SARS-CoV-2: Immunantwort und antivirale Behandlung

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1. Zusammenfassung

1.1 Zusammenfassung

Die COVID-19-Pandemie, ausgelöst durch SARS-CoV-2, führte weltweit zu über 700 Millionen Infektionen und über sieben Millionen Todesfällen (Stand März 2024). Erkenntnisse über die durch Impfung und Infektion generierte Immunantwort sowie über die Wirksamkeit von Medikamenten gegen das Virus waren von zentraler Bedeutung. In der vorliegenden Arbeit haben wir die zelluläre und humorale Immunantwort bei verschiedenen Kohorten nach Impfung und Infektion sowie die Wirksamkeit verschiedener Medikamente gegen SARS-CoV-2 untersucht.

Der Schutz vulnerabler Gruppen vor einer Infektion ist besonders wichtig, da ein erhöhtes Risiko für einen schweren bis tödlichen Verlauf besteht. Wir untersuchten die zelluläre und humorale Immunantwort bei Nieren- und Stammzelltransplantierten nach Booster-Impfung gegen SARS-CoV-2. Beide Kohorten bildeten nach Auffrischungsimpfung eine T-Zell-Immunität und neutralisierende Antikörper aus, die zum Schutz vor einer Infektion mit SARS-CoV-2 beitragen könnten. Des Weiteren konnten wir zeigen, dass ungeimpfte, immunkompromittierte an COVID-19 erkrankte Personen eine zelluläre und humorale Immunantwort aufbauen.

Darüber hinaus analysierten wir die Wirksamkeit von Rekonvaleszentenplasma und monoklonalen Antikörpern zur Behandlung von COVID-19-Patienten. Im Vergleich zur symptomorientierten Therapie führten beide Therapieformen zu einer höheren 30-Tage-Überlebensrate. Patienten, welche mit monoklonalen Antikörpern behandelt wurden, zeigten allerdings eine beeinträchtige zelluläre Langzeitantwort.

Aufgrund der immer neu auftretenden Varianten von SARS-CoV-2 kam es zu einer Verringerung der Wirksamkeit von Rekonvaleszentenplasmen, monoklonalen Antikörpern und Impfstoffen. Wir konnten zeigen, dass die Wirksamkeit neutralisierender Antikörper in Seren geimpfter Personen mit einer SARS-CoV-2 Durchbruchsinfektion gegenüber den Omikron-Varianten BA.1 und BA.5 stark reduziert war. Des Weiteren konnten wir zeigen, dass die Antidepressiva Fluoxetin und Sertralin als alternative Medikamente die Replikation verschiedener SARS-CoV-2-Varianten *in vitro* inhibieren können.

Zusammenfassend konnten im Rahmen der vorliegenden Arbeit wichtige Erkenntnisse über die Immunantwort und die Wirksamkeit antiviraler Medikamente gegen SARS-CoV-2 gewonnen werden.

1.2 Summary

The pandemic, caused by SARS-CoV-2, led to over 700 million infections and over seven million deaths. Insights into the immune response generated by vaccination and infection and the efficacy of drugs against the virus were of central importance. In the present study, we analyzed the cellular and humoral immune response in different cohorts after vaccination and infection as well as the efficacy of different drugs against SARS-CoV-2.

Protecting vulnerable groups from infection is important, because they have an increased risk of a severe to fatal course of COVID-19. We investigated the cellular and humoral immune response in kidney and stem cell transplant recipients after booster vaccination against SARS-CoV-2. Both cohorts developed T-cell immunity and neutralizing antibodies after booster vaccination, which could contribute to protection against infection with SARS-CoV-2. Furthermore, we were able to show that unvaccinated, immunocompromised individuals suffering from COVID-19 build up a cellular and humoral immune response.

In addition, we analyzed the efficacy of the treatment of COVID-19 patients with convalescent plasma and monoclonal antibodies. Both forms of therapy led to a higher 30-day survival rate, compared to symptom-orientated therapy. However, patients treated with monoclonal antibodies showed an impaired long-term cellular response.

