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# 3 Results

## 3.1 Sequencing and phylogenetic analysis of SARS-CoV-2 variants causing breakthrough infections

At the time of sample collection, all study participants had received at least one vaccine dose. Of the control group, 57.1% individuals were double-vaccinated, 35.7% were triple-vaccinated and 7.1% were quadruple-vaccinated. 88% of Delta-infected patients were double vaccinated. Of the patients with Omicron-BA.1 infection, 53.3% were double and 40% triple vaccinated. Omicron-BA.5 infected patients were all boosted with either one booster dose (83.3%) or two booster doses (16.7%).

Clinical isolates of hospitalized patients with SARS-CoV-2 breakthrough infection were sequenced by whole genome sequencing (Figure 1). S region sequences were successfully assembled from 18 patients. These patients were infected with Delta (B.1.617.2) and Omicron sub-lineages BA.1 and BA.5 (Figure 2). The remaining patients were classified based on information about infection waves from healthcare workers and patients at the University Hospital Essen (13). The phylogenetic analysis highlights the continuous evolution of SARS-CoV-2, which poses a challenge for vaccine development.

## 3.2 SARS-CoV-2 binding serum antibody levels

Sera of patients with SARS-CoV-2 breakthrough infection were tested for SARS-CoV-2 subunit 1 (S1) specific IgG antibodies and IgG and IgM antibodies against the nucleocapsid protein (NCP) by an enzyme-linked immunosorbent assay (ELISA) (Figure 1). Overall, 91.7% of samples were positive for S1 specific antibodies (Figure 3A). Next, we measured IgM and IgG antibody levels against NCP of SARS-CoV-2 to distinguish between the early and late humoral responses during infection. Antibody levels against the NCP were significantly lower compared to S1 ($p<0.0001$) (Figure 3A). In total, 20.8% of patient sera were positive for IgM antibodies and 29.2% for IgG antibodies. When dividing patients by breakthrough infection and number of vaccines, there were no significant differences in S1 and NCP IgG levels between groups (Figure 3B). However, patients with Delta breakthrough infection who received two vaccine doses had significantly higher levels of NCP IgM compared to patients with booster vaccination and Omicron BA.1 infection ($p<0.05$) as well as patients with booster vaccination and Omicron BA.5 infection ($p<0.01$).

## 3.3 Neutralizing antibody titers in sera after Delta, BA.1 or BA.5 breakthrough infection

The humoral immunity of COVID-19 patients with SARS-CoV-2 breakthrough infections was further investigated using a cell culture-based neutralization assay. Serum samples from those patients were tested against a SARS-CoV-2 D614G wildtype clinical isolate and Alpha (B.1.1.7), Delta (AY.43) and the Omicron sub-lineages BA.1 and BA.5. Sera from COVID-19 patients as well as sera from non-infected but immunized individuals showed reduced complete neutralization titers ($NT_{100}$) towards BA.1 and BA.5 compared to wildtype, Alpha and Delta (Figure 4A).

Double-vaccinated patients with Delta breakthrough infection displayed a significantly increased neutralizing antibody response against Delta compared to double-vaccinated uninfected controls (median $NT_{100}$ 640 versus 80, $p<0.05$, Figure 4B). In double-vaccinated patients, infection with Omicron sub-lineage BA.1

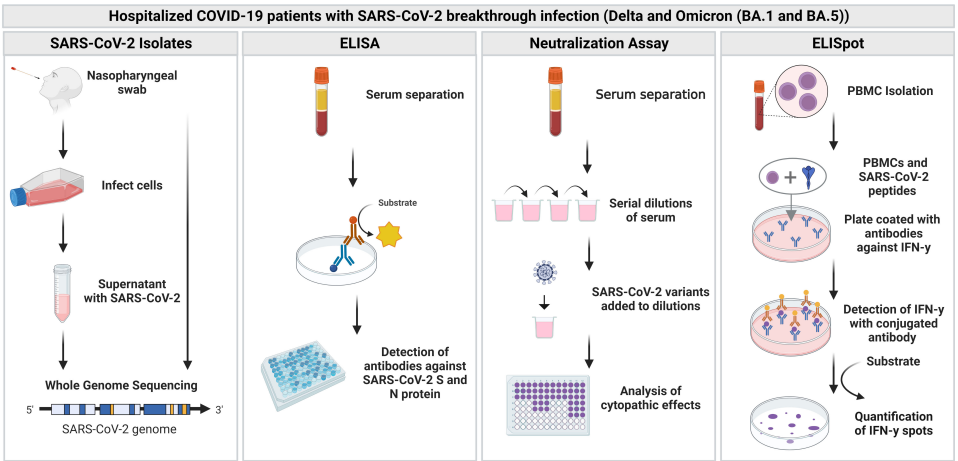


FIGURE 1
Overview of the study. Blood samples and nasopharyngeal swabs were collected from hospitalized patients with SARS-CoV-2 breakthrough infection (Delta and Omicron). Blood samples were further analyzed with an enzyme-linked immunosorbent assay (ELISA), neutralization assay as well as enzyme-linked-immuno-spot (ELISpot) assay. Figure was created with BioRender.com.

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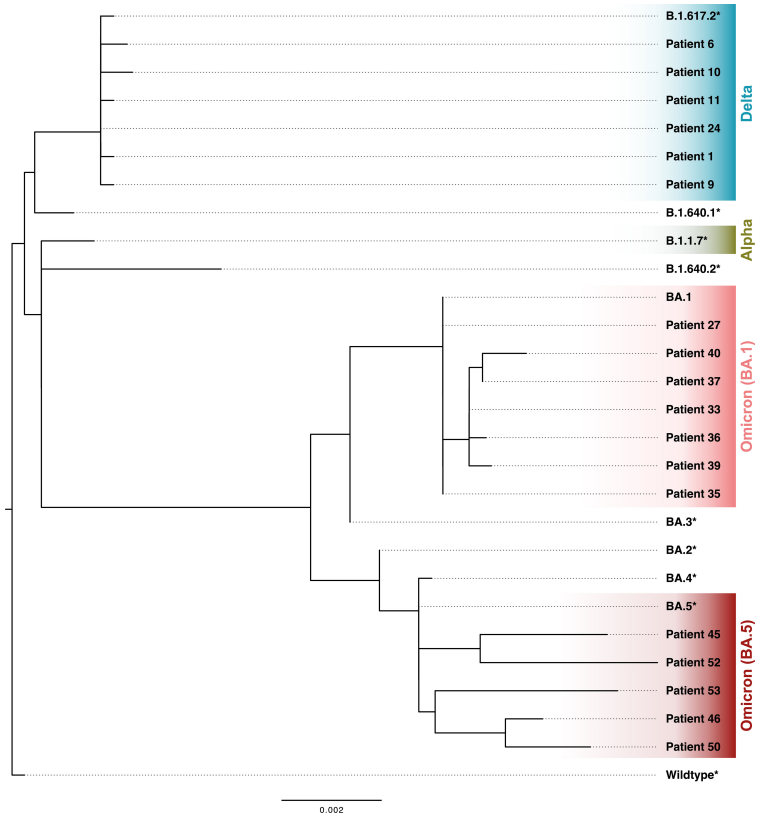


FIGURE 2
Phylogenetic tree of assembled SARS-CoV-2 spike (S) region sequences of clinical isolates of patients with SARS-CoV-2 breakthrough infection. * S region extracted from GISAID reference genomes (GISAID).

boosted immunity against BA.1 just above statistical significance (median $NT_{100}$ of 160 in patients versus 20 in controls, p=0.07) as well as against BA.5 (median $NT_{100}$ of 40 in patients versus <20 in controls, p<0.05) (Figure 4B). A higher median $NT_{100}$ against BA.1 was also observed for boosted Omicron-BA.1 infected patient compared to boosted controls (median $NT_{100}$ of 50 versus 20, p=0.68). Interestingly, results suggest cross-reactive immunity for patients with Omicron-BA.1 infection against Delta, as double-vaccinated had a 6-fold (median $NT_{100}$ of 480 versus 80, p=0.24) and boosted a 4-fold (median $NT_{100}$ of 320 versus 80, p=0.44) higher $NT_{100}$ than control. For individuals with BA.5 infection, we observed no immune boost against BA.5 or other variants.

Next, we investigated neutralization capacity of patient sera against the SARS-CoV-2 clinical isolate from these respective patients compared to wildtype. In total, ten different SARS-CoV-2 isolates from patients infected with sub-lineages of Delta and Omicron could be propagated in cell culture to investigate the respective neutralizing antibody titers. Patients infected with Delta showed similar neutralization efficacy against their isolate compared to wildtype (Figure 4C). In contrast, we found reduced neutralization capacity against isolates from Omicron-infected patients in comparison to wildtype (median $NT_{100}$ of 7.3 versus 80, p=0.25).

In summary, we found that Delta infections exhibit a strong immune boosting effect against the Delta variant. Patients infected with BA.1 showed an increased neutralizing antibody response against both tested Omicron variants. Compared to Delta and BA.1, BA.5 was the least immunogenic variant, as BA.5 infections did not boost immunity against BA.5 or other variants.

## 3.4 Cellular immunity in patients with SARS-CoV-2 breakthrough infection

Cellular immunity against SARS-CoV-2 was measured using an IFN-γ enzyme-linked-immuno-spot (ELISpot) assay. We stimulated peripheral blood mononuclear cells (PBMCs) with the NCP, spike (S) protein of Wuhan wildtype and with selectively mutated regions of Alpha (B.1.1.7), Delta (AY.1) and Omicron (B.1.1.529). An IFN-γ-spots increment of three was considered positive.

Double-vaccinated patients with Delta infection showed the highest positivity in response to NCP stimulation, followed by boosted BA.5-infected patients (56.2% and 33.3%, respectively, Figure 5). As expected, infection-naïve participants did not show a positive NCP response. Among dually vaccinated patients, the

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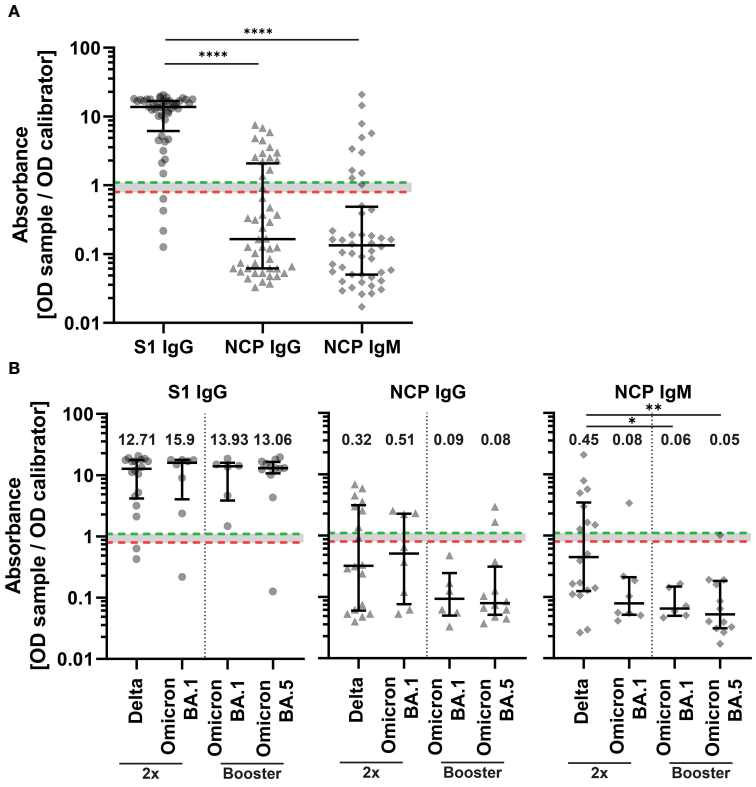


FIGURE 3
Binding serum antibody levels in COVID-19 patients with SARS-CoV-2 breakthrough infection. IgG antibodies against the subunit 1 of spike protein (S1) (Wuhan-Hu-1 isolate) and IgG and IgM antibodies against the nucleocapsid protein (NCP) of all patients (n=48) **(A)** and double vaccinated patients (2x) with Delta (n=18) and BA.1 (n=8) infection and patients with booster vaccination with BA.1 (n=6) and BA.5 (n=12) infection **(B)**. Binding serum antibodies were measured with an enzyme-linked immunosorbent assay (ELISA). An absorbance of <0.8 was regarded as negative (red dotted line), ≥0.8 to <1.1 borderline, and ≥1.1 positive (green dotted line). Differences between groups were analyzed by Kruskal-Wallis test with post-hoc Dunn's multiple comparisons test (* p<0.05; ** p<0.01, **** $p$<0.0001). Horizontal lines indicate median values, while error bars represent the interquartile range.

IFN-γ spots increment was significantly higher for Delta-infected patients than for patients infected with BA.1 (31 versus 5.5, p<0.05) after stimulation with wildtype S protein. A significantly higher response to S wildtype was also observed for BA.5-infected patients compared to double-vaccinated BA.1-infected patients (42.5 versus 5.5, p<0.05). All groups showed a median IFN-γ spots increment below positivity to mutated regions of SARS-CoV-2 variants (Figure 5).

### 3.5 Correlation between SARS-CoV-2 ELISA IgG antibody levels and neutralizing antibody titers as well as cellular IFN-γ response

Next, we analyzed if there is a correlation between neutralizing antibody titers of the respective sera against SARS-CoV-2 wildtype, Alpha, Delta and Omicron (BA.1 and BA.5) and SARS-CoV-2 ELISA IgG antibody levels against S1 (Wuhan-Hu-1 isolate). The neutralizing antibody titers correlated positively with ELISA IgG antibody levels (Figure 6). The highest correlation was observed for neutralizing antibody titers against wildtype and Alpha (Spearman's ρ=0.9, respectively). Compared to wildtype and Alpha, we observed a lower correlation for Delta, Omicron-BA-1 and Omicron-BA.5, with Spearman's rank coefficients of 0.82, 0.79 and 0.72, respectively. Next, we analyzed the correlation between IgG antibodies against S1 and cellular IFN-γ production in response to stimulation with SARS-CoV-2 variants. The results only revealed a correlation between SARS-CoV-2 ELISA IgG antibody levels and the cellular immune response against wildtype (ρ=0.41), but not SARS-CoV-2 variants (Figure 7).

## 4 Discussion

Throughout the COVID-19 pandemic, several SARS-CoV-2 variants with immune-escape mutations have emerged, leading to an increase of SARS-CoV-2 breakthrough infections (5). In the present study, we report on the humoral and cellular immunity in response to Delta and Omicron (BA.1 and BA.5) infection in a

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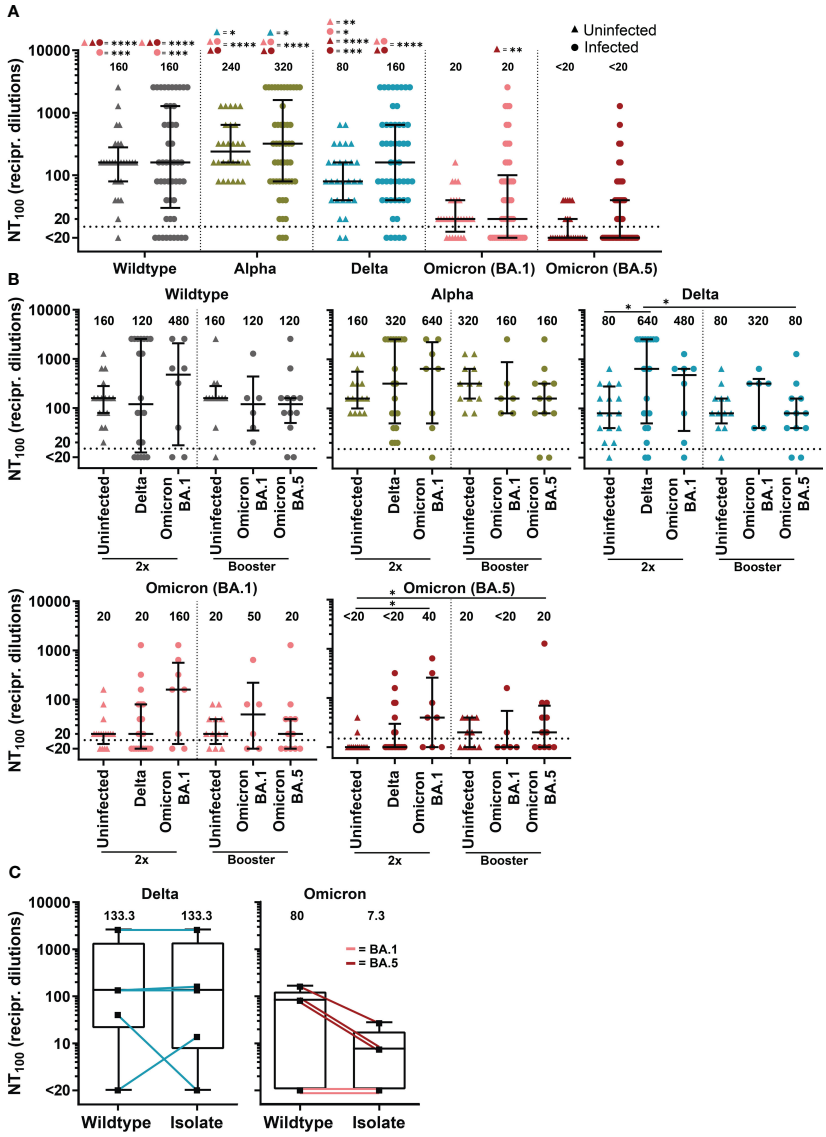


FIGURE 4
Neutralizing antibody titers against SARS-CoV-2 variants of COVID-19 patients with SARS-CoV-2 breakthrough infection and uninfected, vaccinated controls. **(A)** Complete neutralization titer ($NT_{100}$) against clinical isolate with D614G mutation (wildtype), Alpha (B.1.1.7), Delta (AY.43) and Omicron (BA.1 and BA.5) of patients with SARS-CoV-2 breakthrough infection (n=50) compared to vaccinated uninfected control (n=28). **(B)** $NT_{100}$ against clinical isolate with D614G mutation (wildtype), Alpha (B.1.1.7), Delta (AY.43) and Omicron (BA.1 and BA.5) of double vaccinated patients (2x) with Delta (n=20) and BA.1 (n=8) infection and patients with booster vaccination with BA.1 (n=6) and BA.5 (n=12) infection compared to uninfected control with two vaccine doses (n=16) and booster vaccination (n=12). **(A, B)** Differences between groups were analyzed by Kruskal-Wallis test with post-hoc Dunn's multiple comparisons test (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$). **(C)** $NT_{100}$ of sera from patients with breakthrough infection with Delta and Omicron (BA.1 and BA.5) against their equivalent clinical isolate compared to wildtype. Differences between groups were analyzed by Wilcoxon signed-rank test. **(A–C)** Horizontal lines indicate median values, while error bars represent the interquartile range.

group of vaccinated patients with SARS-CoV-2 breakthrough infections. We compared the results to vaccinated uninfected controls, to assess the additive effect of the infection on immunity.

Of note, the neutralizing antibody titers against Omicron sub-variants BA.1 and especially BA.5 were strongly reduced when compared to Alpha, Delta or wildtype. These findings are consistent with recently published data using pseudovirus-neutralization assays, showing a substantial immune escape of BA.5 sub-variant against antibodies of vaccinated individuals or individuals infected with BA.1 or BA.2 (29–31).

In line with recent studies, we showed that Delta and Omicron-BA.1 infections lead to a strain-specific boost of neutralizing immunity (32, 33). Previous data indicated that Delta breakthrough infections increase Delta specific neutralization titers to levels comparable to

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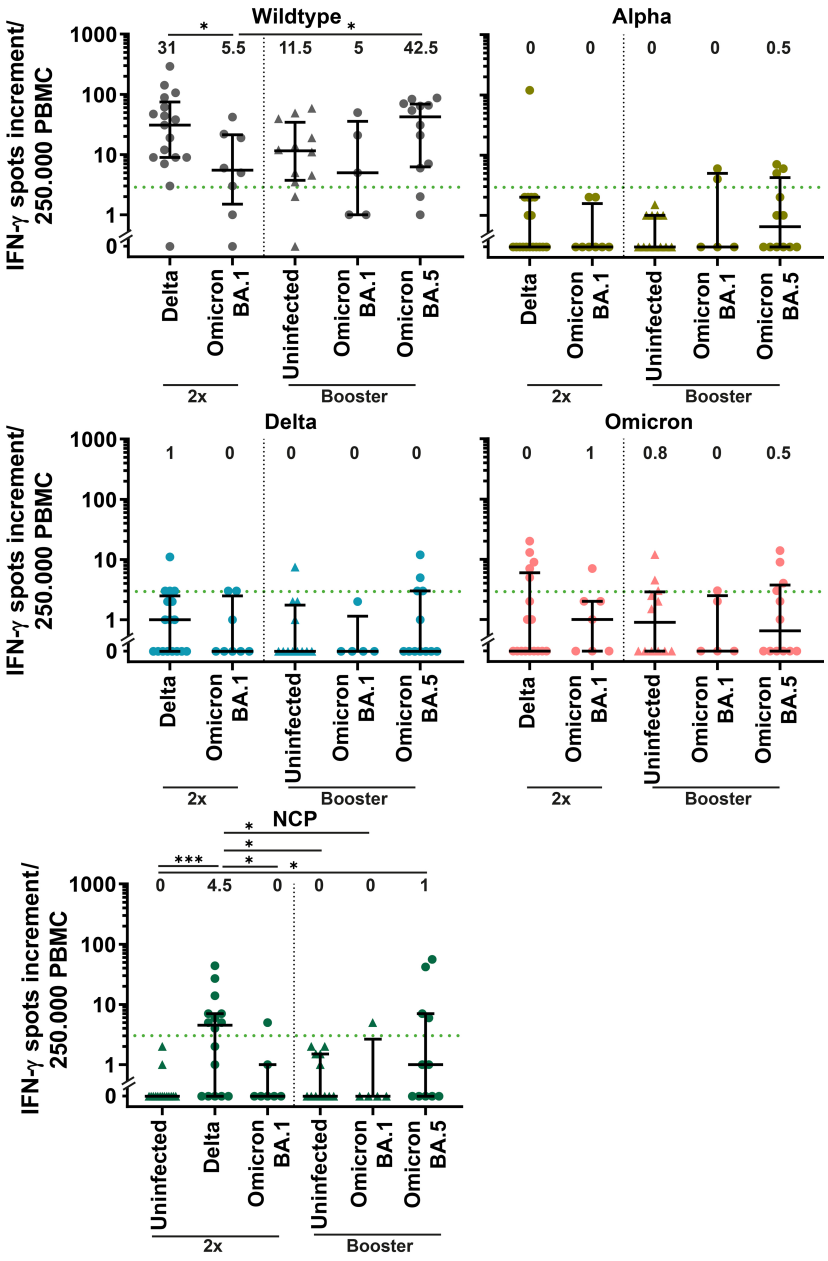


FIGURE 5
Cellular response against SARS-CoV-2 variants in COVID-19 patients with SARS-CoV-2 breakthrough infection. Cellular immunity was assessed by an IFN-γ enzyme-linked-immuno-spot (ELISpot) assay using peripheral blood mononuclear cells (PBMCs) and is displayed for double vaccinated patients (2x) with Delta (n=18) and BA.1 (n=8) infection and patients with booster vaccination with BA.1 (n=5) and BA.5 (n=12) infection compared to uninfected control with two vaccine doses (n=15) and booster vaccination (n=12). PBMCs were stimulated with S protein of Wuhan wildtype, nucleocapsid protein (NCP) and selectively mutated regions Alpha (B.1.1.7), Delta (AY.1) and Omicron (B.1.1.529). A spot increment of three was considered positive (green dotted line). Differences between groups were analyzed by Kruskal-Wallis test with post-hoc Dunn's multiple comparisons test (* $p<0.05$; *** $p<0.001$). Horizontal lines indicate median values, while error bars represent the interquartile range.

wildtype neutralization (32). In our study, Delta infection markedly increased neutralizing antibody titers against Delta in double-vaccinated patients, even with a 5.3-fold higher neutralizing antibody titer against Delta compared to wildtype. Omicron-BA.1 breakthrough infection enhanced the neutralizing antibody titer against BA.1 and Delta (33). Notably, the neutralizing antibody titer of sera from uninfected controls was 8-fold reduced against BA.1 when compared to wildtype. In contrast, in double-vaccinated patients with BA.1 infection the ratio between BA.1 and wildtype neutralizing antibody titers reduced to 3 and in boosted patients to 2.4.

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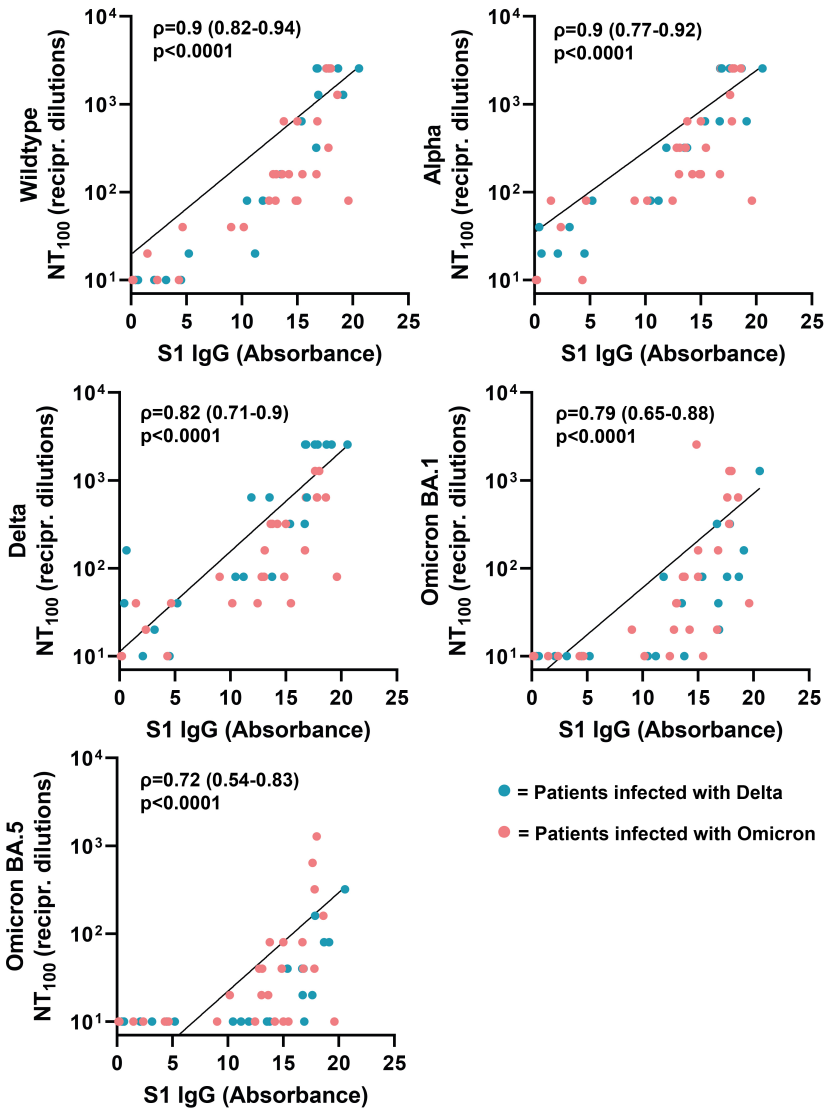


FIGURE 6
Correlation between serum antibody levels and neutralizing antibody titers against SARS-CoV-2 variants. Correlation coefficients (ρ) and *p*-values were calculated using Spearman's rank analysis.

Additionally, our study provides insight into the immunity in BA.5 breakthrough infections. We found no evidence of a boosting effect on humoral immunity for this sub-variant, which could increase the likelihood of reinfections in people who have recovered from BA.5 infection. Our results suggest that BA.5 sub-variant is capable not only of bypassing humoral immunity boosted by SARS-CoV-2 infection, but also leads to a weak enhancement of humoral immunity itself. In contrast to our data, recent data indicated an enhanced neutralization against BA.5 following BA.5 infection in triple-vaccinated individuals (34). In the study by Wang et al. (34), serum samples were collected from already recovered patients at a mean of 32 days after infection, whereas in our study the sera were collected during the acute phase at hospitalization.

Interestingly, we found a weak IFN-γ response after stimulating PBMCs with selectively mutated regions of SARS-CoV-2 variants. One reason could be that the participants were still early in the infection and a measurable T-cell immunity against the mutated regions had not yet developed. Overall, all patient groups had a high positivity after cellular stimulation with S protein of Wuhan wildtype, regardless of vaccination status and variant responsible for breakthrough infection. PBMCs of patients with Omicron-BA.5 breakthrough infection showed the strongest IFN-γ response against Wuhan wildtype, followed by patients with Delta infection.

One limitation of this study are differences between cohorts regarding to demographic characteristics. For instance, among the BA.1-infected patients, 87.5% were under 70 years of age in the

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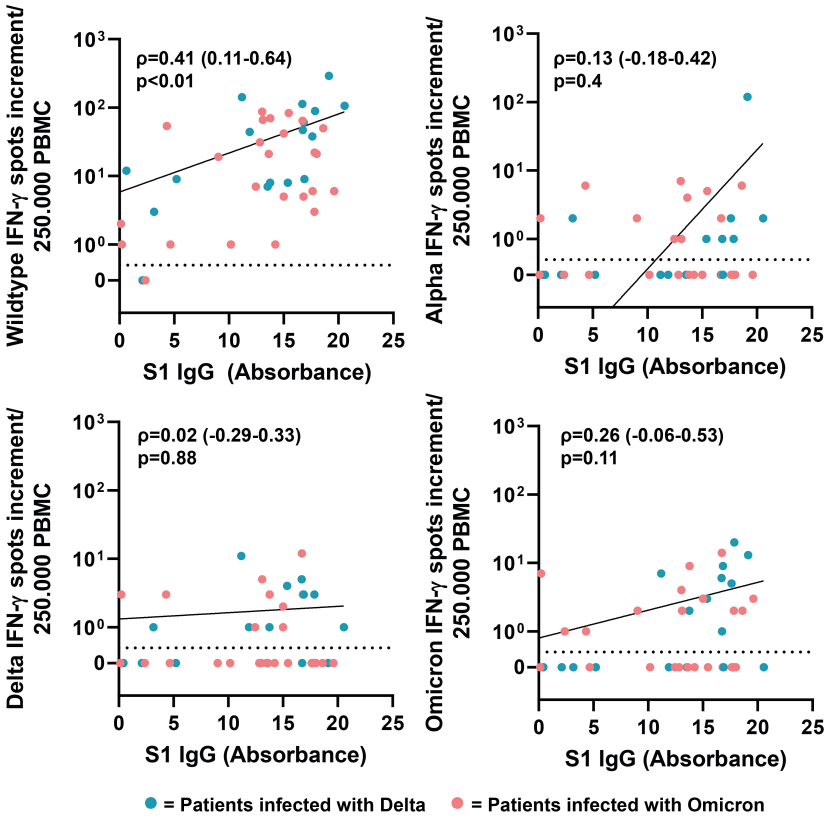


FIGURE 7
Correlation between serum antibody levels and cellular IFN-γ response after stimulation with SARS-CoV-2 variants. Correlation coefficients (ρ) and *p*-values were calculated using Spearman's rank analysis.

dually vaccinated group compared to 33.3% of boosted patients. That might be an explanation for the weaker humoral immune enhancement through BA-1 infection we observed in the group with booster vaccination. For instance, data has shown a reduced antibody neutralization response for elderly above 70 years after vaccination or infection (35, 36). Furthermore, the uninfected control group received a higher percentage of Spikevax (Moderna) vaccines than the patient groups, which could have influenced the results. However, studies found a similar high neutralization potential for individuals vaccinated with Spikevax (Moderna), Comirnaty (BioNTech/Pfizer) and a combination of vaccines (37).

In conclusion, we found strongly reduced neutralizing antibody titers against Omicron sub-variants BA.1 and BA.5. Furthermore, humoral immunity was boosted through Delta and Omicron-BA.1 infections in hospitalized double-vaccinated patients and patients with booster vaccination. This finding does not apply to BA.5 infections, in which we found no enhancing effect on humoral immunity. Despite BA.5 breakthrough infection, those patients may still be vulnerable for reinfections with BA.5 or other newly emerging SARS-CoV-2 variants. Further studies are needed to investigate the humoral and cellular immune response after breakthrough infection with BA.5 and its role in protecting from subsequent breakthrough infections.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: PRJEB59607 (ENA; https://www.ebi.ac.uk/ena/browser/view/PRJEB59607).

## Ethics statement

The studies involving human participants were reviewed and approved by Ethik-Kommission der Medizinischen Fakultät der Universität Duisburg-Essen. The patients/participants provided their written informed consent to participate in this study.

## Author contributions

MB, LB, MA, MO, LT, LS, IK, AT, JG, PB, SC and MW performed the experiments. MB, LB, MA and MO were involved in sample collection. MB, AT, SD, UD, OW, FM and ML analyzed the data. AK, AS, HR and MB planned the study. AK and MB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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## 4.4 SARS-CoV-2-specific humoral and cellular immunity in two renal transplants and two hemodialysis patients treated with convalescent plasma

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2021

Contributions:

- Experimental work: 20 %

Monika Lindemann, Adalbert Krawczyk, Veronika Lenz, Ulf Dittmer, Peter A. Horn, and Oliver Witzke conceived and designed the study. Experiments were performed by Laura Thümmler, Sina Schwarzkopf, Leonie Brochhagen (née Schipper), Maren Bormann and Lukas van de Sand. Sebastian Dolff, Margarethe Konik, Hana Rohn, Maximillian Platte, Marianne Breyer, Hannes Klump, Dietmar Knop, Veronika Lenz, Christian Temme, Peter A. Horn, and Oliver Witzke took care of the patients or convalescent collected samples and interpreted the data. Monika Lindemann, Adalbert Krawczyk, Sebastian Dolff, Peter A. Horn, and Oliver Witzke wrote the manuscript.

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RESEARCH ARTICLE

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# SARS-CoV-2-specific humoral and cellular immunity in two renal transplants and two hemodialysis patients treated with convalescent plasma

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## Abstract

When patients with chronic kidney disease are infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) they can face two specific problems: virus-specific immune responses may be impaired and remdesivir, an antiviral drug described to shorten recovery, is contraindicated. Antiviral treatment with convalescent plasma (CP) could be an alternative treatment option. In this case report, we present two kidney transplant recipients and two hemodialysis patients who were infected with SARS-CoV-2 and received CP. Antibodies against the receptor-binding domain in the S1 subunit of the SARS-CoV-2 spike protein were determined sequentially by immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) and neutralization assay and specific cellular responses by interferon-gamma ELISpot. Before treatment, in both kidney transplant recipients and one hemodialysis patient antibodies were undetectable by ELISA (ratio < 1.1), corresponding to low neutralizing antibody titers (≤1:40). ELISpot responses in the four patients were either weak or absent. After CP treatment, we observed an increase of SARS-CoV-2-specific antibodies (IgG ratio and neutralization titer) and of specific cellular responses. After intermittent clinical improvement, one kidney transplant recipient again developed typical symptoms on Day 12 after treatment and received a second cycle of CP treatment. Altogether, three patients clinically improved and could be discharged from the hospital. However, one 83-year-old multimorbid patient deceased. Our data suggest that the success of CP therapy may only be temporary in patients with chronic kidney disease; which requires close monitoring of viral load and antiviral immunity and possibly an adaptation of the treatment regimen.

KEYWORDS
cellular immunity, convalescent plasma, COVID-19, ELISpot, hemodialysis, kidney transplantation

## 1 | INTRODUCTION

In patients with chronic kidney disease and infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) treatment can be complicated because their immune function is suppressed due to medication to prevent allograft rejection and/or the underlying kidney disease. Thereby, the formation of specific antibodies and of T-cell immunity is impaired; which can result in a prolonged persistence of SARS-CoV-2 (for up to 2 months[1]). Furthermore, remdesivir, an antiviral nucleoside analog that shortened the time to recovery in adults hospitalized with coronavirus 2019 (COVID-19) disease,[2] is contraindicated in this special cohort. Antiviral treatment with convalescent plasma (CP) could be an alternative treatment option. Data on patients with chronic kidney disease infected with SARS-CoV-2 and receiving CP treatment are still limited. We are aware of only 14 described kidney transplant recipients who received CP.[3–7] Whereas clinical improvement after CP has been shown for all six kidney transplant recipients included in three studies,[3–5] in the fourth study[6] a mortality rate for solid organ recipients (including six with kidney allograft) in the range of recipients without CP treatment[8–10] was reported (23%[6] vs. 24%–32%,[8–10] respectively). In the fifth study describing HIV-infected kidney transplant recipients[7] one of the two patients died after having received CP treatment. However, the previous reports did not present data on the course of SARS-CoV-2-specific antibodies or cellular responses in the patients.

It was the aim of the current study to follow-up up virus-specific humoral and cellular immunity in patients with chronic kidney disease who were infected with SARS-CoV-2 and received CP therapy. We functionally analyzed the antibodies (by neutralization assay) and measured specific cellular responses by the highly sensitive ELISpot method, using various protein antigens of SARS-CoV-2 as specific stimuli. Finally, in one transplant recipient who again developed typical COVID-19 symptoms after initial clinical improvement, we had the chance to modify the treatment regimen and to apply the second cycle of CP therapy.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients and blood donors

The current case report includes two renal transplant recipients and two hemodialysis patients (Table 1) and their respective CP donors. Within the study period (July 27 to September 9, 2020), all SARS-CoV-2 infected renal transplant and hemodialysis patients with an estimated glomerular filtration rate (eGFR) < 30 ml/min/1.73 $m^2$ were included. The four patients included in the current study had chronic kidney disease according to the eGFR of 7–29 ml/min/1.73 $m^2$. Both transplant recipients received tacrolimus, mycophenolate mofetil, and prednisone, both hemodialysis patients dexamethasone. The kidney transplant recipients were treated with prednisone to prevent organ rejection (which was not changed due to COVID-19 infection), whereas the dialysis-requiring patients were specifically treated with dexamethasone for 5 days to prevent an exaggerated immune response during COVID-19 infection. Treatment with CP started when patients with chronic kidney disease without detectable immunoglobulin G (IgG) antibodies against SARS-CoV-2 showed increasing oxygen demand/clinical deterioration (RTX01, RTX02, and HD01) or when oxygen supply via nasal cannula was no longer sufficient in a patient with chronic kidney disease with detectable antibodies (HD02). One patient suffered from moderate (RTX02) and three from severe COVID-19 disease.[11] More detailed information on the patients and the therapy used can be found in Table 1. One cycle of CP consisted of three units, separated with the Amicus™ (Fresenius Kabi), each containing 200–280 ml, which was applied at Days 1, 3, and 5. The study was approved by the local ethics committee (20-9256-BO for the patients and 20-9225-BO for the donors) and the study participants provided written informed consent. The procedures were in accordance with the institutional and national ethical standards as well as with the Helsinki Declaration of 1975, as revised in 2013. Four donors were selected based on their SARS-CoV-2 IgG ratio after polymerase chain reaction (PCR)-confirmed SARS-CoV-2 infection and additional parameters like blood group and weight (Table 2). Details on the donor selection criteria have been described recently.[12]

### 2.2 | Antibody enzyme-linked immunosorbent assay

To assess SARS-CoV-2-specific humoral immunity, IgG antibodies in donor and patient sera were determined by a CE marked anti-SARS-CoV-2 IgG semi-quantitative enzyme-linked immunosorbent assay (ELISA; Euroimmun), according to the manufacturer's instructions. The ELISA plates were coated with recombinant SARS-CoV-2 spike (S) 1 protein (receptor binding domain). Serum samples were analyzed automatically at a 1:100 dilution, using the Immunomat™ (Virion\Serion). Results are given as a ratio (patient sample/control sample). An antibody ratio of ≥1.1 was considered positive, of ≥0.8 to <1.1 borderlines and of <0.8 negative.