Due to the emerging variants of SARS-CoV-2, the efficacy of convalescent plasma, monoclonal antibodies and vaccines has been reduced. We were able to show that the efficacy of neutralizing antibodies in sera of vaccinated persons with a SARS-CoV-2 breakthrough infection was greatly reduced towards the omicron variants BA.1 and BA.5. Furthermore, we could show that the antidepressants fluoxetine and sertraline as alternative drugs can inhibit the replication of different SARS-CoV-2 variants *in vitro*.

In summary, this study has provided important insights into the immune response and the efficacy of antiviral drugs against SARS-CoV-2.

2. Einleitung

2.1 Coronaviren

Die zur Familie *Coronaviridae* gehörenden Viren sind membranumhüllt und beinhalten eine Einzelstrang-Ribonukleinsäure (engl.: *ribonucleic acid*, RNA) in Plusstrangorientierung. Elektronenmikroskopische Aufnahmen zeigen, dass die in der Membran verankerten Glycoproteine wie ein Strahlenkranz (lat.: *corona*) um die Virionen erscheinen (Almeida & Tyrrell, 1967). Diese morphologische Eigenschaft führte dazu, dass die *Coronaviridae* 1968 als eigene Familie klassifiziert wurden (Holmes, 1999; Almeida & Tyrrell, 1967). Bereits 1965 identifizierten David A. J. Tyrell und seine Mitarbeiter humane Coronaviren bei Menschen mit Erkältung (Tyrrell & Bynoe, 1966). In der Familie der *Coronaviridae* wird seit 2018 zwischen den Unterfamilien *Letovirinae* und *Orthocoronavirinae* unterschieden. Coronaviren sind in der Lage ein breites Wirtsspektrum zu infizieren, was zu verschiedenen Erkrankungsbildern bei Menschen, Katzen, Schweinen, Vögeln und Walen führt. Humanpathogene Coronaviren finden sich in der Unterfamilie der *Orthocoronavirinae* in den Gattungen der Alpha- und Betacoronaviren. Infektionen mit den Coronaviren 229E, NL63, OC43 und HKU1 führen beim Menschen zumeist zu Erkältungserkrankungen sowie Infektionen des oberen Respirationstraktes. In seltenen Fällen kommt es zu Infektionen des unteren Respirationstraktes.

Bislang sind drei Coronaviren bekannt, welche zu schwerwiegenden Infektionen und pandemischer Ausbreitung führten. Infektionen mit dem schweren akuten respiratorischen Syndrom Coronavirus (engl.: *severe acute respiratory syndrome-coronavirus*, SARS-CoV) traten erstmals 2002 auf. Die Infektionen breiteten sich pandemisch bis Juli 2003 aus, mit rund 8000 Infizierten und einer Mortalitätsrate von 9,6 % (WHO, 2015). Lange Zeit war der Ursprung des Virus unklar, jedoch gab es Vermutungen, dass es sich bei SARS-CoV um eine Zoonose handelt. Untersuchungen von Isolaten aus Schleichkatzen und Marderhunden wiesen eine hohe Sequenzhomologie zu SARS-CoV auf und so konnten diese als Zwischenwirte identifiziert werden (Cheng *et al.*, 2007; Guan *et al.*, 2003). Im Jahr 2005 wurde die fast identische Sequenz von SARS-CoV in Fledermäusen entdeckt (Lau *et al.*, 2005). Aufgrund der Sequenzanalysen von SARS-CoV bei Tieren wird vermutet, dass eine Übertragung der mutierten Viren von Fledermäusen der Gattung Hufeisennasen auf

Schleichkatzen erfolgte, welche das Virus nach weiteren Mutationen auf den Menschen übertrugen (Muralidar *et al.*, 2020; Pekar *et al.*, 2022).