**TABLE 1** Clinical characteristics of patients with chronic kidney disease

| ID | Sex/age/ blood group | CP interval[a] (days) | CP units/ cycles | (Pre-existing) comorbidity/cause of death | COVID-19 therapy | Severity of COVID-19 disease/ outcome (discharge from hospital[b]) |
|---|---|---|---|---|---|---|
| RTX01 | F/63/O | 3 | 6/2 | RTX 1997 and 2001, chronic antibody-mediated rejection, hypertension, asthma bronchial, reactive arthritis | Oxygen administration via nasal cannula | Severe[c]/A (d28) |
| RTX02 | F/62/A | 13 | 3/1 | RTX 14.08.2020 (13 days before SARS-CoV-2 infection), steroid-induced diabetes, hypertension | No oxygen necessary (minimum oxygen saturation 92%) | Moderate (CT: pneumonia, but clinically asymptomatic)/A (d16) |
| HD01 | F/83/A | 4 | 2[d]/1 | HD since 02/2012, type II diabetes, coronary heart disease, atrial fibrillation, apoplexy, acute event of fall (no evidence of stroke)/COVID-19 pneumonia | Oxygen administration first via nasal cannula, then 50–60 L/min high-flow ventilation ($FiO_2$ 60%), dexamethasone | Severe[c]/D |
| HD02 | F/78/O | 7 | 3/1 | HD since 01/2020, type II diabetes, hypertension, chronic obstructive pulmonary disease, adipositas | Oxygen administration via nasal cannula, dexamethasone | Severe[c]/A (d8) |

Abbreviations: A, alive; COVID, coronavirus; CP, convalescent plasma; CT, computed tomography; D, deceased; HD, hemodialysis; RTX, renal transplantation; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

[a]Interval between the onset of symptoms or positivity to SARS-CoV-2 polymerase chain reaction and treatment with CP.

[b]Discharge from hospital given as days after initiation of CP treatment.

[c]Oxygen supplementation but no mechanical ventilation.

[d]The patient deceased due to COVID-19 pneumonia after having received two CP units.

**TABLE 2** Characteristics of convalescent plasma donors

| ID | Sex | Age | Blood group | Antibody ratio | Neutralizing antibody titer | HLA antibodies |
|---|---|---|---|---|---|---|
| D-RTX01 | F | 55 | O | 5.83 | 1:1280 | neg |
| D-RTX02 | M | 53 | A | 7.33 | 1:320 | neg |
| D-HD01 | M | 40 | A | 10.44 | 1:160 | neg |
| D-HD02 | F | 48 | O | 3.39 | 1:320 | neg |

Abbreviations: D, donor; neg, negative.

### 2.3 | Virus neutralization assay

The function of specific antibodies was measured by a cell-culture based neutralization assay, using Vero E6 cells (ATCC® CRL-1586™) and a clinical isolate of SARS-CoV-2 in a biosafety level 3 laboratory.[12,13] Neutralization capacity was determined by endpoint dilution assay, expressed as 50% tissue culture infective dose ($TCID_{50}$)/ml. Serial dilutions (1:20 to 1:1280) of the respective sera were preincubated with 100 $TCID_{50}$ of SARS-CoV-2 for 1 h at 37°C and added afterward to confluent Vero E6 cells cultured in 96-well microtiter plates. On Day 3 after infection, the cells were stained with crystal violet (Roth) solved in 20% methanol (Merck) and the appearance of cytopathic effects (CPE) was analyzed by light microscopy. The neutralizing titer was defined as the reciprocal of the highest serum dilution at which no CPE breakthrough in any of the triplicate cultures was observed.

### 2.4 | ELISpot assay

To assess SARS-CoV-2-specific cellular immunity, we performed ELISpot assays, using peptide pools of the S1/S2 protein, the S1 protein, and the membrane (M) protein (PepTivator®, Miltenyi Biotec) and an S1 protein antigen of SARS-CoV-2 (Sino Biological). The peptide pools consist mainly of 15-mer sequences with 11 amino acids overlap. We tested 250,000 peripheral blood mononuclear cells per cell culture and measured interferon-gamma (IFN-γ) production after 19 h, as published recently in detail.[12] Spot numbers were analyzed by an ELISpot reader (AID Fluorospot; Autoimmun Diagnostika GmbH). Mean values of duplicate cell cultures were considered. SARS-CoV-2-specific spots were determined as stimulated minus nonstimulated (background) values (spots increment). We defined threefold higher SARS-CoV-2-specific spots versus background together with at least three spots above background as a positive response. This cut-off was set based on negative control values as specified previously.[12]

## 3 | RESULTS

In both kidney recipients and one hemodialysis patient with undetectable SARS-CoV-2-specific IgG (ratio < 1.1) and low neutralizing antibody titers (≤ 1:40; RTX01, RTX02, and HD01; Table 1) we observed an increase of antibody titers (Figure 1A–C). A 63-year-old female who was transplanted twice (RTX01) initially showed a clinical response to CP therapy, but at Day 12 again developed typical symptoms of COVID-19 disease (fever and shortage of air; Figure 2A,B). Therefore, she received another cycle of CP therapy (from the same donor). SARS-CoV-2 antibodies increased after both CP cycles and SARS-CoV-2 viral load decreased ($C_t$ value to the PCR increased from 17.8 to 25.8 after the first and to 34.9 after the second CP cycle; Figure 2C). The patient could be discharged from the hospital on Day 28 after initiation of CP treatment. Since Day 13 after initiation of CP therapy oxygen supplementation via nasal cannula could be completely stopped and at Day 29 viral load became undetectable. The second kidney transplant recipient (RTX02), a 62-year-old female who received her graft 13 days before the detection of SARS-CoV-2 infection, also showed a decrease of the viral load ($C_t$ value to the PCR increased from 21.6 to 30.3 after the CP cycle and to 35.4 on Day 16 after initiation of CP treatment, when the patient was discharged from the hospital). On Day 39 after CP therapy, SARS-CoV-2 viral load became undetectable in the nasopharyngeal swab. The third patient, an 83-year-old multimorbid female (HD01), showed no clinical improvement despite increasing neutralizing antibody titers and decreasing C-reactive protein and deceased due to COVID-19 pneumonia on Day 4 after initiation of CP therapy. She had been on hemodialysis for 8 years, suffered from diabetes mellitus, coronary heart disease, had apoplexy in 2010, and an acute event of fall.

The antibody ratios in these first three patients before the CP therapy were 0.15, 0.14, and 0.17, and the respective neutralizing antibody titers 1:20, <1:20, and 1:40. After CP therapy, antibodies in the patients reached a maximum ratio of 3.07, 2.19, and 3.70, corresponding to a neutralizing titer of up to 1:640, 1:160, and 1:640, respectively. In the donors, the antibody ratios were 5.83, 7.33, and 10.44, and the neutralizing titers 1:1280, 1:320, and 1:160, respectively.

The fourth patient, a 78-year-old female with pre-existing antibodies (HD02), showed rapid clinical improvement and could be discharged from the hospital on Day 8 after initiation of CP treatment. Before CP treatment, SARS-CoV-2 was detectable by PCR at a low level ($C_t$ value of 31.1). On Day 14 after CP therapy, viral load was undetectable. The patient also showed an increase of specific immunity (ratio 5.96 → 7.01; neutralizing titer 1:640 → 1:1280; Figure 1D). However, SARS-CoV-2-specific antibodies in the CP

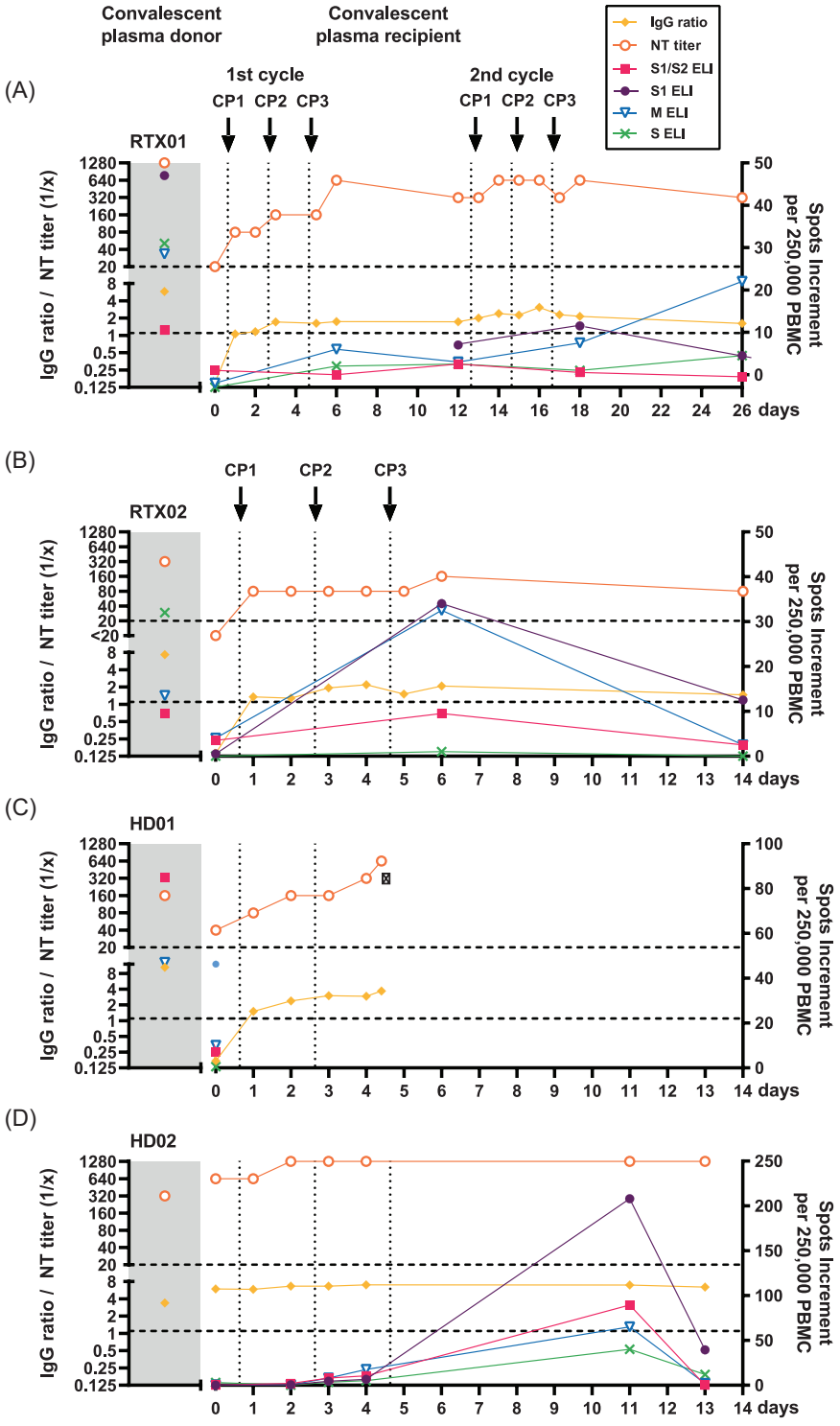


**FIGURE 1** (See caption on next page)

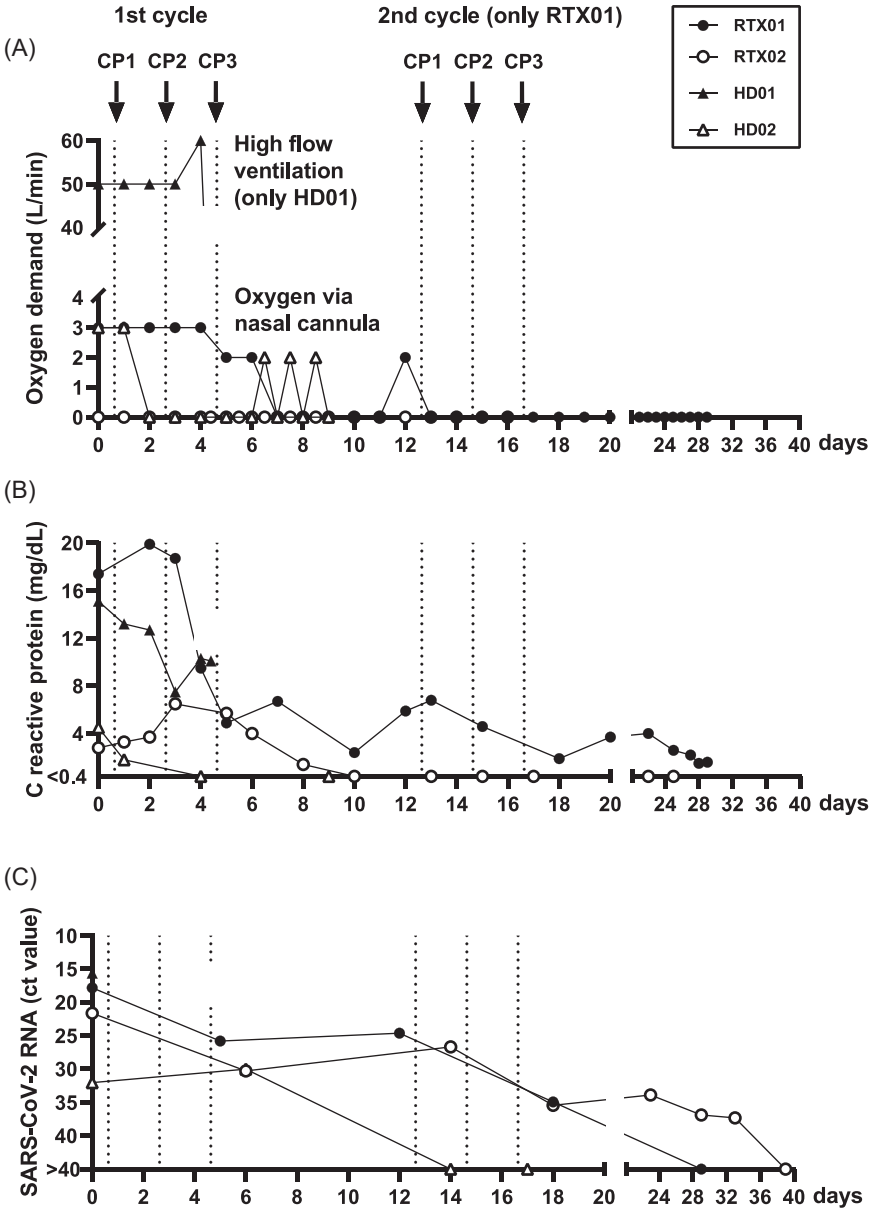


**FIGURE 2** Course of oxygen demand, C-reactive protein, and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral load in four patients with chronic kidney disease infected with SARS-CoV-2 and receiving convalescent plasma treatment. We here present data on two kidney transplant recipients (RTX01, RTX02) and two patients on hemodialysis (HD01, HD02) which were tested up to Day 39 after receiving convalescent plasma (CP). Vertical dotted lines indicate the time points of convalescent plasma applications (CP1, CP2, and CP3). Of note, only RTX01 received two cycles of CP while the remaining three patients received one cycle. A ct value of SARS-CoV-2 RNA > 40 was considered negative

**FIGURE 1** Course of specific humoral and cellular immunity in four patients with chronic kidney disease infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and receiving convalescent plasma treatment. Antibodies were determined by an S1 specific immunoglobulin G (IgG) enzyme-linked immunosorbent assay (Euroimmun) and by cell-culture based neutralization assay (NT titer). Cellular responses were analyzed by an interferon-gamma (IFN-γ) ELISpot assay, using peptide pools of the S1/S2, S1, and M protein and an S1 protein antigen as specific stimuli (depicted as S1/S2, S1, M, and S ELI). We here present data on two kidney transplant recipients (RTX01, RTX02) and two patients on hemodialysis (HD01, HD02) and compared their immune responses with those of the corresponding donors of convalescent plasma (CP; shaded area). SARS-CoV-2-specific antibody data (IgG ratio and NT titer) are given on the left *Y*-axis and ELISpot data on the right one. Horizontal dashed lines represent the cut-off values for positive reactions (IgG ratio of 1.1 and NT titer of 1:20). Vertical dotted lines indicate the time points of convalescent plasma applications (CP1, CP2, and CP3). Related data points are connected. PBMC, peripheral blood mononuclear cells

donor of the fourth patient were lower than in the patient (ratio: 3.39, neutralizing titer: 1:320).

Cellular immunity could be followed-up by IFN-γ ELISpot, using four different SARS-CoV-2-specific antigens (peptide pools of the S1/S2, S1, and M protein and an S1 protein antigen). Before CP treatment, one patient was negative to the ELISpot (HD02) and three showed weak responses (RTX01, RTX02, and HD01). Three patients could be followed-up after CP treatment. In these three patients, IFN-γ production to the ELISpot intermittently increased, reaching a maximum at Days 6–14 after CP therapy.

## 4 | DISCUSSION

Our data show an increase of specific humoral and cellular immunity in two kidney transplant recipients and two hemodialysis patients with SARS-CoV-2 infection after treatment with CP. This may represent the natural course of infection. However, the increase of immune responses occurred very close in time to the administration of CP; which suggests that there may be a causal relationship between treatment with CP and the increase in humoral and cellular immune responses. CP contains neutralizing antibodies as well as anti-inflammatory cytokines and other immunomodulatory proteins. This combination could improve virus control in immunocompromised patients.[3] CP therapy thus could bridge the phase of acute COVID-19 disease. However, presumably due to drug-induced immunosuppression or impaired kidney function, the immune responses were not as long-acting as expected. In one patient with two prior kidney transplantations (RTX01) two cycles of therapy were necessary for successful treatment. It can be supposed that the patient herself was unable to mount an adequate antibody response and that the passively transferred antibodies partly bound the virus that resides in the affected organs and in the respective lymphoid tissue.[14] Theoretically, it is possible that CP therapy mitigates the native humoral immune response and leaves an individual vulnerable to subsequent reinfection with SARS-CoV-2.[3,15] This phenomenon appears more likely in immunosuppressed versus otherwise healthy individuals. Concerning ELISpot data, we observed a maximum of IFN-γ responses shortly after completion of the CP cycle. Of note, cellular immunity is regarded as important for recovery from SARS-CoV-2 infection[16] and appears as short-lived in the current cohort. As CP therapy is a form of passive immunization, an increase in cellular responses is not expected at first glance. After an initial increase, IFN-γ production decreased again, which could reflect the fact that proinflammatory immune responses shifted to anti-inflammatory responses.[17] It has already been shown that there was a reduction in proinflammatory cytokines like IL-6 and an increase in anti-inflammatory cytokines after CP was administered.[18–20] Moreover, chronic kidney disease suppressed T-cell function, which could impede long-term protection against reinfection.[3,21]

Three out of four patients with chronic kidney disease showed clinical improvement; which is in the range of previous reports.[3–6] However, due to the low patient number, it was beyond the aim of our study to answer the question of CP therapy was effective. This answer can only be given by large randomized clinical studies such as the Randomized Evaluation of COVID-19 Therapy (RECOVERY) trial[22]; which is currently underway.

In conclusion, our data suggest that despite an increase of SARS-CoV-2-specific immunity the success of CP therapy may only be temporary in patients with chronic kidney disease. Thus, short-term treatment control (monitoring of viral load and antiviral immunity) appears mandatory for this patient group. If necessary, the treatment regimen has to be adapted.

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### CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

### ETHICS STATEMENT

The study was approved by the local ethics committee (20-9256-BO for the patients and 20-9225-BO for the donors) and the study participants provided written informed consent.

### AUTHOR CONTRIBUTIONS

Monika Lindemann, Adalbert Krawczyk, Veronika Lenz, Ulf Dittmer, Peter A. Horn, and Oliver Witzke conceived and designed the study. Laura Thümmler, Sina Schwarzkopf, Leonie Schipper, Maren Bormann, and Lukas van de Sand performed the experiments and analyzed the data. Sebastian Dolff, Margarethe Konik, Hana Rohn, Maximillian Platte, Marianne Breyer, Hannes Klump, Dietmar Knop, Veronika Lenz, Christian Temme, Peter A. Horn, and Oliver Witzke took care of the patients or convalescent plasma donors and participated in the collection and interpretation of data. Monika Lindemann had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analyses. Monika Lindemann, Adalbert Krawczyk, Sebastian Dolff, Peter A. Horn, and Oliver Witzke wrote the manuscript. All authors gave final approval of the manuscript.

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## 4.5 Cellular and Humoral Immunity against Different SARS-CoV-2 Variants Is Detectable but Reduced in Vaccinated Kidney Transplant Patients

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2022

Contributions:

- Experimental work: 20 %

Monika Lindemann conceptualized the study. Experiments were performed by Laura Thümmler, Neslinur Fisenkci, Maren Bormann, Mona Otte, and Mira Alt. Laura Thümmler and Monika Lindemann analyzed the data. Monika Lindemann, Anja Gäckler, Hana Rohn, Sandra Ciesek, Marek Widera, Peter A. Horn, and Adalbert Krawczyk, provided resources. The manuscript was prepared by Laura Thümmler, and Monika Lindemann. The manuscript was edited by Laura Thümmler, Monika Lindemann, Ulf Dittmer, Peter A. Horn, Oliver Witzke, and Adalbert Krawczyk.

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Prof. Dr. Adalbert Krawczyk

vaccines

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# Cellular and Humoral Immunity against Different SARS-CoV-2 Variants Is Detectable but Reduced in Vaccinated Kidney Transplant Patients

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**Abstract:** In kidney transplant (KTX) patients, immune responses after booster vaccination against SARS-CoV-2 are inadequately examined. We analyzed these patients a median of four months after a third/fourth vaccination and compared them to healthy controls. Cellular responses were analyzed by interferon-gamma (IFN-γ) and interleukin-2 (IL-2) ELISpot assays. Neutralizing antibody titers were assessed against SARS-CoV-2 D614G (wild type) and the variants alpha, delta, and omicron by a cell culture-based neutralization assay. Humoral immunity was also determined by a competitive fluorescence assay, using 11 different variants of SARS-CoV-2. Antibody ratios were measured by ELISA. KTX patients showed significantly lower SARS-CoV-2-specific IFN-γ responses after booster vaccination than healthy controls. However, SARS-CoV-2-specific IL-2 responses were comparable to the T cell responses of healthy controls. Cell culture-based neutralizing antibody titers were 1.3-fold higher in healthy controls for D614G, alpha, and delta, and 7.8-fold higher for omicron ($p < 0.01$). Healthy controls had approximately 2-fold higher concentrations of potential neutralizing antibodies against all 11 variants than KTX patients. However, more than 60% of the KTX patients displayed antibodies to variants of SARS-CoV-2. Thus, KTX patients should be partly protected, due to neutralizing antibodies to variants of SARS-CoV-2 or by cross-reactive T cells, especially those producing IL-2.

**Keywords:** ELISpot; T cells; vaccination



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## 1. Introduction

Since the first appearance of severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) in December 2019, more than 500 million people have been infected with SARS-CoV-2 and more than 6 million people have died from coronavirus disease 19 (COVID-19) (June 2022) [1].

Immunocompromised individuals, such as cancer patients, solid organ recipients, and individuals with comorbidities, have a higher mortality and morbidity rate from COVID-19 [2–4]. Individuals who belong to vulnerable groups benefit from vaccination against

SARS-CoV-2 to protect themselves from infection. They can also be protected indirectly by vaccinating individuals around them, as this significantly reduces the risk of infection [5–7].

However, studies displayed only weak or no vaccination responses after SARS-CoV-2 infection and two mRNA vaccinations in immunosuppressed patients who also suffer more frequently from vaccine breakthrough infection [8–10]. Previous studies have shown that multiple vaccinations against SARS-CoV-2 can lead to an increase in the immune response of immunocompromised individuals [8,10–12]. So far, there is insufficient data on whether booster vaccination leads to adequate immune responses, especially with regard to the currently predominant SARS-CoV-2 variants delta and omicron.

In the present study, we focused on cellular and humoral immunity to SARS-CoV-2 and its variants in immunosuppressed and immunocompetent vaccinated individuals after at least three mRNA vaccinations. We analyzed cellular immunity by a fluorescence ELISpot assay, which can detect the secretion of IFN-$\gamma$ and IL-2 simultaneously, as well as by colorimetric SARS-CoV-2-specific IFN-$\gamma$ and IL-2 ELISpot assays. Neutralizing antibody titers to SARS-CoV-2 D614G (wild type) and its alpha, delta, and omicron variants were analyzed by a cell culture-based neutralization assay. Moreover, potential neutralizing antibodies to variants and mutants of SARS-CoV-2 were determined by competitive fluorescence assay. SARS-CoV-2-specific IgG antibodies were measured by semiquantitative ELISA.

## 2. Materials and Methods

### *2.1. Volunteers*

The patient cohort comprised 32 kidney transplant (KTX) patients after booster vaccination against SARS-CoV-2 (Table 1) and without SARS-CoV-2 infection at the timepoint of blood collection. Kidney transplantation was performed at a median of 2 years (range 0.4–11.8) before blood collection. The group included 12 males and 20 females with a median age of 54 years (range 21–76). Of the 32 KTX patients, 31 were vaccinated with Comirnaty® (BioNTech/Pfizer, Mainz, Germany) and one with Spikevax® (Moderna, Cambridge, Massachusetts). Twenty-four of the KTX patients were triple-vaccinated and eight were quadruple-vaccinated. The booster vaccination took place a median of 111 days (range 43–212) before testing. The majority of patients received an immunosuppressive regimen consisting of tacrolimus, mycophenolate, and prednisone. Immunosuppressive therapy was also provided at the time of blood collection and beyond.

**Table 1.** Overview of the study cohort.

| Characteristics [1] | Kidney Transplant Recipients | Healthy Controls |
|---|---|---|
| sex | 12 males<br>20 females | 5 males<br>12 females |
| age, y | 54 (21–76) | 53 (35–65) |
| tacrolimus | 32 (100%) | Ø |
| mycophenolate | 26 (81%) | Ø |
| belatacept | 2 (6%) | Ø |
| prednisone | 32 (100%) | Ø |
| interval kidney transplantation—blood collection | 2 years (0.4–11.8) | Ø |
| interval vaccination—blood collection | 111 days (43–212) | 182 days (69–213) |

[1] The data indicate either the median (range) or absolute numbers (percentage). The characteristics of both groups did not differ significantly, as analyzed by Fisher's exact test (sex: $p = 0.8$) or Mann–Whitney test (age: $p = 0.5$; interval vaccination—blood collection: $p = 0.1$), respectively. Ø: no medication/ no data available.

We included 17 healthy volunteers after the third vaccination without SARS-CoV-2 infection prior to blood collection as a control group. Of the 17 healthy volunteers, 11 were vaccinated with Spikevax® (Moderna, Cambridge, MA, USA) and six were vaccinated with Comirnaty® (BioNTech/Pfizer, New York, NY, USA). The group consisted of 5 males and

12 females and the median age was 53 years (range 35–65). The cohort was tested at a median of 182 days (range 69–213) after the third vaccination.

This study was approved by the ethics committee of the University Hospital Essen, Germany (20-9753-BO), and all volunteers provided informed consent to participate. It has been performed in accordance with the ethical standards noted in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

### *2.2. CoV-iSpot for Interferon-γ and Interleukin-2*

In 31 samples (21 KTX patients, 10 healthy controls), we simultaneously stained for IFN-γ and IL-2 using the CE-marked CoV-iSpot (AID, Strassberg, Germany), as previously described [13]. This fluorescence ELISpot (Fluorospot) contains a peptide mix of the wild type SARS-CoV-2 spike protein. Duplicates of 200,000 peripheral blood mononuclear cells (PBMC) were grown with or without adding the peptide mix (S-pool). The cut-off definition was described previously [14]. We chose 5 as cut-off for positivity for IFN-γ and for IL-2. Among the positive controls, we found an average of 410 spots (range 50–880) in KTX patients for IFN-γ and 463 spots (range 50–1100) for IL-2. In the healthy controls, we found an average of 679 spots (range 486–904) for IFN-γ and 545 spots (range 422–660) for IL-2 in the positive controls.

### *2.3. In-House ELISpot Assay*

To further analyze SARS-CoV-2-specific cellular immunity, we used IFN-γ and IL-2 ELISpot assays separately, as previously described [13]. Briefly, 250,000 PBMC of 32 KTX patients and 17 healthy controls were cultured in the presence or absence of either PepTivator® SARS-CoV-2 wild type protein S1/S2, protein S1 (600 pmol/mL of each peptide, Miltenyi Biotec, Bergisch Gladbach, Germany), of the wild type protein S1 (4 µg/mL, Sino Biological, Wayne, PA, USA.) or the omicron variant of the protein S1 (SARS-CoV-2 B.1.1.529, 4 µg/mL, Sino Biological) in 150 µL of AIM-V®. Spot numbers were analyzed by an ELISpot reader (AID Fluorospot, Autoimmun Diagnostika GmbH, Strassberg, Germany). The average values of duplicate cell cultures were included. SARS-CoV-2-specific spots were determined as the stimulated minus non-stimulated values (spots increment). We chose a spot increment of 3 for positivity for IFN-γ as well as for IL-2. In the positive controls, we saw on average 432 spots (range 200–600) in KTX patients and 464 spots (range 250–600) in healthy controls for IFN-γ. For IL-2, we saw on average 508 spots (range 200–600) in KTX patients and 517 spots (range 400–600) in healthy controls.

### *2.4. Cells and Viruses*

A549-AT cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS), 4 mM L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37 °C and 5% $CO_2$. The clinical SARS-CoV-2 isolates D614G (wild type), alpha, delta, and omicron were obtained from nasopharyngeal swabs of COVID-19 patients at our hospital. The SARS-CoV-2 spike gene was sequenced and the corresponding variants were determined after sequence analysis with the WHO list of variants of concern [15]. The viruses were propagated on A549-AT cells and stored at −80 °C. Viral titers were determined using a standard endpoint dilution assay and calculated as 50% tissue culture infective dose (TCID50)/mL, as previously described [16].

### *2.5. Assessment of Neutralizing Antibodies by Cell Culture-Based Neutralization Assay*

To assess the neutralizing antibody titers of sera from 28 KTX patients and 11 healthy controls, we used a standard endpoint dilution assay, as described previously [13,17,18]. From the respective sera, serial dilutions (1:20 to 1:2560) were incubated with 100 $TCID_{50}$ of SARS-CoV-2 D614G (wild type), alpha (B.1.1.7), delta (B.1.617.2) or omicron (BA.1) for one hour at 37 °C. Thereafter, the dilutions were added to confluent A549-AT cells [18] in 96-well microtiter plates. After three days of incubation, cells were stained with crystal violet (Roth, Karlsruhe, Germany) solved in 20% methanol (Merck, Darmstadt, Germany).

Cells were evaluated for the presence of cytopathic effects (CPE) by light microscopy. The neutralizing titer was defined as the reciprocal of the highest serum dilution at which no CPE was observed in any of the three test wells. A549-AT cells overexpress carboxypeptidase angiotensin-I-converting enzyme 2 (ACE2) receptor and the cellular transmembrane protease serine 2 (TMPRSS2), enabling enhancement of CPE and high SARS-CoV-2 susceptibility. A549-AT cells were cultivated in minimum essential media (MEM), supplemented with 10% (*v/v*) FCS, penicillin (100 IU/mL), and streptomycin (100 µg/mL) at 37 °C in an atmosphere of 5% $CO_2$ (all Life Technologies Gibco, Darmstadt, Germany).

### *2.6. Assessment of Neutralizing Antibodies by Competitive Immunofluorescence*

For the detection of potential neutralizing antibodies against wild type SARS-CoV-2 and 11 variants of SARS-CoV-2, we used a commercial competitive immunofluorescence assay (Bio-Plex Human SARS-CoV-2 Variant Neutralization Antibody 11-Plex Panel, BIO-RAD, Hercules, CA, USA), as described previously [13]. This competitive immunofluorescence assay works like a binding inhibition assay. Magnetic beats covered with different SARS-CoV-2 spike variants are incubated with soluble, biotin-conjugated ACE2 receptors in the presence of patient sera. Neutralizing serum antibodies compete for binding to the immobilized spike proteins with biotinylated ACE2 receptors. Detection of bound ACE2 receptors is achieved by the addition of streptavidin–phycoerythrin (SA-PE), which binds to the biotinylated ACE2 receptor. The benefit of this method is to detect antibodies that can bind to different mutants and variants of SARS-CoV-2. The upper limit of the system is 1000 ng/mL. We chose 175 ng/mL as the cut-off for positivity, which was defined for a similar testing system [19].

### *2.7. Antibody ELISA*

SARS-CoV-2-specific antibodies were detected by a CE-marked Anti-SARS-CoV-2 IgG semiquantitative ELISA (Euroimmun, Lübeck, Germany), according to the manufacturer's instructions, as described previously [14]. The ELISA plates were coated with wild type recombinant SARS-CoV-2 spike protein (S1 domain). Serum samples were analyzed automatically at a dilution of 1:100, using the Immunomat (Virion\Serion, Würzburg, Germany). An antibody ratio >1.1 was considered positive, ≥0.8 to <1.1 borderline, and <0.8 negative.

### *2.8. Statistical Analysis*

Statistical analysis was performed using GraphPad Prism 9.4.0 (San Diego, CA, USA) software. We used Mann–Whitney tests and Spearman correlation to analyze the numerical variables. To compare the categorical variables, we used Fisher's exact test. Two-sided $p$ values < 0.05 were considered significant.

## 3. Results

### *3.1. T Cell Responses in Kidney Transplant Patients and Healthy Volunteers*

We examined the cellular immune response in KTX patients and healthy volunteers after booster vaccination and detected significant differences in the commercial CoV-iSpot upon stimulation with the S pool of wild type SARS-CoV-2 (Figure 1). Of the 21 KTX patients, six showed a positive response for IFN-γ, and seven showed a positive response for IL-2. There was a positive reaction only in one KTX patient in the ELISpot measuring simultaneous secretion of IFN-γ and IL-2. Of the 11 healthy controls, seven showed a positive reaction for IFN-γ, seven for IL-2, and two for the simultaneous secretion of IFN-γ and IL-2. The spot increment for IFN-γ and the simultaneous secretion of IFN-γ and IL-2 differed significantly between KTX patients and healthy volunteers (IFN-γ: $p = 0.005$; IFN-γ and IL-2: $p = 0.001$).

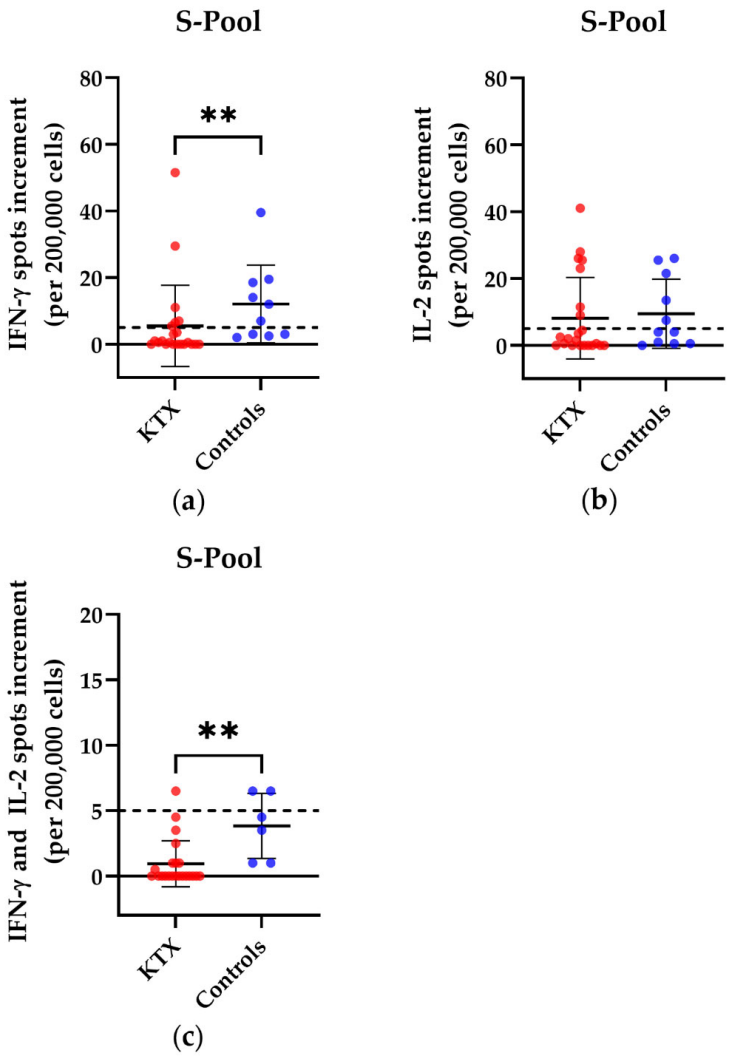


**Figure 1.** SARS-CoV-2-specific CoV-iSpot responses in kidney transplant (KTX) patients and healthy volunteers after booster vaccination. Distribution of (**a**) IFN-γ, (**b**) IL-2, and (**c**) simultaneous IFN-γ and IL-2 CoV-iSpot responses after stimulation with the S pool of the wild type SARS-CoV-2. Please note the different scales. Red circles show data of the KTX patients, while blue circles indicate data of the healthy volunteers. Two-tailed Mann–Whitney tests were used to compare the responses (** $p < 0.01$). Mean values are represented by horizontal lines, while the standard deviation is represented by error bars. The horizontal line shows the zero line. The dashed line indicates the cut-off.

Using our in-house ELISpot, we observed in KTX patients versus healthy controls significantly lower numbers of IFN-γ spots after stimulation with S1/S2, S1 or with a recombinantly expressed S1 protein (called S1 Sino hereinafter) (S1/S2: $p < 0.0001$; S1: $p < 0.0001$; S1 Sino: $p = 0.0005$) (Figure 2a,c,f). We also detected significantly lower numbers of IFN-γ spots after stimulation with a recombinant S1 protein of the omicron (B 1.1.529) variant ($p = 0.0005$) (Figure 2g). For IL-2, we could not observe significant differences between KTX patients and healthy volunteers. For IFN-γ, six of the 32 patients displayed a positive reaction towards the S1/S2 peptide mix, seven towards the S1 peptide mix, five to the S1 Sino, and five to the recombinant S1 protein of the omicron variant. For IL-2, 11 of the 32 KTX patients displayed a positive reaction towards S1/S2, 12 towards S1, 13 towards

S1 Sino, and 12 to the recombinant S1 protein of the omicron variant. Of the 17 healthy controls, 12 exhibited a positive response to the S1/S2 peptide mix, 15 to the S1 peptide mix, ten to the S1 Sino, and 11 to the recombinant S1 protein of the omicron variant. For IL-2, 11 of the 17 healthy volunteers showed a positive reaction towards S1/S2, 10 towards S1, 5 towards S1 Sino, and 9 to the recombinant S1 protein of the omicron variant. We could not detect significant differences in the cellular immune response between KTX patients after the third vaccination and KTX patients after the fourth vaccination.