Ein weiteres, zoonotisches Virus, welches zu schweren Erkrankungen der Atemwege führt, ist das *middle east respiratory syndrome-coronavirus* (MERS-CoV). Das Virus wurde im September 2012 in einem schwer an Pneumonie erkrankten Patienten in Saudi-Arabien isoliert und identifiziert (Zaki *et al.*, 2012). Es zeigte sich, dass bereits im April 2012 zwei Patienten in Jordanien mit MERS-CoV infiziert waren, jedoch war das Virus zu dem Zeitpunkt noch unbekannt und wurde retrospektiv identifiziert (Al-Abdallat *et al.*, 2014). Wie bereits bei SARS-CoV stammt auch bei MERS-CoV das ursprüngliche Virus aus Fledermäusen, jedoch dienen hier Dromedare als Zwischenwirte (Memish *et al.*, 2014; Reusken *et al.*, 2015; Saeed *et al.*, 2017). Seit 2012 wurden in insgesamt 27 Ländern rund 2500 Infektionen mit einer Mortalitätsrate von 35 % verzeichnet (Goldstein & Weiss, 2017; Okba *et al.*, 2017; WHO, 2022).

Im Dezember 2019 trat in Wuhan, China, erstmals eine neue schwere Atemwegserkrankung unbekannten Ursprungs auf. Als Erreger konnte das neue Coronavirus 2019-nCOV, später *severe acute respiratory syndrome-coronavirus type 2* (SARS-CoV-2) genannt, identifiziert werden (Peng Zhou *et al.*, 2020). Innerhalb weniger Wochen entwickelte sich die *coronavirus disease 2019* (COVID-19) genannte Infektionskrankheit zur massiven Pandemie. Bis Dezember 2023 gab es über 700 Millionen bestätigte Fälle von COVID-19, fast sieben Millionen Menschen verstarben aufgrund der Infektion mit SARS-CoV-2 (WHO, 2023). Viele Länder haben Restriktionen wie Ausgangssperren, die Pflicht zum Tragen von *filtering face piece 2* (FFP-2) Masken, Kontaktbeschränkungen und Abstandsregeln, erlassen, um die Pandemie einzudämmen. Zusätzlich wurde im Dezember 2021 der erste Impfstoff gegen SARS-CoV-2 in Europa zugelassen (Paul-Ehrlich-Institut, 2021). Die Restriktionen, Impfungen und bereits durchgemachte Infektionen mit SARS-CoV-2 führten zu einer rückläufigen Zahl an Infektionen und einer sinkenden Mortalitätsrate. Dennoch kommt es weiterhin zu Infektionen mit neuen Varianten von SARS-CoV-2, zu Impfdurchbruchsinfektionen und zu Langzeitfolgen einer SARS-CoV-2-Infektion, genannt „Post-COVID-19“, sodass COVID-19 weiterhin ein globales Gesundheitsproblem darstellt (Choutka *et al.*, 2022; Gazit *et al.*, 2022; Salehi-Vaziri *et al.*, 2022).

2.2 Aufbau von Coronaviren

Die Virionen sind membranumhüllt, sphärisch und haben einen Durchmesser von 120 bis 160 nm. Das im Inneren gelegene Nucleocapsid besteht aus der einzelsträngigen RNA mit Plusstrangorientierung und den an ihr assoziierten N-Proteinen (Lai & Cavanagh, 1997). Dieser helikal angeordnete Komplex weist einen Durchmesser von 10 bis 20 nm auf. Das Nucleocapsid ist von der Hüllmembran umgeben, in welcher das aminoterminal O-glykosylierte M-Protein eingelagert ist (Rota *et al.*, 2003). Das carboxyterminale Ende des M-Proteins interagiert mit definierten Aminosäuren des N-Proteins, wodurch eine Proteinwechselwirkung zwischen dem Nucleocapsid und der Innenseite der Hüllmembran besteht (Kuo & Masters, 2003; Neuman *et al.*, 2006). In der Hüllmembran ebenfalls eingelagert liegt das S-Protein vor, ein 180 bis 200 kDa großes, N-glykosyliertes Protein, welches als Trimer vorliegt und zum Erscheinungsbild der *Corona* führt (Holmes, 1999; Zhu *et al.*, 2020). Ein weiteres, nur in geringen Mengen vorliegendes Membranprotein ist das E-Protein. Es ist 9 bis 12 kDa groß, nicht glykosyliert und bildet pentamere Komplexe (Kuo & Masters, 2003) (Abbildung 1).