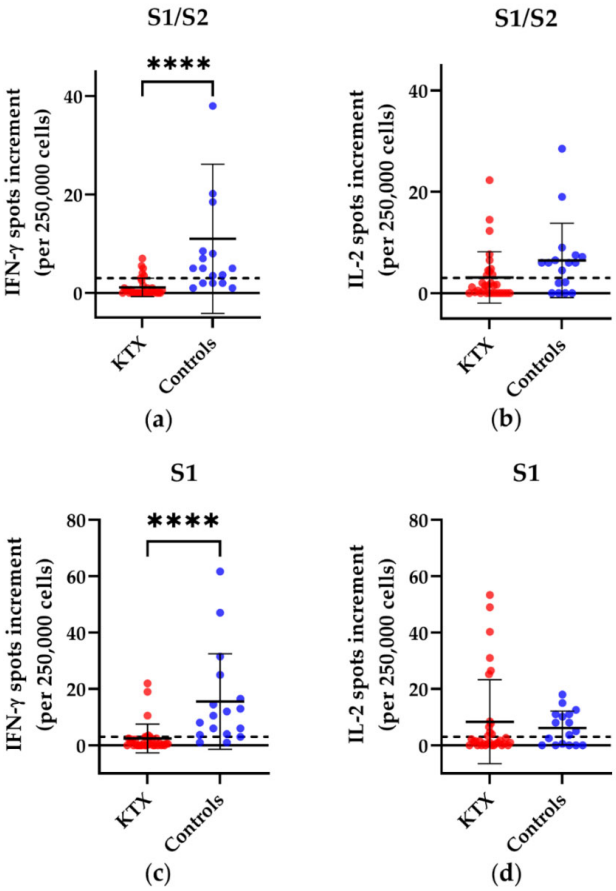


**Figure 2.** *Cont.*

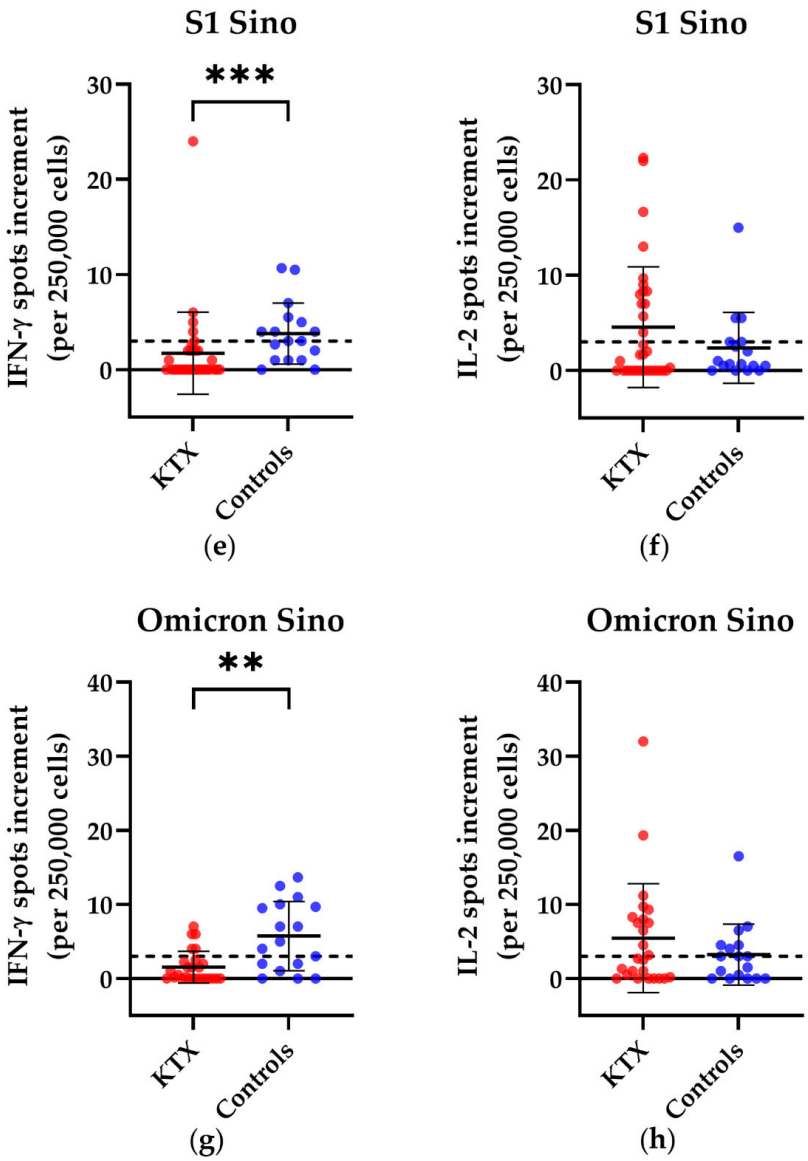


**Figure 2.** SARS-CoV-2-specific responses in kidney transplant (KTX) patients and healthy controls after booster vaccination, using our in-house ELISpot assay. Distribution of (**a**) IFN-γ and (**b**) IL-2 ELISpot responses after stimulation with an S1/S2 peptide mix, with an S1 peptide mix (**c**,**d**), S1 Sino (**e**,**f**) and S1 Sino of the omicron variant (**g**,**h**). Red circles show data of the KTX patients, while blue circles indicate data of the healthy volunteers. Two-tailed Mann–Whitney tests were used to compare the responses (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Mean values are represented by horizontal lines, while the standard deviation is represented by error bars. The horizontal line shows the zero line. The dashed line indicates the cut-off.

Summarizing the cellular data, KTX patients showed significantly lower SARS-CoV-2-specific responses for IFN-γ, but similar mean values for IL-2, compared to healthy controls.

### *3.2. Humoral Immunity in Kidney Transplant Patients and Healthy Controls*

We examined the neutralizing antibodies by a cell culture-based neutralization assay and evaluated whether immunocompromised individuals could generate similar levels of neutralizing antibodies against the wild type SARS-CoV-2, alpha variant, delta variant, and

omicron (BA.1) variant as the healthy controls. KTX patients showed significantly lower titers of neutralizing antibodies than the healthy controls against all tested variants (wild type: $p = 0.0001$; alpha: $p = 0.003$; delta: $p < 0.0001$; omicron: $p = 0.0002$) (Figure 3). We could not detect significant differences between 24 KTX patients after third vaccination vs. eight KTX patients after fourth vaccination (wild type: $p = 0.7$; alpha: $p = 0.9$; delta: $p = 0.9$; omicron: $p = 0.6$).

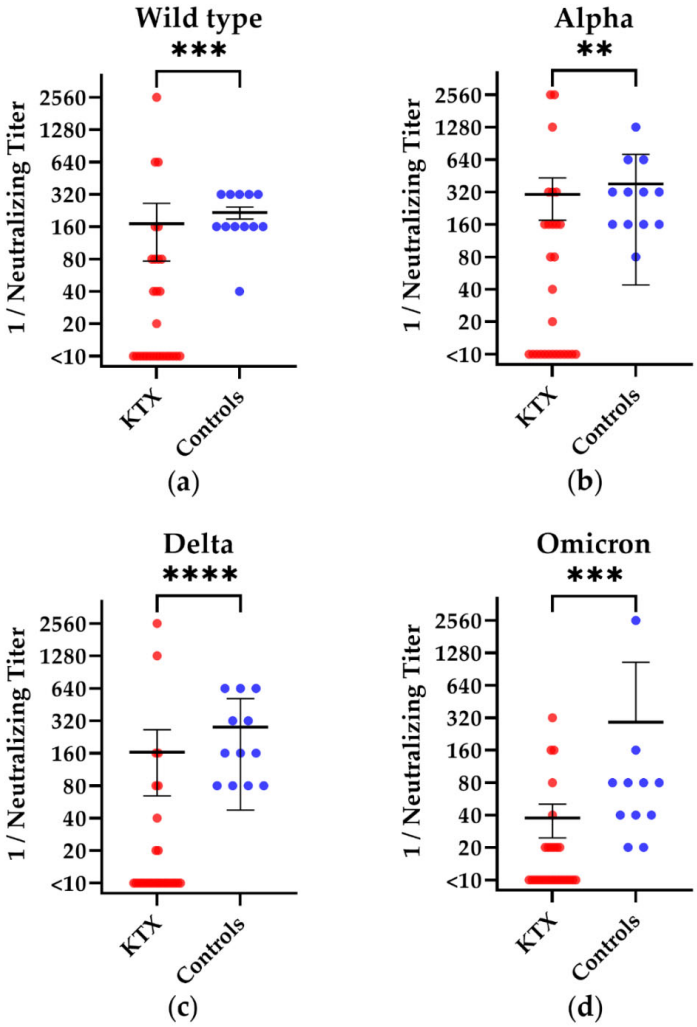


**Figure 3.** Titer of SARS-CoV-2-specific neutralizing antibodies in kidney transplant (KTX) patients and healthy volunteers. The reciprocal of the titer of neutralizing anti-SARS-CoV-2 (**a**)D614G (wild type), (**b**) alpha, (**c**) delta, and (**d**) omicron (BA.1) antibodies is shown on the *y*-axis. Red circles show data of the KTX patients, while blue circles indicate data of the healthy volunteers. Two-tailed Mann–Whitney tests were used to compare the responses (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Mean values are represented by horizontal lines, while the standard deviation is represented by error bars.

We also examined if vaccination can lead to a humoral immune response towards different variants and mutations of SARS-CoV-2 by a competitive immunoassay. KTX patients showed significantly lower concentrations of potential neutralizing antibodies for all tested mutations, namely, alpha, beta, gamma, delta (plus), epsilon, eta, iota, kappa, lambda, mu, and omicron (B 1.1529), compared to the healthy controls (Figure 4). In detail,

18 of the 32 patients responded to the D614G mutation, which can be found in the variants delta and omicron (Figure 4i); 22 of the 32 responded towards the K417N mutation (omicron variant, Figure 4j); and 20 of the 32 showed a positive reaction towards the N501Y mutation (omicron, Figure 4k). All 17 healthy volunteers displayed a positive response towards the D614G mutation, K417N, and N501Y. We detected significant differences between KTX patients and healthy volunteers after booster vaccination in neutralizing antibodies against all variants/mutations examined ($p < 0.001$). The comparison between 24 KTX patients after the third vaccination and eight KTX patients after the fourth vaccination did not display significant differences.

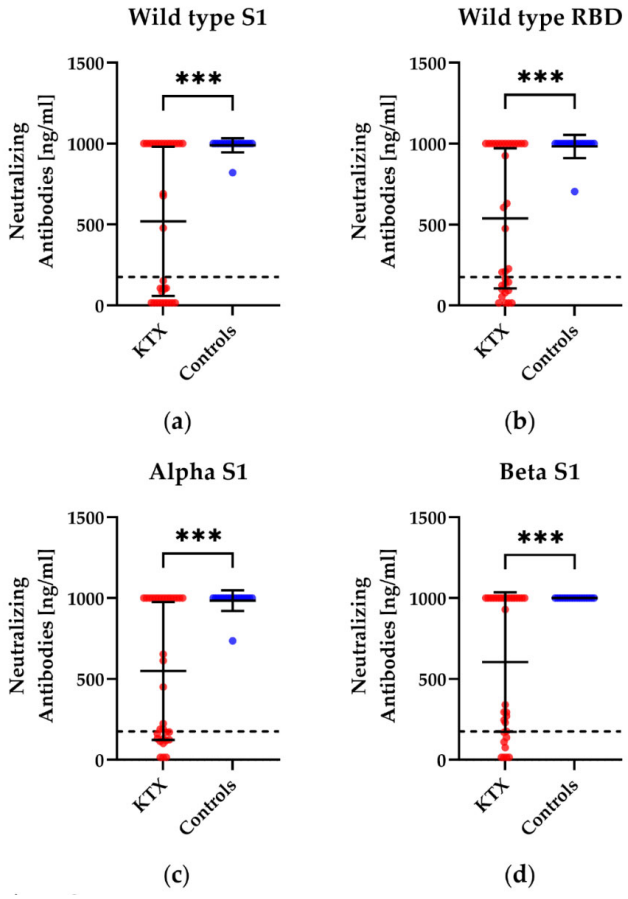


**Figure 4.** *Cont.*

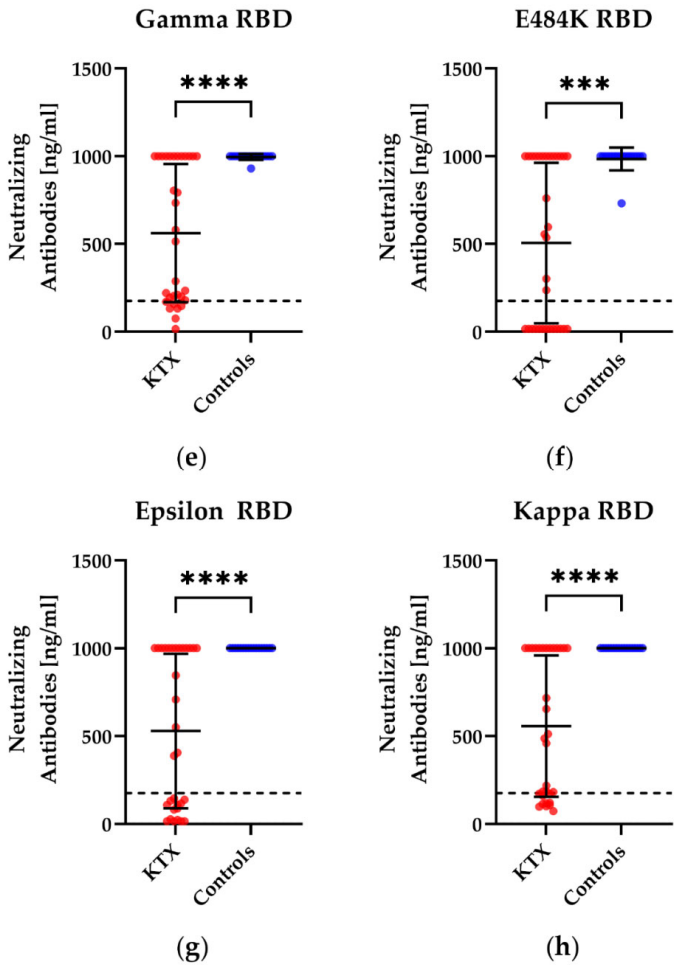


**Figure 4.** *Cont.*

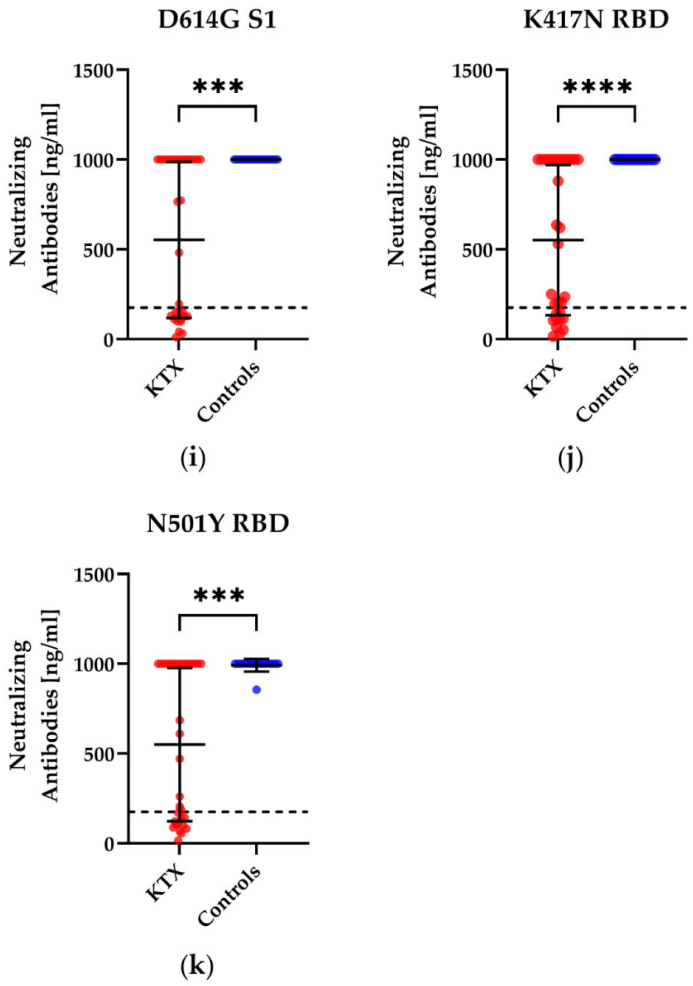


**Figure 4.** Concentration of potential neutralizing antibodies towards different variants of the subunit 1 of spike protein (S1) or the receptor-binding domain (RBD) of SARS-CoV-2 in kidney transplant (KTX) patients and healthy volunteers (controls) after booster vaccination. Humoral responses after booster vaccination against (**a**) wild type S1, (**b**) wild type RBD, (**c**) alpha S1, (**d**) beta S1, (**e**) gamma RBD, (**f**) E484K RBD, (**g**) epsilon RBD, (**h**) kappa RBD, (**i**) D614G S1, (**j**) K417N RBD and (**k**) N501Y RBD. The mutation D614G can be found in the delta and omicron variants, while K417N and N501Y are mutations in the omicron variant. Red circles show data of the KTX patients, while blue circles indicate the data of healthy volunteers. Two-tailed Mann–Whitney tests were used to compare the responses (*** $p < 0.001$, **** $p < 0.0001$). Mean values are represented by horizontal lines, while the standard deviation is represented by error bars. The dashed line indicates the cut-off.

In addition, we measured the antibody ratio in 32 KTX patients and in 17 healthy controls. We detected a significantly lower antibody ratio in KTX patients compared to healthy volunteers (mean ratio of 3.7 vs. 9.3, $p < 0.0001$) (Figure 5). We observed no significant differences between 24 KTX patients after the third vaccination and eight KTX patients after the fourth vaccination ($p = 0.7$).

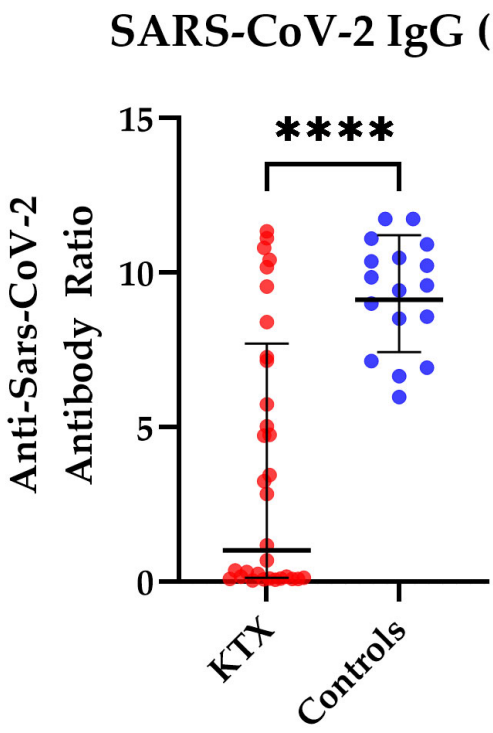


**Figure 5.** SARS-CoV-2-specific IgG antibody responses in kidney transplant (KTX) patients and healthy controls. SARS-CoV-2-specific IgG antibody responses are shown as antibody ratios, which determines a quotient of antibodies in the patient samples and in a control sample. Red circles show data of the KTX patients, while blue circles indicate the data of healthy volunteers. Two-tailed Mann–Whitney tests were used to compare the responses (**** $p < 0.0001$). Mean values are represented by horizontal lines, while the standard deviation is represented by error bars.

## 4. Discussion

We observed significant differences in cellular immunity between KTX patients and healthy controls after booster vaccination. While the lower response in the IFN-γ ELISpot was expected, comparable results in the IL-2 ELISpot were at first glance surprising. However, Schrezenmeier et al. found an increase in IL-2-secreting T cells after booster vaccination in KTX patients, whereas the IFN-γ response remained reduced [20]. This is in agreement with our results.

The results of the cell culture based neutralization assay showed comparable mean values of antibodies against the wild type, alpha, and delta, which were moderately decreased in KTX patients as compared to the healthy controls, who had a 1.3-fold higher mean value. For omicron (BA.1), however, differences between KTX patients and healthy controls were more pronounced ($p < 0.0001$). Here, the healthy controls had a 7.8-fold higher mean value of neutralizing antibody titers [21]. This could be due to the fact that these vulnerable groups are still specially protected from possible contact with the virus.

Our results demonstrate that both KTX patients and healthy controls displayed neutralizing antibodies towards variants and mutations of SARS-CoV-2 after booster vaccination against SARS-CoV-2. However, based on the detection of specific antibodies, a protective effect can hardly be assumed. Previous studies have shown that only about 40% of KTX patients develop a humoral immune response after the third vaccination [20,22]. In our study, the measured values were above the cut-off in about 64% of the KTX patients. This could indicate a better response to booster vaccination. An impact of the KTX patients after the fourth vaccination can be excluded, as they do not show any significant differences to KTX patients after the third vaccination.

We detected strongly reduced antibody ratios in KTX patients, which is consistent with the results of previous studies [20,22,23]. However, a study by Bensouna et al. observed an increase in the humoral immune response 30 days after the third vaccination. However, in our study, testing took place at a median of 111 days after vaccination. Other reasons for the lower humoral immune response could be treatment with mycophenolate mofetil or impaired germinal center immunity in immunosuppressed individuals [24].