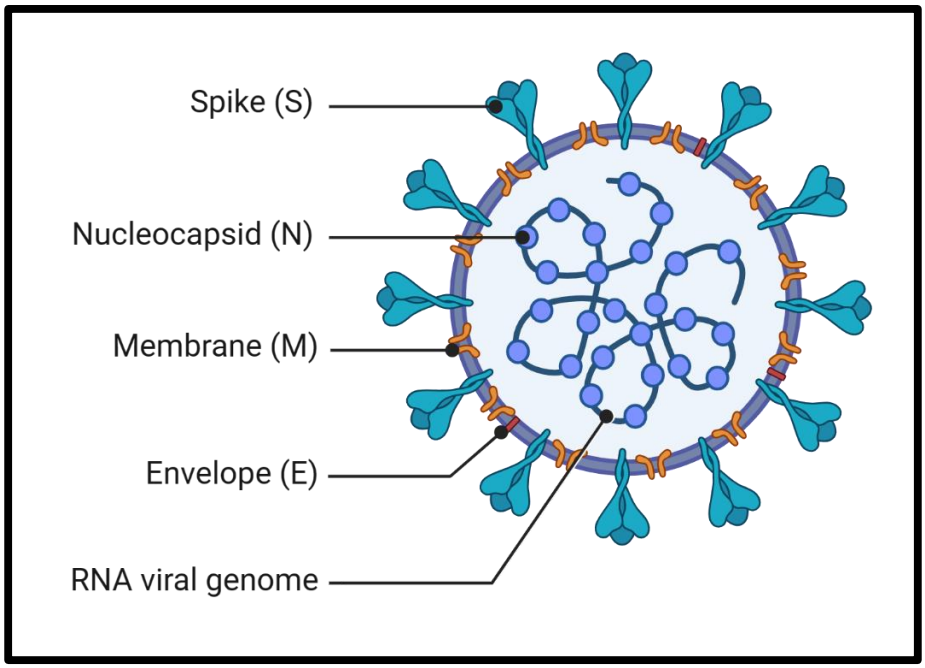

Abbildung 1: Struktur der Virionen von SARS-CoV-2. SARS-CoV-2 weist vier Strukturproteine auf, das im Inneren gelegene Nucleocapsid, bestehend aus RNA und daran assoziierten *Nucleocapsid*-Proteinen (N), den *Membrane*-Proteinen (M), den trimeren *Spike*-Proteinen (S) und den in geringer Anzahl vorhandenen *Envelope*-Proteinen (E). (Modifizierte Abbildung von BioRender.com [https://app.biorender.com/biorender-templates] [2024]).

2.3 Replikation von SARS-CoV-2

SARS-CoV-2 nutzt das Angiotensin-konvertierende Enzym 2 (engl.: *angiotensin-converting enzyme 2*, ACE2) als Rezeptor zur Vermittlung des Viruseintritts in die Wirtszelle (Walls *et al.*, 2020). ACE2 wird unter anderem von Gefäßendothelzellen, Epithelzellen des Gastrointestinaltraktes sowie Atemwegsepithelien exprimiert. Das S-Protein von SARS-CoV-2 besteht aus zwei Untereinheiten. Die S1-Untereinheit enthält die Rezeptor-bindende Domäne (engl.: *receptor-binding domain*, RBD) und ist für die Bindung an ACE2 verantwortlich, wohingegen die S2-Untereinheit der Fusion des Virus mit der Zellmembran dient. SARS-CoV-2 bindet über die in der S1-Untereinheit enthaltene RBD an ACE2, wodurch es zu einer Konformationsänderung im S-Protein kommt (Moreira *et al.*, 2020). Diese Konformationsänderung bewirkt, dass die transmembrane Serinprotease 2 (engl.: *transmembrane protease serine subtype 2*, TMPRSS2) die S1-Untereinheit abspaltet und die S2-Untereinheit weiter prozessiert, wodurch es zur Fusion des Virus mit der Zelle kommt (Maison *et al.*, 2023). Die prozessierte S2‘-Untereinheit lagert sich in die Zytoplasmamembran der Zelle ein und zwei in der Untereinheit enthaltene Heptadwiederholungen (engl.: *heptad repeat*, HR) 1 und 2 interagieren miteinander. HR1 und HR2 bilden ein Bündel aus sechs Helices, wodurch die Virus- und die Zellmembran näher aneinander gezogen werden und es zur Verschmelzung beider Membranen kommt (Xia *et al.*, 2020). Fehlt TMPRSS2 auf der Zellmembran der Wirtsoberfläche, kann das Virus mittels Endozytose aufgenommen werden, wobei Cathepsin L die Spaltung des S-Proteins induziert (Shang *et al.*, 2020; Peng Zhou *et al.*, 2020) (Abbildung 2).