One limitation of the present study is a lack of data on memory B cells. Notably, other studies showed impaired humoral immunity after mRNA vaccination [25]. Furthermore, it could be demonstrated that a humoral immune response is generated when immunosuppressants are paused [26]. In the cohort studied in our paper, no pausing of immunosuppressive medication was performed. Subsequent studies are needed to comprehensively analyze the memory B cell response in mRNA-vaccinated patients with immunosuppressive treatment.

Our data indicate that there is inadequate immunization in vulnerable groups when compared to healthy controls. In a previous study, we also observed an insufficient humoral immune response in HSCT patients after the third vaccination [13]. Accordingly, other studies of the humoral immune response after SARS-CoV-2 vaccination in vulnerable groups, such as organ transplant and cancer patients, also showed a reduced immune response [27–29]. For these individuals, it is recommended to follow all the related safety precautions and to monitor the humoral immune response on a regular basis.

## 5. Conclusions

In conclusion, cellular immunity of KTX patients was significantly lower compared to healthy controls for IFN-$\gamma$. For IL-2, KTX patients had a similar mean value of spots increment as the healthy controls. It might be possible that IL-2-secreting T cells also contribute to protection against SARS-CoV-2 infection. However, these cells are not measured by most standard tests. More than half of the KTX patients generated levels of potential neutralizing antibodies to variants of SARS-CoV-2. KTX patients developed neutralizing antibodies, even if they were significantly lower than the titers of healthy controls. Nevertheless, our data suggest that KTX patients are at least partly protected against SARS-CoV-2, either by neutralizing antibodies to variants of SARS-CoV-2 or by cross-reactive T cells.

**Author Contributions:** Conceptualization, M.L.; methodology, L.T., N.F., M.B., M.O. and M.A.; software, L.T.; validation, M.L. and A.G.; formal analysis, L.T. and M.L.; investigation, M.L.; resources, M.L., A.G., H.R., S.C., M.W., P.A.H. and A.K.; data curation, L.T. and M.L.; writing—original draft preparation, L.T. and M.L.; writing—review and editing, L.T., M.L., U.D., P.A.H., O.W. and A.K.; visualization, L.T.; supervision, M.L., P.A.H. and O.W.; project administration, M.L. and P.A.H.; funding acquisition, P.A.H. All authors have read and agreed to the published version of the manuscript.

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## 4.6 Glycyrrhizin Effectively Inhibits SARS-CoV-2 Replication by Inhibiting the Viral Main Protease

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2021

Contributions:

- Experimental work: 50 %

Adalbert Krawczyk and Lukas van de Sand conceptualized the study. Experiments were performed by Maren Bormann, Leonie Brochhagen (née Schipper), Carina Elsner and Lukas van de Sand. Lukas van de Sand and Mira Alt analyzed and visualized the data. Ulf Dittmer and Oliver Witzke provided resources. The manuscript was prepared by Adalbert Krawczyk and Lukas van de Sand. The manuscript was edited by Adalbert Krawczyk, Maren Bormann, Eike Steinmann, Carina Elsner, Daniel Todt, Christiane Silke Heilingloh, and Ulf Dittmer.

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viruses

MDPI

*Article*

# Glycyrrhizin Effectively Inhibits SARS-CoV-2 Replication by Inhibiting the Viral Main Protease

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**Abstract:** The outbreak of SARS-CoV-2 developed into a global pandemic affecting millions of people worldwide. Despite one year of intensive research, the current treatment options for SARS-CoV-2 infected people are still limited. Clearly, novel antiviral compounds for the treatment of SARS-CoV-2 infected patients are still urgently needed. Complementary medicine is used along with standard medical treatment and accessible to a vast majority of people worldwide. Natural products with antiviral activity may contribute to improve the overall condition of SARS-CoV-2 infected individuals. In the present study, we investigated the antiviral activity of glycyrrhizin, the primary active ingredient of the licorice root, against SARS-CoV-2. We demonstrated that glycyrrhizin potently inhibits SARS-CoV-2 replication in vitro. Furthermore, we uncovered the underlying mechanism and showed that glycyrrhizin blocks the viral replication by inhibiting the viral main protease $M^{pro}$ that is essential for viral replication. Our data indicate that the consumption of glycyrrhizin-containing products such as licorice root tea of black licorice may be of great benefit for SARS-CoV-2 infected people. Furthermore, glycyrrhizin is a good candidate for further investigation for clinical use to treat COVID-19 patients.

**Keywords:** SARS-CoV-2; glycyrrhizin; main protease



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## 1. Introduction

The newly emerged coronavirus, which was designated as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is the causative agent of the COVID-19 disease. Even presymptomatic patients or patients with mild symptoms are able to infect other people. The most common symptoms are headache, fever, cough, loss of taste and smell, sore throat and rhinorrhea. Highly effective and well-tolerated medication for hospitalized and non-hospitalized patients is urgently needed. Besides compounds that were initially approved for the treatment of other viral infections such as remdesivir [1], traditional herbal medicine substances were discussed as promising candidates for the complementary treatment of viral diseases and recently suggested for the treatment of COVID-19. Lastly, an in-silico simulation study proposed an antiviral activity of glycyrrhizin against SARS-CoV-2, but this hypothesis remains experimentally unproven up to now [2]. Glycyrrhizic acid is a triterpene saponin and found in high concentrations in the root of the Glycyrrhiza glabra plant. It was described as an antiviral active ingredient of the licorice root and exhibits antiviral activity against herpes simplex viruses [3], the human immunodeficiency virus as well as human and animal coronaviruses [4]. In the present study,

we investigated aqueous licorice root extract for its antiviral activity against SARS-CoV-2 in vitro, identified the active compound glycyrrhizin and uncovered the respective mechanism of how glycyrrhizin inhibits viral replication. We demonstrated that glycyrrhizin, the primary active ingredient of the licorice root, potently inhibits SARS-CoV-2 replication in vitro. Subsequently, we examined the underlying mechanism of antiviral efficacy and demonstrated that glycyrrhizin blocks the viral replication by inhibiting the viral main protease. Our experiments highlight glycyrrhizin as a potential antiviral compound that should be further investigated for the treatment of COVID-19.

## 2. Materials and Methods

### *2.1. Licorice Root Extract*

Dried licorice roots (Teeversand Naturideen, Hennstedt, Germany) were brewed in PBS at a concentration of 8 mg/mL (*w/v*). The fluid was subsequently sterile filtered with a syringe filter (Minisart®NML Plus 0.2 µm, Sartorius, Goettingen, Germany) to obtain an aqueous licorice root extract.

### *2.2. Glycyrrhizin Acid Ammonium-Nitrate*

For cell culture experiments, glycyrrhizin acid ammonium-nitrate (Roth, Karlsruhe, Germany) was dissolved in DMEM containing 2% (*v*/*v*) FCS, penicillin (100 IU/mL) and streptomycin (100 µg/mL) at 37 °C and adjusted with NaOH to pH 7. Otherwise, glycyrrhizin was dissolved in water and stored at 37 °C.

### *2.3. Cells and Virus*

Vero E6 cells (American Type Culture Collection, ATCC, Manassas, Virginia, USA; ATCC®CRL-1586™) were maintained in Dulbecco's modified Eagle's medium (DMEM Life Technologies Gibco, Darmstadt, Germany), supplemented with 10% fetal calf serum (FCS; Life Technologies Gibco, Darmstadt, Germany), penicillin (100 U/mL) and streptomycin (100 µg/mL) at 5% $CO_2$ and 37 °C. The clinical SARS-CoV-2 isolate was obtained from a nasopharyngeal swab of a patient suffering from COVID-19 disease at our hospital. The virus was propagated in Vero E6 cells cultured in DMEM supplemented with 10% (*v*/*v*) FCS, penicillin (100 IU/mL), streptomycin (100 µg/mL), ciprofloxacin (10 µg/mL) and amphotericin B (2.5 µg/mL) and stored at −80 °C. Viral titers were determined using a standard endpoint dilution assay and calculated as 50% tissue culture infective dose ($TCID_{50}$)/mL, as previously described [5].

### *2.4. Antiviral Activity of Aqueous Licorice Root Extract and Glycyrrhizin*

The antiviral activity of aqueous licorice root extract and glycyrrhizin was determined in cell culture using endpoint dilution. For this purpose, serial dilutions of licorice root extract or glycyrrhizin (0.004–4 mg/mL) were pre-incubated with 100 $TCID_{50}$ of SARS-CoV-2 for 1 h at 37 °C and subsequently incubated on confluent Vero E6 cells grown in 96-well microtiter plates (combined pre- and post-entry approach). After 48 h, the cells were stained with crystal violet (Roth, Karlsruhe, Germany) solved in 20% methanol (Merck, Darmstadt, Germany) and analyzed for cytopathic effects (CPE). The neutralizing titer was determined as the concentration required for reducing virus-induced CPE by 100%.

Moreover, the antiviral activity of glycyrrhizin was investigated under post-entry conditions (post-entry treatment). Therefore, Vero E6 cells were infected with 100 $TCID_{50}$ SARS-CoV-2 for 4 h and subsequently treated with various glycyrrhizin concentrations ranging from 0.002–4 mg/mL (post-entry conditions). After 2 days of incubation, the cells were stained with crystal violet (Roth, Karlsruhe, Germany) solved in 20% methanol (Merck, Darmstadt, Germany) and the CPEs were scored using light microscopy.

### *2.5. Cell Viability Assay*

Cytotoxicity of licorice root extract and glycyrrhizin towards Vero E6 cells was determined by using the "Orangu cell counting solution" (Cell guidance systems, Cambridge,

UK) according to the manufacturer's protocol. Orangu™ is a colorimetric assay for the determination of viable cell numbers in cytotoxicity assays. Orangu™ utilizes WST-8, a water-soluble tetrazolium salt that is reduced by dehydrogenase activities in viable cells. The amount of the orange-colored formazan dye formed is directly proportional to the number of living cells [6]. Briefly, descending licorice root extract or glycyrrhizin concentrations (0.002–4 mg/mL) were incubated (37 °C, 5% $CO_2$) with $1 \times 10^5$ Vero E6 cells per ml (and 10,000 cells/well of a 96-well plate) grown in 96-well microtiter plates. At four distinct time points (5 min, 12 h, 24 h and 4 h), medium (DMEM containing 2% (*v*/*v*) FCS (PAA), penicillin (100 IU/mL) and streptomycin (100 µg/mL)) was changed and 10 µL of Orangu™ cell counting solution was added for 120 min of incubation (37 °C, 5% $CO_2$). Cell viability was measured at an absorbance of 450 nm using Mithras LB 940 (Berthold Technologies, Oak Ridge, TN, USA) and normalized to untreated control cells.

### *2.6. Determination of $EC_{50}$ of Glycyrrhizin*

To further investigate the antiviral efficacy of glycyrrhizin, we determined the half-maximal effective concentration ($EC_{50}$) sufficient to inhibit viral replication by 50%. Confluent Vero E6 cells grown in 6-well plates were infected with $1 \times 10^4$ $TCID_{50}$ SARS-CoV-2 and treated with various concentrations of glycyrrhizin ranging from 0.0625 to 4 mg/mL. After 48 h of incubation, the supernatants were harvested and the viral loads were determined using endpoint dilution. The experiment was performed in triplicates and $EC_{50}$ was calculated via linear regression using GraphPad Prism 8.0.1. (Graph Pad Software, San Diego, CA, USA).

### *2.7. Quantitative SARS-CoV-2 RT-PCR*

Confluent Vero E6 cells grown in 24-well plates were infected with 500 $TCID_{50}$ and simultaneously treated with 1 mg/mL of glycyrrhizin. Supernatants were collected at seven different time points (0 h, 4 h, 8 h, 24 h, 28 h, 32 h and 48 h) post-infection. Viral RNA was purified from the supernatants with the "High Pure Viral RNA Kit" (Roche Diagnostics, Basel, Switzerland), and the genomic SARS-CoV-2 RNA was quantified via RT-qPCR. Therefore, primer targeting the viral M or N gene were used [7]. M and N gene copy numbers were assessed using a 1:10 plasmid dilution series as reference (details and sequence information available upon request).

### *2.8. Determination of Main Protease Inhibition*

The inhibition of SARS-CoV-2 $M^{pro}$ by glycyrrhizin was measured using the "Fluorogenic 3CL Protease, MBP-tagged (SARS-CoV-2) Assay Kit" (BPS Bioscience, San Diego, CA, USA). Briefly, 90 ng of recombinant SARS-CoV-2 $M^{pro}$ were incubated with two different concentrations of glycyrrhizin (30 µM and 2000 µM, dissolved in water) in a 96-well microtiter plate at room temperature for 30 min. As control, the protease inhibitor GC376 was used. The enzyme activity was measured using Mithras LB 943 (Berthold Technologies, Oak Ridge, TN, USA) at 360 nm excitation and 460 nm emission after overnight incubation of the inhibitor-$M^{pro}$ mixtures with the added substrate (Dabcyl-KTSAVLQ↓SGFRKM-E(Edans)-NH2) at room temperature. Data were analyzed using GraphPad Prism 8.0.1 (Graph Pad Software, San Diego, CA, United States). Statistical analysis of inhibition of the protease activity was performed with non-parametric ANOVA (Kruskal–Wallis) and post hoc Dunn's multiple-comparisons test. Comparisons were considered significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

## 3. Results

### *3.1. Antiviral Activity of Licorice Root Extract*

Licorice root extract is of great importance in traditional medicine and was shown to be effective against coronaviruses and other viruses [3,4]. In the present study, we investigated the antiviral efficacy of licorice root as a part of the Glycyrrhiza glabra plant against SARS-CoV-2. Initially, we investigated the antiviral activity of an aqueous licorice root extract

against SARS-CoV-2 in vitro. Various concentrations of the extract (0.004–4 mg/mL) were pre-incubated with a viral load of 100 $TCID_{50}$ of SARS-CoV-2 for 1 h and subsequently added to confluent Vero E6 cells. Plaque formation was visualized after 48 h of infection using crystal violet. Aqueous licorice root extract showed antiviral effects even at a subtoxic concentration of 2 mg/mL (Figure 1A,B). This concentration is lower than the normal consuming dilution, e.g., in tea (12.5 mg/mL). Although licorice root tea may represent a good candidate for complementary use, the identification and characterization of the active compound is of great importance for a potential clinical application.

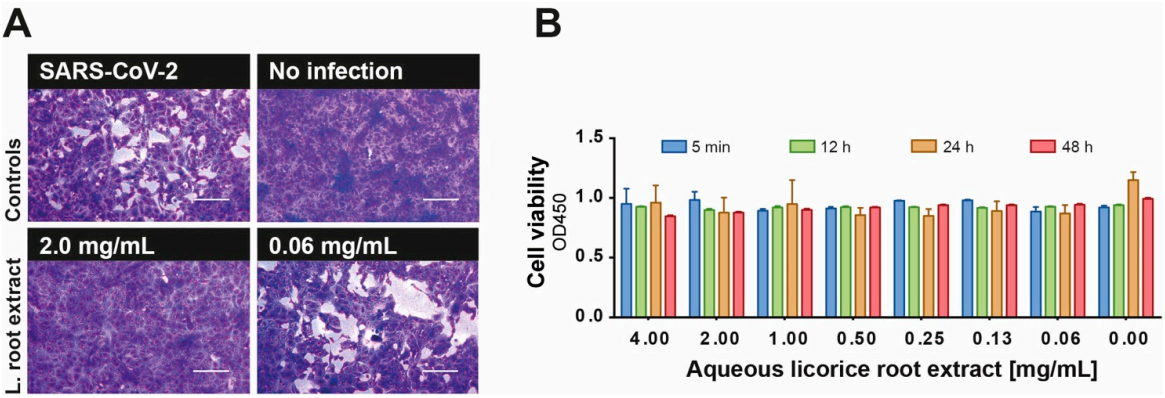


**Figure 1.** Potent inhibition of SARS-CoV-2 replication by the licorice root extract. (**A**) Decreasing aqueous licorice root extract concentrations (0.004–4 mg/mL) were pre-incubated with 100 $TCID_{50}$/mL SARS-CoV-2 for 1 h at 37 °C and applied to a confluent layer of Vero E6 cells. After 48 h of incubation, cell cultures were stained with crystal violet and analyzed for plaque formation. Representative pictures of protected and infected cell cultures at the indicated concentrations are shown. Bars represent 200 µm. (**B**) Cytotoxicity of aqueous licorice root extract towards Vero E6 cells was tested using "Orangu cell counting solution". Different concentrations of the extract were incubated with a confluent layer of Vero E6 cells and the cell viability was evaluated at four different time points (5 min, 12 h, 24 h, 48 h). All experiments were performed in biological replicates. Error bars represent the standard error of the mean (SEM).

### *3.2. Effective Inhibition of SARS-Cov-2 Replication by Glycyrrhizin*

Glycyrrhizin, the main ingredient of the licorice root, was shown to exhibit antiviral activity against viruses such as herpes simplex virus, human immunodeficient virus and other coronaviruses [3,4]. Therefore, we hypothesized that aqueous licorice root extract may inhibit SARS-CoV-2 replication due to its high content of glycyrrhizin. For this reason, we investigated its antiviral activity of glycyrrhizin against SARS-CoV-2 in vitro under pre- and post-entry conditions. Descending concentrations of glycyrrhizin (0.002–4 mg/mL) were pre-incubated with 100 $TCID_{50}$ SARS-CoV-2 for 1 h at 37 °C and the mixtures were subsequently incubated on confluent Vero E6 cells for 48 h (combined pre- and post-entry conditions). Additionally, Vero E6 cells were inoculated with 100 $TCID_{50}$ SARS-CoV-2 for 4 h before the glycyrrhizin-containing inoculation medium with various glycyrrhizin concentrations (0.002–4 mg/mL end-concentration) was added (post-entry conditions). Plaque formation was evaluated after 48 h post infection (p.i.). SARS-CoV-2 replication was completely blocked by glycyrrhizin at a concentration of 0.5 mg/mL (combined pre- and post-entry conditions) or 1 mg/mL (post-entry conditions) (Figure 2A).

In order to exclude the possibility of toxic-associated effects of glycyrrhizin to Vero E6 cells, we analyzed the impact of various concentrations of glycyrrhizin on the cell viability. No cytotoxic effect could be observed even at a concentration of 4 mg/mL (Figure 2B).

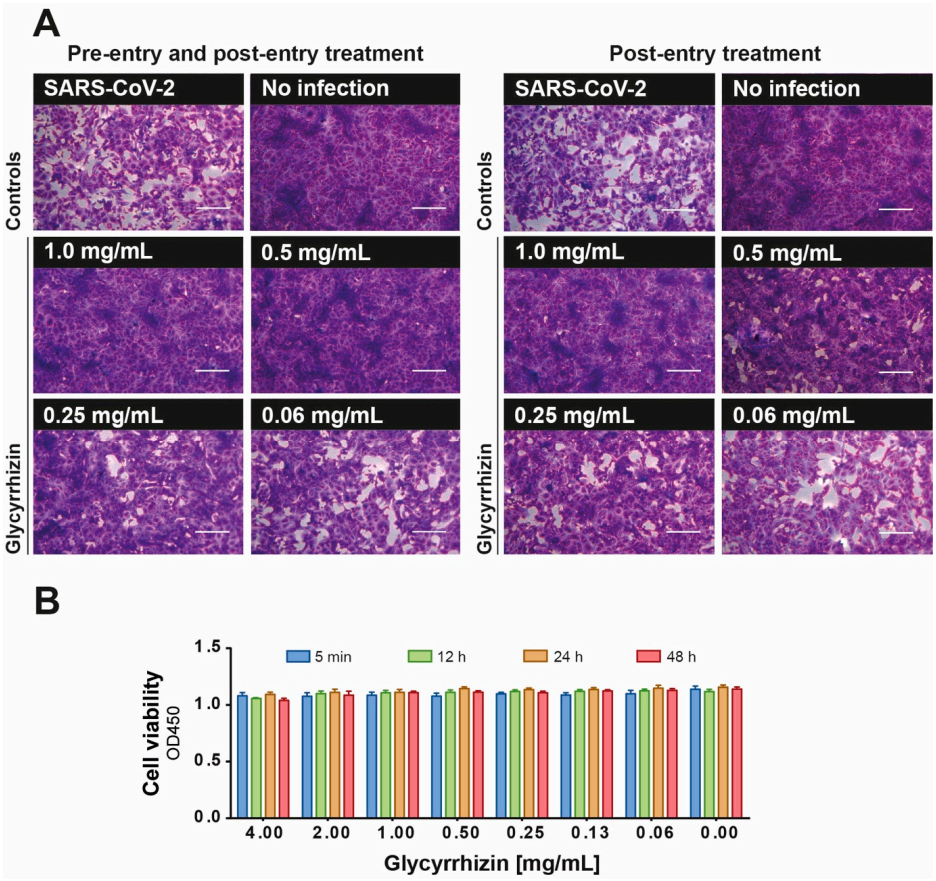


**Figure 2.** Antiviral efficacy of glycyrrhizin against SARS-CoV-2. The antiviral efficacy of glycyrrhizin against SARS-CoV-2 was investigated under combined pre- and post-entry or post-entry conditions. (**A**) Descending concentrations of glycyrrhizin (0.002–4 mg/mL) were pre-incubated with 100 $TCID_{50}$ SARS-CoV-2 for 1 h at 37 °C and subsequently added to confluent Vero E6 cells in 96-well microtiter plates for 48 h (combined pre- and post-entry conditions). In a second approach, Vero E6 cells were inoculated with 100 $TCID_{50}$ SARS-CoV-2 for 4 h before the glycyrrhizin-containing inoculation medium with various glycyrrhizin concentrations (0.002–4 mg/mL end-concentration) was added (post-entry conditions). Plaque formation was evaluated after 48 h post infection (p.i.). Bars represent 200 µm. (**B**) Cytotoxicity of glycyrrhizin used at the indicated concentrations towards Vero E6 cells was tested using "Orangu cell counting solution". Different concentrations of glycyrrhizin were incubated with a confluent layer of Vero E6 cells and the cell viability was evaluated at four different time points (5 min, 12 h, 24 h, 48 h). All experiments were performed in biological replicates. Error bars represent the standard error of the mean (SEM).

### *3.3. Quantitative Analysis of the Antiviral Activity of Glycyrrhizin Against SARS-Cov-2*

Next, we determined the half-maximal effective concentration ($EC_{50}$) of glycyrrhizin against SARS-CoV-2 and investigated the impact of glycyrrhizin on SARS-CoV-2 replication on RNA level. Therefore, confluent Vero E6 cells were infected with 10,000 $TCID_{50}$ SARS-CoV-2 and treated with various concentrations of glycyrrhizin ranging from 0.0625 to 4 mg/mL. After 48 h, the cell culture supernatants were titrated and the $EC_{50}$ value was determined. The $EC_{50}$ was calculated with 0.44 mg/mL, uncovering glycyrrhizin as a potent compound effective against SARS-CoV-2 (Figure 3). The initial finding was supported by quantifying the SARS-CoV-2 RNA from the supernatants of SARS-CoV-2 infected cells treated with glycyrrhizin. Vero E6 cells were infected with $1 \times 10^4$ $TCID_{50}$ SARS-CoV-2 and treated with 1 mg/mL glycyrrhizin. SARS-CoV-2 RNA levels were quantified at 0, 4, 8, 24, 28, 32 and 48 h post infection. Untreated Vero E6 cells served as control. Glycyrrhizin treatment significantly reduced SARS-CoV-2 RNA levels in cell

culture supernatants, indicating a potent antiviral activity of glycyrrhizin against SARS-CoV-2 (Figure 4). Taken together, we demonstrated that glycyrrhizin exhibited a high antiviral activity against SARS-CoV-2 and completely inhibited the viral replication at subtoxic concentrations.

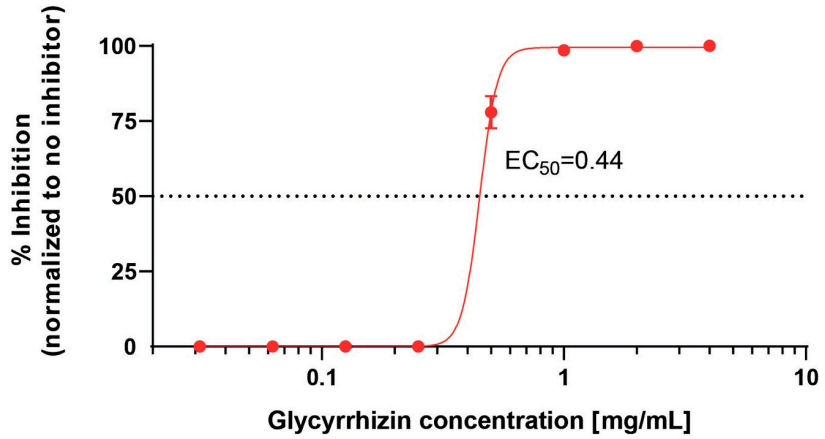


**Figure 3.** Dose-dependent activity of glycyrrhizin against SARS-CoV-2. Vero E6 cells were infected with 10,000 $TCID_{50}$ and treated with serial dilutions of glycyrrhizin (0.004−4 mg/mL), respectively. After 48 h of infection, cell culture supernatants were harvested and the viral loads were determined using endpoint dilution. The experiment was performed in technical triplicates. The $EC_{50}$ value was calculated using GraphPad Prism. Error bars display the standard error of the mean (SEM).

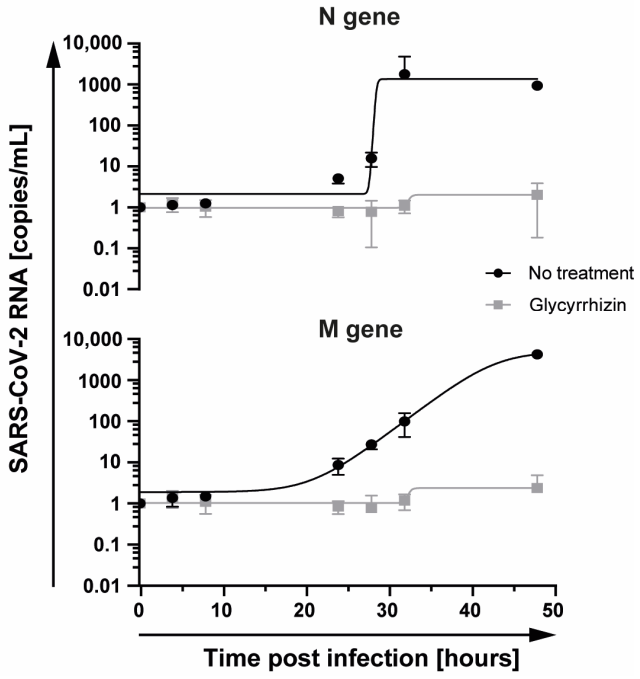


**Figure 4.** Glycyrrhizin treatment reduces SARS-CoV-2 RNA levels in cell culture supernatant. Vero E6 cells were infected with 500 $TCID_{50}$ SARS CoV-2 and treated with glycyrrhizin at 1 mg/mL. Total RNA was extracted from the supernatants harvested at 0, 4, 8, 24, 28, 32 and 48 h post infection. SARS-CoV-2 RNA was quantified by determining the amounts of the M and N gene using RT-qPCR. All experiments were performed in biological replicates. Error bars displays the standard error of the mean (SEM).

*3.4. Inhibition of the Viral Main Protease by Glycyrrhizin*

Next, we investigated the underlying mechanism of how glycyrrhizin may interfere with the virus replication. Recently, protease inhibitory activity of glycyrrhizin was predicted using in silico simulations [8]. The SARS-CoV-2 main protease $M^{pro}$, also known as the 3CL protease, plays a vital role in processing the viral polyproteins that are translated from SARS-CoV-2 RNA. This process is essential for the virus replication. Glycyrrhizin was suggested as a possible inhibitor of the viral main protease $M^{pro}$, but this hypothesis has not been experimentally proven, yet [9]. Thus, we investigated whether glycyrrhizin may inhibit the proteolytic activity of the SARS-CoV-2 $M^{pro}$ by using a 3CL protease activity kit [10]. Different glycyrrhizin concentrations (30 µM and 2000 µM) were dissolved in 0.5 M DTT Buffer containing Mpro at a concentration of 3 ng/µL and incubated for 30 min at room temperature. As control, the protease inhibitor GC376 was used at 100 µM. Subsequently, the 3CL Protease (SARS-CoV-1/SARS-CoV-2) substrate was added and the mixture was incubated overnight. Protease activity was measured by determining the enzymatic reaction of the substrate at OD 360nm/460nm (*exc/em*). Glycyrrhizin completely inhibited $M^{pro}$ activity at a concentration of 2000 µM (1.6 mg/mL) and reduced its activity by 70.3% at a concentration of 30 µM (0.024 mg/mL) (Figure 5). For the first time, we could demonstrate that glycyrrhizin inhibits the SARS-CoV-2 main protease (3CL protease), thereby potently blocking the viral replication.

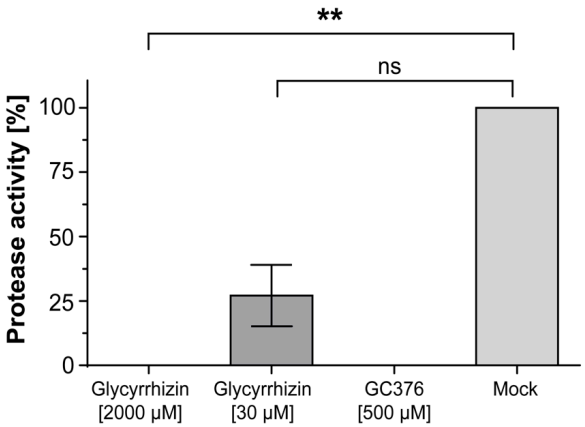


**Figure 5.** Glycyrrhizin potently inhibits the viral main protease ($M^{pro}$). The inhibition of SARS-CoV-2 $M^{pro}$ by glycyrrhizin was measured using the "Fluorogenic 3CL Protease, MBP-tagged (SARS-CoV-2) Assay Kit". Different glycyrrhizin concentrations (30 µM and 2000 µM) and the complete protease inhibitor GC376 (100 µM) were dissolved with 90 ng $M^{pro}$ in 30 µL 0.5 M DTT Buffer and incubated for 30 min at room temperature. Subsequently, the 3CL Protease (SARS-CoV-1/SARS-CoV-2) substrate was added and the protease activity was measured after overnight incubation at a wavelength of 360 nm/460nm (*exc/em*). The experiment was performed in triplicates. Measurement was performed using Mithras LB 943 after 12 h incubation. Statistical analysis was undertaken with a non-parametric ANOVA test. Comparisons were considered significant at ** $p < 0.01$ and n.s. = no significance. All experiments were performed in biological replicates. Error bars represent the standard derivation of the mean (SD).

## 4. Discussion

The newly emerged coronavirus SARS-CoV-2 is continuing to spread around the world thereby causing major public health issues. Remdesivir is currently the only medication approved by the FDA to treat COVID-19. Corticosteroids such as dexamethasone are used to treat the sickest patients with COVID-19 suffering from a hyperinflammatory immune response [11]. Despite some recently approved vaccines in the last months, there is still a great medical need for novel, well tolerated and broadly accessible interventions. Herbal medicine revealed to be a promising option for the treatment of various viral infections. In the present study, we investigated the antiviral efficacy of licorice root and its main

ingredient glycyrrhizin against SARS-CoV-2. We demonstrated that glycyrrhizin potently inhibits SARS-CoV-2 replication in cell culture at subtoxic concentrations and identified the underlying mechanism.

Traditional medicine was shown to be of great importance in the development of modern medication. The primary active compound of the licorice root, glycyrrhizin, was evaluated as a liver protective in the treatment of chronic hepatitis B and C patients [12,13]. Of note, glycyrrhizin was clinically evaluated in the context of pharmacokinetic analyses and described to be a safe and well-tolerated compound [14]. The pharmacological effects include antioxidative and anti-inflammatory, corticosteroid-like activities [15]. The side effects of glycyrrhizin, such as hypertension, fatal arrhythmias and renal failure after several weeks of consumption, are well known and are therefore predictable and controllable [16]. Clinical trials with Stronger Neo-Minophagen C (SNMC), which is an intravenous pharmaceutical with glycyrrhizin as the primary active compound, showed liver protective results in hepatitis B patients [12]. In prior studies, different mechanisms causing the beneficial effect of glycyrrhizin were discussed. The anti-inflammatory and mineralocorticoid potency could be explained by the inhibition of 11-beta-hydroxysteroid dehydrogenase (11βHSDH) leading to a higher cortisol levels [17]. The potent antiviral activity as well as anti-inflammatory properties highlight glycyrrhizin as an excellent candidate for further clinical investigations in COVID-19 treatment.

The pharmacokinetic in rats showed that enterohepatic metabolism and biliary excretion play a major role in the drug clearance, and higher bioavailability of glycyrrhizin is given after intravenous or intraperitoneal than oral application [18]. After oral administration of 1500 mg of glycyrrhizin acid in healthy humans, the mean plasma concentration after 24 h was 4 mg/mL, which is more than four times higher than the calculated $EC_{50}$ value determined for the inhibition of SARS-CoV-2 replication in our study [19]. These findings demonstrate that antiviral active levels of glycyrrhizin are reachable and well tolerated in humans. The drug's half-time ($t_{1/2}$) of glycyrrhizin in healthy males was described as 3.5 h[18]. In contrast, in human patients with chronic hepatitis C glycyrrhizin's half-live after intravenous application was around 9 h [13]. In this study group, pharmacokinetics was linear up to 200 mg and steady-state was reached after 12 doses of 200 mg glycyrrhizin per day[19]. However, the glycyrrhizin dose required to reach a therapeutically effective concentration in the respiratory tract needs to be determined in subsequent studies. A case report described compassionate use of glycyrrhizin among other potential antivirals for the treatment of COVID-19 [20]. The patient received 150 mg glycyrrhizin 3 times per day for 8 days. The patient's condition started improving after 12 h of treatment [20]. Although the patient recovered from disease, further controlled studies are needed to prove the therapeutic effects of glycyrrhizin in COVID-19.

Glycyrrhizin was discussed in in silico simulations as a potential protease inhibitor [8,9]. Beside the viral main protease ($M^{pro}$), the human transmembrane serine protease (TMPRSS2) is another discussed target of glycyrrhizin. TMPRSS2 was shown to cleave the SARS-CoV-2 spike protein thereby facilitating the entry of the virus into the host cell [21]. However, since there was only a slight difference in antiviral activity of glycyrrhizin between pre- and post-entry conditions, and only a minor affinity was simulated for the interaction between glycyrrhizin and TMPRSS2 in former in silico studies [21], we concluded that glycyrrhizin blocks SARS-CoV-2 replication mainly via a mechanism different from inhibiting TMPRSS2. Thus, we focused on the SARS-CoV-2 main protease ($M^{pro}$) as a potential target for glycyrrhizin [22]. $M^{pro}$ is essential for processing the viral polyproteins that are translated from the viral RNA and, thus, for virus replication [22]. Therefore, the inhibition of the SARS-CoV-2 main protease would inhibit the viral replication. Our experiments uncovered the inhibitory activity of glycyrrhizin against $M^{pro}$ and confirm former data from different in silico simulations [8,22]. An inhibitory activity of glycyrrhizin towards TMPRSS2 or other enzymes cannot be completely excluded. However, since there was only a slight difference in the concentration of glycyrrhizin required for complete inhibition of viral replication under combined pre- and post-entry (0.5 mg/mL) or post-entry

(1 mg/mL) conditions, we concluded that glycyrrhizin blocks SARS-CoV-2 replication mainly by inhibiting the main protease $M^{pro}$.

Taken together, we demonstrated that glycyrrhizin, the primary active ingredient of the licorice root, potently blocks SARS-CoV-2 replication by inhibiting the viral main protease. Our experiments highlight glycyrrhizin as a potential antiviral compound that should be further investigated for the treatment of COVID-19.

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# 5 Discussion

The SARS-CoV-2 pandemic had a devastating impact worldwide, causing millions of COVID-19 cases and deaths (World Health Organization, 2023d). The fast transmission of SARS-CoV-2 required a rapid response of scientists worldwide on different levels. These ranged from the development of effective preventive measures to therapies, which required an understanding of immunity induced by infection, treatment, and vaccination. Over the course of the pandemic, the SARS-CoV-2 virus continuously evolved, leading to an increase of highly transmissible VOCs harboring numerous immune escape mutations (European Centre for Disease Prevention and Control, 2023). The constant evolution of SARS-CoV-2 makes the continuous research for effective treatment strategies even more important.

In the context of the present work, we investigated the effectiveness of commercially available UVC-LED boxes for disinfection of SARS-CoV-2 contaminated surfaces to evaluate the potency of UVC irradiation to prevent fomite transmission. Furthermore, we investigated the antiviral activity of the traditional medicinal herbs turmeric root and licorice root against SARS-CoV-2 as well as the immune responses in immunocompetent and immunocompromised individuals.

## 5.1 Effectiveness UVC light in disinfecting SARS-CoV-2

SARS-CoV-2 can be transmitted through inhalation of virus-containing particles and through virus-contaminated objects (Marr and Tang, 2021, Duval et al., 2022, Bak et al., 2021). In the present work, two commercially available disinfection boxes for personal items, which were equipped with UVC-LEDs on the sides or in the lid, were tested for their ability to inactivate SARS-CoV-2 (Bormann et al., 2021b). Glass, metal, and plastic carriers that had previously been inoculated with a high viral load of SARS-CoV-2 were placed at different distances from the LEDs and irradiated for different lengths of time. We showed that both UVC-LED boxes were effective in inactivating SARS-CoV-2 on all used materials.

When the carriers were placed at 1 cm from the LEDs, the disinfection box equipped with side LEDs emitting UVC light at 254 nm (box 1) inactivated SARS-CoV-2 to the detection limit at doses ranging from 44 to 147 mJ/cm$^2$ in 3 to 10 minutes. The disinfection box with LEDs in the lid emitting UVC light at 280 nm (box 2) inactivated the virus to the detection limit when the carriers were placed at the bottom in 10 minutes (unable to identify dose). Box 2 was equipped with a mirror and the light reflection may have contributed to an even disinfection of the carriers placed at the bottom (de Sternberg Stojalowski and Fairfoull, 2021). However, at 1 cm from the LEDs, this box did not achieve complete SARS-CoV-2 inactivation at the highest dose of 70.2 mJ/cm$^2$.

Other data showed an effective inactivation of SARS-CoV-2 (99.9 %) on stainless steel, glass, and plastic after UVC irradiation at 254 nm with doses ranging from 10.3 to 23.7 mJ/cm$^2$ using a monochromatic UVC lamp (Gidari et al., 2021). In another study, a chamber with UVC mercury lamps (254 nm) was used to disinfect dried SARS-CoV-2 on plastic and the virus was reduced to below detection levels at a dose of 7.6 mJ/cm$^2$ (Storm et al., 2020). In contrast to our work, these studies did not dilute the virus with an organic matrix mimicking respiratory secretions prior to irradiation, which may explain the higher effective doses in our study. The organic matrix might protect the virus against UVC inactivation, as has previously been reported for sunlight exposure (Sloan et al., 2021).

Besides respiratory secretions and the dose of UVC applied the wavelength of the UVC light can be a decisive factor for disinfection efficacy. In our study, the peak wavelength emission for box 1 was measured at 254 nm and for box 2 at 280 nm. Studies showed that LEDs at 254 nm are more effective in inactivating SARS-CoV-2 than LEDs with longer wavelengths (Mariita and Peterson, 2021, Matsuura et al., 2022). These findings may explain the less effective disinfection capacity of box 2 when the carriers were placed at 1 cm from the LEDs. In comparison to UVC light, UVA and UVB light is less effective in inactivating SARS-CoV-2 (Biasin et al., 2022, Heilingloh et al., 2020).

In addition to disinfecting personal items such as smartphones, coins or credit cards, commercially available UVC-LED chambers may also be useful in the hospital setting for disinfecting personal protective equipment. UVC disinfection may be a valuable tool for reusing or disposing contaminated protective equipment such as masks. Data indicated that UVC treatment with a dose of 0.45 J/cm$^2$ effectively decontaminated N95 respirators, surgical masks, and cotton fabric masks from SARS-CoV-2 (Metolina et al., 2022).