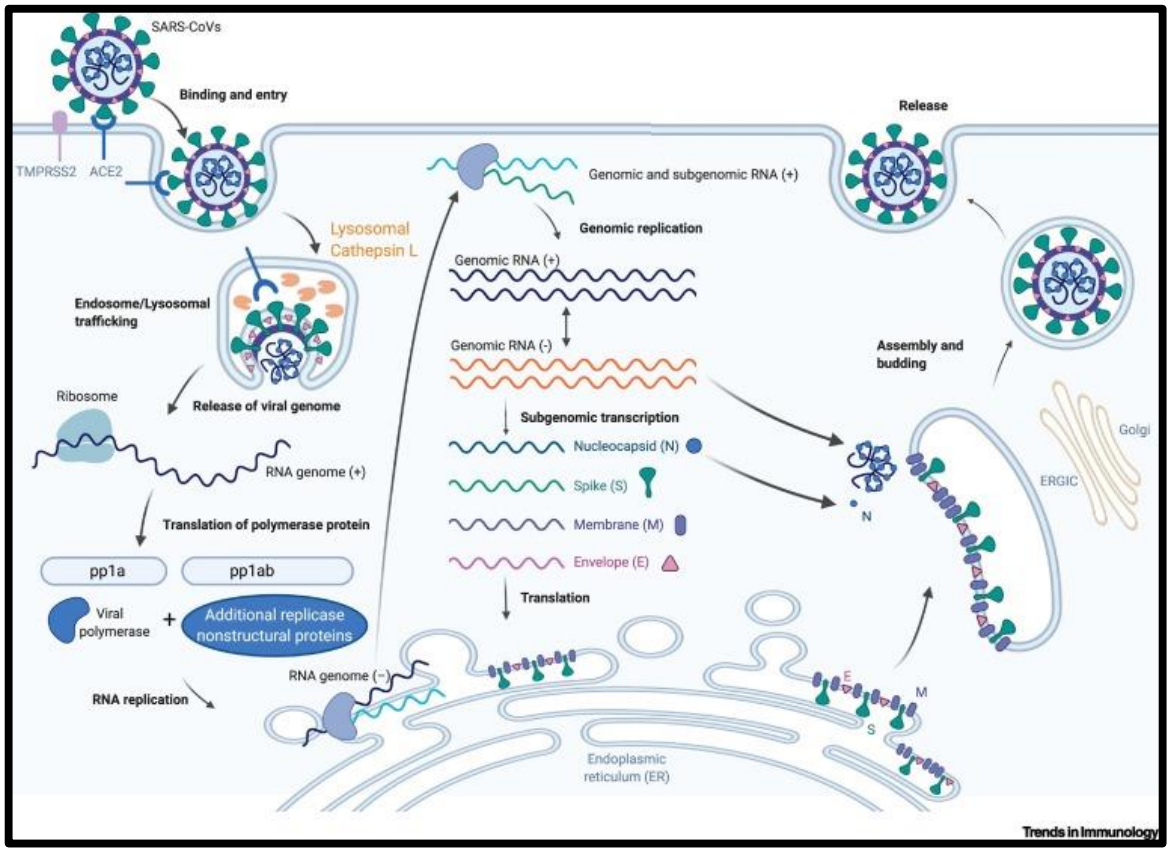

Abbildung 2: Replikationszyklus von SARS-CoV-2. Nach Bindung des Spike-Proteins an ACE2 gelangt das Virus über Membranfusion oder Endozytose in die Wirtszelle. Das RNA-Genom wird in das Zytoplasma der Wirtszelle freigesetzt, wo die Translation der Polymerase, Replicase und weiterer Nichtstrukturproteinen stattfindet. Der bei der RNA-Replikation entstehende negative RNA-Strang dient als Matrize der genomischen und subgenomischen RNA. Aus der subgenomischen RNA entstehen durch Transkription und Translation die vier Strukturproteine Nucleocapsid (N), Spike (S), Membrane (M) und Envelope (E). Im endoplasmatischen Retikulum-Golgi-Zwischenkompartiment (engl.: *endoplasmic-reticulum-Golgi intermediate compartment*, ERGIC) verbinden sich die vier Strukturproteine zu neuen Virionen, welche anschließend von der Zelle freigesetzt werden. (Harrison *et al.*, 2020).