We showed that UVC-LED boxes can effectively inactivate SARS-CoV-2, making them an affordable and environmentally friendly option for disinfecting personal items. Factors such as wavelength, dose, use of reflective material, and the position of the LEDs and contaminated objects proved to be critical for the effectivity of these boxes.

## 5.2 Natural products as antivirals against SARS-CoV-2

Natural products have a wide range of bioactive ingredients that can be used for the development of medicines (Español et al., 2021). Especially in developing countries, natural products may be an accessible treatment option for COVID-19. In the present work, we investigated the antiviral activity of curcumin from turmeric root and glycyrrhizin from licorice root against SARS-CoV-2 (Bormann et al., 2021a, van de Sand et al., 2021).

For both natural products, we determined the half-maximal effective concentration ($EC_{50}$) for the neutralization of SARS-CoV-2 on Vero E6 cells. We found an $EC_{50}$ of 7.9 µg/mL (21.5 µM) for curcumin and an $EC_{50}$ of 440 µg/mL (534.7 µM) for glycyrrhizin. Furthermore, we showed

that both compounds effectively reduced SARS-CoV-2 RNA levels in cell culture supernatants. Another study found an $EC_{50}$ ranging from 3.6 to 6 µg/mL for curcumin, depending on whether Vero E6 cells were treated with curcumin after infection, before infection or simultaneously (Marín-Palma et al., 2021).

Given that curcumin potently neutralized SARS-CoV-2, we hypothesized that inhibition of viral entry by curcumin is a possible mechanism of action (Bormann et al., 2021a). This theory is supported by the finding that SARS-CoV-2 was more strongly inhibited when cells were treated with curcumin either before or simultaneously with infection than after infection (Marín-Palma et al., 2021). Inhibition of cellular entry of SARS-CoV-2 through curcumin might be achieved by preventing the binding of the RBD to ACE2. Indeed, a binding assay showed that curcumin can significantly inhibit the binding of the RBD to ACE2 starting from a concentration of 2.5 µg/mL (Goc et al., 2021). The authors also found that curcumin can decrease the activity of TMPRSS2, which is involved in the activation of the S protein upon binding of RBD to ACE2. Other studies investigated the ability of curcumin to suppress SARS-CoV-2 replication by inhibiting $M^{pro}$. Guijarro-Real et al. (2021) showed that 75 µg/mL (203.6 µM) curcumin decreased residual $M^{pro}$ activity to 28.1 %. However, as turmeric showed complete inhibition of $M^{pro}$, the authors suggested that other components of turmeric may play a stronger role than curcumin in inhibiting $M^{pro}$. In contrast to the findings of Guijarro-Real et al. (2021), Bahun et al. (2022) found a strong inhibition of $M^{pro}$ by curcumin with an $EC_{50}$ of 11.9 µM.

In the present work, we discovered glycyrrhizin as an inhibitor of $M^{pro}$ (van de Sand et al., 2021). 30 µM of glycyrrhizin reduced protease activity by 70.3 % and complete inhibition was achieved at a concentration of 2000 µM. Contradictory to these findings, in another study glycyrrhizin failed to achieve 50 % reduction of $M^{pro}$ (He et al., 2022). Nevertheless, the authors showed that glycyrrhizin, as previously described for curcumin, prevents cell entry by inhibiting binding of the S protein to ACE2. Consistent with these results, other data suggest that glycyrrhizin blocks cellular binding by targeting the S protein with high affinity (Li et al., 2021).

In addition to the antiviral effect of curcumin and glycyrrhizin, both compounds are associated with an anti-inflammatory activity in the literature. As a storm of proinflammatory cytokines has been observed in patients with severe COVID-19 (Liu et al., 2020a, Silva et al., 2023), the anti-inflammatory activity of these compounds might have a beneficial effect on disease progression. Curcumin encapsuled nanoparticles showed an inhibition of the release of cytokines and chemokines *in vitro* by deactivating nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling (Sharma et al., 2022). Clinical trials indicated a decreased serum and expression level of IL-6 and IL-1ß after treatment of COVID-19 patients with nano-curcumin (Asadirad et al., 2022, Valizadeh et al., 2020). Furthermore, an increase of regulatory T (Treg) cells was observed in COVID-19 patients after nano-curcumin treatment as well as a reduction of T helper 17 (Th17) cells and their related inflammatory cytokines

(Tahmasebi et al., 2021a, Tahmasebi et al., 2021b). Similar to curcumin, glycyrrhizin attenuated the release of the proinflammatory cytokines IL-1ß, IL-6 and IL-8 *in vitro* (Gowda et al., 2021).

Glycyrrhizin can be administered orally and intravenously in humans. After oral administration of a clinical dose of 75 mg glycyrrhizin to healthy volunteers, an average peak plasma concentration of 24.8 ng/mL was found (Suzuki et al., 2017). Before absorption via the intestine, glycyrrhizin is metabolized to glycyrrhetinic acid. After an oral dose of 75 mg glycyrrhizin, the average peak plasma concentration of glycyrrhetinic acid was 200.3 ng/mL (Suzuki et al., 2017). Since glycyrrhizin is poorly absorbed from the gastro-intestinal tract, higher plasma levels can be achieved by intravenous administration. Directly after intravenous administration of 80 mg glycyrrhizin, plasma levels of about 30 µg/mL can be achieved in humans (Yamamura et al., 1992). After an oral dose of 10 g curcumin, only one of six healthy volunteers had detectable levels of curcumin in plasma (Vareed et al., 2008). However, in all volunteers, curcumin glucuronides and sulfates were detected with a mean peak concentration of 2.3 µg/mL (Vareed et al., 2008). In another study, average peak serum concentrations of 1.8 µM were observed after administration of 8 g curcumin (Cheng et al., 2001). The low blood levels of curcumin can be explained by its low solubility and rapid metabolic degradation and conjugation upon oral administration (Vareed et al., 2008, Bolger et al., 2022).

Both curcumin and glycyrrhizin have low bioavailability, which can be increased using various novel drug delivery systems. These drug delivery systems are based on nanostructured carriers including nanoparticles, micelles, nanogels, liposomes, microemulsions and nanoemulsions (Dourado et al., 2021, Cai et al., 2016). Nanosystems have the ability to enhance drug solubility and diffusion and to protect drugs from metabolization or chemical degradation (Pires and Santos, 2018).

Several clinical trials have investigated the effect of curcumin, mainly in the form of nano-curcumin, on disease progression in COVID-19 patients. A meta-analysis summarized the results from randomized controlled trials published until October 2022 (Shafiee et al., 2023). The pooled result of seven studies showed that curcumin significantly reduced the risk of mortality and improved the recovery from COVID-19. However, no definitive conclusion can be drawn due to small sample sizes and risk of bias. Large-scale randomized controlled trials are needed to confirm the results.

Both curcumin and glycyrrhizin have been described as safe and well-tolerated compounds. Turmeric root and licorice root are generally recognized as safe by the U.S. Food & Drug Administration (FDA) (2023a, 2023b). Doses of up to 12 g curcumin per day were described as well tolerated and safe in clinical trials (Shojaei et al., 2023). For glycyrrhizin, doses of 2 mg per kg body weight over 8 weeks were described as safe (van Gelderen et al., 2000). However, it should be noted that excessive consumption of glycyrrhizin over a long period can lead to

hyperaldosteronism due to inhibition of 11-beta-hydroxysteroid dehydrogenase type-2 (11βHSDH) (McHugh et al., 2021). Symptoms of hyperaldosteronism include hypertension, headache, and muscle weakness (McHugh et al., 2021).

In conclusion, due to their antiviral and anti-inflammatory activity, curcumin and glycyrrhizin are promising compounds for the complementary treatment of COVID-19. Studies indicate that their antiviral activity is based on inhibiting the cellular entry of SARS-CoV-2, viral replication, or both. Further studies are needed to investigate their exact mechanism of action and to evaluate how the compounds affect the progression of COVID-19.

## 5.3 Immunity against SARS-CoV-2

Various VOCs emerged in Germany during the COVID-19 pandemic, including Delta in December 2020, which was replaced by Omicron in November 2021 as the main circulating variant (European Centre for Disease Prevention and Control, 2023). The reduced neutralizing activity of the sera from primary vaccinated individuals led to an increase of vaccine breakthrough infections, particularly with the Omicron variant (Tuekprakhon et al., 2022, Edara et al., 2022, Cele et al., 2022, Christensen et al., 2022). In the present study, we investigated the humoral and cellular immune responses of hospitalized COVID-19 patients during breakthrough infection with Delta and the Omicron sub-variants BA.1 and BA.5 (Bormann et al., 2023). Insights into infection-induced immunity are important for understanding whether infections, similar to vaccinations, can induce protective immunity against SARS-CoV-2.

Our data demonstrated an overall strongly reduced neutralizing activity of sera from vaccinated individuals or patients with SARS-CoV-2 breakthrough infection against BA.1 and BA.5, independent of the number of vaccinations and the variant causing the infection. Consistent with these results, other studies showed that sera from vaccinated or previously infected individuals exhibited decreased neutralization against BA.1 and BA.5 compared to the ancestral strain (Tuekprakhon et al., 2022, Hachmann et al., 2022, Cao et al., 2022). In our study and in the literature, the BA.5 variant showed the strongest immune escape compared to previous Omicron sub-variants and other SARS-CoV-2 variants. Meanwhile, several new sub-lineages of Omicron emerged with additional spike mutations with immune-evading potential, including BA.2.75.2, BQ.1.1 and XBB.1 (Hodcroft, 2021). Sera of patients with monovalent and bivalent mRNA boosters showed an even stronger reduced neutralizing activity against these sub-lineages compared to BA.5 (Hoffmann et al., 2023, Zou et al., 2023, Davis-Gardner et al., 2023).

We found variant specific immune responses when we examined the immunity of patients with breakthrough infections. Patients with Delta breakthrough infection showed higher levels of nucleocapsid protein (NCP) binding IgM antibodies and IFN-γ spots when stimulating peripheral blood mononuclear cells (PBMCs) with NCP compared to patients with BA.1 or BA.5

infection. Similarly, Błaszczuk et al. (2022) found a higher level of NCP antibodies after Delta infection compared to Omicron infection. These findings are coherent with data showing a delayed and lower immune response after Omicron breakthrough compared to Delta breakthrough infections (Koutsakos et al., 2022). The lower immune response against Omicron might be an indication of a reduced pathogenicity of the variant compared to Delta. Indeed, studies showed a reduced number of hospitalizations and case fatality rate for Omicron BA.1, BA.2 and BA.4/5 infected patients compared to Delta patients (Wolter et al., 2022b, Sievers et al., 2022). In addition to a reduced pathogenicity of Omicron, pre-existing immunity from vaccination may also explain the milder progression (Sigal, 2022). However, Omicron infections resulted in a strongly lower number of patients requiring supplemental oxygen than Delta infections in a setting where all patients were vaccinated (Goga et al., 2022).

Delta and Omicron-BA.1 breakthrough infections both boosted the neutralizing immunity in our study. In agreement with previous data, Delta infections markedly increased the neutralizing antibody titers against Delta (Servellita et al., 2022). Furthermore, we and others found enhanced neutralizing antibody titers against Omicrom-BA.1 in sera from patients with BA.1 breakthrough infections (Quandt et al., 2022). In agreement with our results, Seaman et al. (2022) sowed that Omicron-BA.1 breakthrough infections induce a broad response against other variants, including Delta. Interestingly, patients with BA.5 breakthrough infection showed no boosted neutralizing antibody titer against BA.5 or other variants in our study. Therefore, we hypothesized that these patients may be more susceptible to reinfection with BA.5 or other emerging SARS-CoV-2 variants. Recently published data indicated that BA.4/5 infections after three doses of monovalent mRNA vaccination may enhance neutralizing antibody titers against BA.4/5 (Wang et al., 2023). However, in contrast to the BA.5 infected patients in our study, the convalescent patients were younger (median age of 46 vs. 69). In addition, in our study the serum was collected during the acute phase of infection whereas the sera in the study published by Wang et al. (2023) were collected at 32 days after infection. This could explain the different neutralizing properties of patient sera.

Studies investigating the immune response after bivalent mRNA booster vaccination demonstrated that the neutralizing antibody titer against the ancestral antigen was higher than the titer against the adjusted antigen (Chalkias et al., 2022, Zou et al., 2023, Collier et al., 2023). In our study, the strain-specific neutralizing antibody titer induced by Omicron breakthrough infection was generally lower than in patients with Delta breakthrough infection. A short follow-up time might overlook more long-term benefits of an Omicron infection or bivalent mRNA booster vaccination on immunity (Tang et al., 2023). As the Omicron sub-variants exhibit more antigenic differences from the ancestral strain than the Delta variant (Mykytyn et al., 2022), a stronger immune response against these variants might develop over a longer period of time.

When we stimulated PBMCs with peptides covering selectively the mutated regions in the S protein of Alpha, Delta, and Omicron, we observed only a weak IFN-γ response. One reason for this finding may be that the samples were collected too early after the infection to detect strong T cell immunity. Karsten et al. (2022) found a strong response of T cells against the S protein after infection and vaccination. In line with these data, we found a median IFN-γ spots increment above positivity after stimulation with the S protein of the ancestral strain. A recent study showed that human leukocyte antigen (HLA) class I and class II presented T-cell epitopes of the S protein are highly conserved, in contrast to the loss of a large proportion of neutralizing B-cell epitopes with the emergence of Omicron (Alexander et al., 2022). The authors concluded that CD8+ and CD4+ T-cell recognition of Omicron sub-lineages may be largely intact. This finding suggests that T cells play an essential role in maintaining immunity against Omicron.

As immunocompromised patients are a risk group for a fatal course of COVID-19 (Zhang et al., 2023, Gao et al., 2020, Caillard et al., 2021), it is important to assess the efficacy of vaccination in these individuals. Immunosuppressive drugs can impair the immune function of these patients by suppressing the generation of protective antibodies, B cells and T cells (Lederer et al., 2022). In the present work, we investigated the humoral and cellular immunity of immunosuppressed KTX patients after the first or second booster dose compared to immunocompetent individuals against the ancestral SARS-CoV-2 strain and SARS-CoV-2 variants (Thümmler et al., 2022).

We showed that KTX patients had a significantly lower cellular IFN-γ response to wildtype and Omicron S protein compared to healthy controls, whereas the IL-2 response was not different. Consistent with this finding, administration of a first booster dose in KTX patients resulted in increased levels of IL-2 and IL-4 secreting $CD4^+$ T cells, but not IFN-γ secreting $CD4^+$ T cells (Schrezenmeier et al., 2021). Generally, transplant recipients can benefit from booster vaccination by increasing SARS-CoV-2 neutralizing antibodies and SARS-CoV-2 specific B and T cells (Hall et al., 2021, Peled et al., 2022, Benotmane et al., 2021, Schrezenmeier et al., 2021). However, when compared to healthy controls, the immune response remains largely impaired in KTX patients (Stumpf et al., 2021, Rincon-Arevalo et al., 2021). In accordance with the literature, we demonstrated that KTX patients had reduced neutralizing antibodies compared to healthy controls after booster vaccination. KTX patients showed significantly decreased neutralizing antibody titers to SARS-CoV-2 variants, especially Omicron. Consistently, other studies found that organ transplant recipients have a poor neutralizing antibody response against Omicron after booster vaccination (Kumar et al., 2022, Moal et al., 2023). Certain types of immunosuppressants, including mycophenolate, which was used in 81% of the KTX patients in our study, may contribute to a markedly impaired immune response and low seroconversion rate (Stumpf et al., 2021). However, 64 % of the patients in our study

were able to generate potential neutralizing antibodies against SARS-CoV-2 variants. Overall, we concluded that KTX patients might be partly protected against SARS-CoV-2 by IL-2 producing T cells or neutralizing antibodies.

In addition to vaccination-induced immune responses, special attention must also be paid to the efficacy of antiviral therapy in immunocompromised patients. In our work, we assessed the humoral and cellular immunity in two KTX and two hemodialysis patients who received convalescent plasma treatment during infection with SARS-CoV-2 (Lindemann et al., 2021). The RKI generally advises against the use of convalescent plasma in COVID-19 patients due to insufficient data on its efficacy against Omicron (Robert Koch Institut, 2023b). Antiviral therapy with convalescent plasma may be considered for immunosuppressed patients, especially in the absence of an alternative treatment option (Robert Koch Institut, 2023b). Paxlovid® (nirmatrelvir/ ritonavir) and Veklury® (remdesivir) are authorized in the European Union for treatment of COVID-19. However, the use of Paxlovid® and Veklury® is contraindicated in patients with severe renal impairment with an estimated glomerular filtration rate (eGFR) of less than 30 mL/min (European Medicines Agency, 2023c, European Medicines Agency, 2023d).

The patients in our study showed an increase of S binding IgG antibodies, neutralizing antibodies and IFN- γ secreting T cells against SARS-CoV-2 closely after convalescent plasma treatment. Three patients experienced clinical improvement and were discharged from the hospital and one patient deceased. However, due to the small number of patients, we were unable to make a definitive conclusion about the efficacy of convalescent plasma. A recent meta-analysis found a reduced mortality in immunocompromised patients receiving convalescent plasma, highlighting convalescent plasma as a beneficial treatment option for this high-risk group (Senefeld et al., 2023).

To conclude, we showed that vaccine breakthrough infections are able to enhance vaccination-acquired immunity against SARS-CoV-2. However, the magnitude of the immune enhancing effect is dependent on the variant causing the breakthrough infection. Immunocompromised patients might be partly protected against SARS-CoV-2 through booster vaccination. In addition, this high-risk group might benefit from convalescent plasma treatment through enhanced SARS-CoV-2 specific immunity.

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# 7 Appendix

## 7.1 List of abbreviations

| | |
|---|---|
| ACE2 | Angiotensin-converting enzyme 2 |
| ARDS | Acute respiratory distress syndrome |
| CDC | Centers for Disease Control and Prevention |
| COVID-19 | Coronavirus disease 2019 |
| E | Envelope |
| $EC_{50}$ | half-maximal effective concentration |
| EMA | European Medicines Agency |
| FDA | U.S. Food & Drug Administration |
| FP | Fusion peptide |
| HR | Heptad repeat |
| IFNs | Interferons |
| IL | Interleukin |
| JAK-I | Janus kinase inhibitor |
| KTX | Kidney transplant |
| LEDs | Light emitting diodes |
| M | Membrane |
| mAbs | Monoclonal antibodies |
| MERS-CoV | Middle east respiratory syndrome-coronavirus |
| MODS | Multiple organ dysfunction syndrome |
| $M^{pro}$ | Main protease |
| mRNA | Messenger RNA |
| N | Nucleocapsid |
| nAbs | neutralizing antibodies |
| ORF | Open reading frame |
| PBMCs | Peripheral blood mononuclear cells |
| PHEIC | Public health emergency of international concern |
| RBD | Receptor-binding domain |
| RdRp | RNA-dependent RNA polymerase |
| RKI | Robert Koch Institute |
| RNA | Ribonucleic acid |
| S | Spike |
| SARS-CoV | Severe acute respiratory syndrome coronavirus |
| SARS-CoV-2 | Severe acute respiratory syndrome coronavirus 2 |
| STIKO | Standing Committee on Vaccination |
| TMPRSS2 | Transmembrane serine protease 2 |
| UV | Ultraviolet |
| VOCs | Variants of concern |
| WHO | World Health Organization |
| WHO | World Health Organization |

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## 7.3 List of publications

**Bormann, M.**, M. Alt, L. Schipper, L. van de Sand, M. Otte, T. L. Meister, U. Dittmer, O. Witzke, E. Steinmann, and A. Krawczyk. 2021. 'Disinfection of SARS-CoV-2 Contaminated Surfaces of Personal Items with UVC-LED Disinfection Boxes', *Viruses*, 13.

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## 7.5 Curriculum vitae

The curriculum vitae is not included for reasons of data protection.

## 7.6 Erklärungen

Hiermit erkläre ich, gem. § 6 Abs. (2) g) der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema *„COVID-19 – Immune defense and antiviral strategies for prevention and treatment“* zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von *Maren Bormann* befürworte und die Betreuung auch im Falle eines Weggangs, wenn nicht wichtige Gründe dem entgegenstehen, weiterführen werde.

Essen, den _____ _____
Prof. Dr. Adalbert Krawczyk

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