Das einzelsträngige, plusstrangorientierte RNA-Genom umfasst etwa 30.000 Basen und zählt damit zu den größten Genomen bei RNA-haltigen Viren. Am 5‘-Ende ist es durch eine *Cap*-Gruppe modifiziert, das 3‘-Ende ist polyadenyliert. Dem 5‘-Ende folgend liegen die offenen Leserahmen (engl.: *open reading frame*, ORF) 1 und 2, welche für die Polyproteine pp1a und pp1ab codieren (Chan *et al.*, 2020a). Nur in 20 – 30 % der Fälle kommt es zur nötigen ribosomalen Leserasterverschiebung, wodurch die Haarnadelschleife zwischen den beiden ORF überwunden wird und pp1ab translatiert wird (Bhatt *et al.*, 2021). Die Polyproteine pp1a und pp1ab werden durch die in ihrer Sequenz enthaltenen Proteasen nach der Transkription in die Nichtstrukturproteine (engl.: *nonstructural proteins*, NSP) 1 – 11 bzw. 1 – 16 gespalten (Chan *et al.*, 2020a). An den ORF 2 schließen sich die Leserahmen für die vier Strukturproteine S, E, M und N sowie sechs akzessorische Proteine an (Peng Zhou *et*

al., 2020). Die plusstrangorientierte RNA dient als *messenger*-RNA (mRNA) und wird von der Wirtszelle translatiert. Aus den Polyproteinen wird unter anderem eine RNA-Polymerase transkribiert, welche den Plusstrang in negativsträngige, genomische RNA sowie subgenomische RNA synthetisiert. Der genomische RNA-Strang dient als Matrize für weitere Plusstrang-RNA-Genome, die subgenomischen RNA dienen als Matrize für verschiedene Proteine wie dem S, E, M und N-Protein (Chan *et al.*, 2020a). Die RNA lagert sich helikal mit den N-Proteinen zusammen und bildet das Nucleocapsid. Die Nucleocapside binden anschließend an die carboxyterminalen Domänen der M- und E-Proteine, welche in der Membran des endoplasmatischen Retikulums (ER) eingelagert sind. Dies führt zum *Budding*-Prozess, bei dem das Nucleocapsid von der Hüllmembran, welche S-, M- und E-Proteine enthält, umgeben wird (Sheikh *et al.*, 2020). Die neu entstandenen Virionen gelangen ins Lumen des ER und werden über die Golgi-Vesikel aus der Zelle ausgeschleust.

2.4 Epidemiologie und Übertragung von SARS-CoV-2

Dem humanen SARS-CoV-2 ähnelnde Viren wurden bereits vor Ausbruch der Pandemie in Fledermäusen der Gattung Große Hufeisennasen nachgewiesen. Es konnte eine Sequenzhomologie von 96 % mit dem bei Fledermäusen vorkommenden SARS-ähnlichen Coronavirus BatCov RaTG13 gezeigt werden (M. Y. Wang *et al.*, 2020). Bisherige Theorien gehen von Mutationen im Genom der Coronaviren von Fledermäusen aus, welche eine Übertragung auf Zwischenwirte wie Pangoline, Marderhunde oder Frettchen ermöglichten (Muralidar *et al.*, 2020; Pekar *et al.*, 2022). Durch engen Kontakt zu infizierten Tieren auf Lebendtiermärkten kam es vermutlich zu zoonotischen Infektionen des Menschen. Das Virus mutierte innerhalb des Menschen weiter, sodass es zu einer Übertragung von Mensch zu Mensch kam (Abbildung 3).

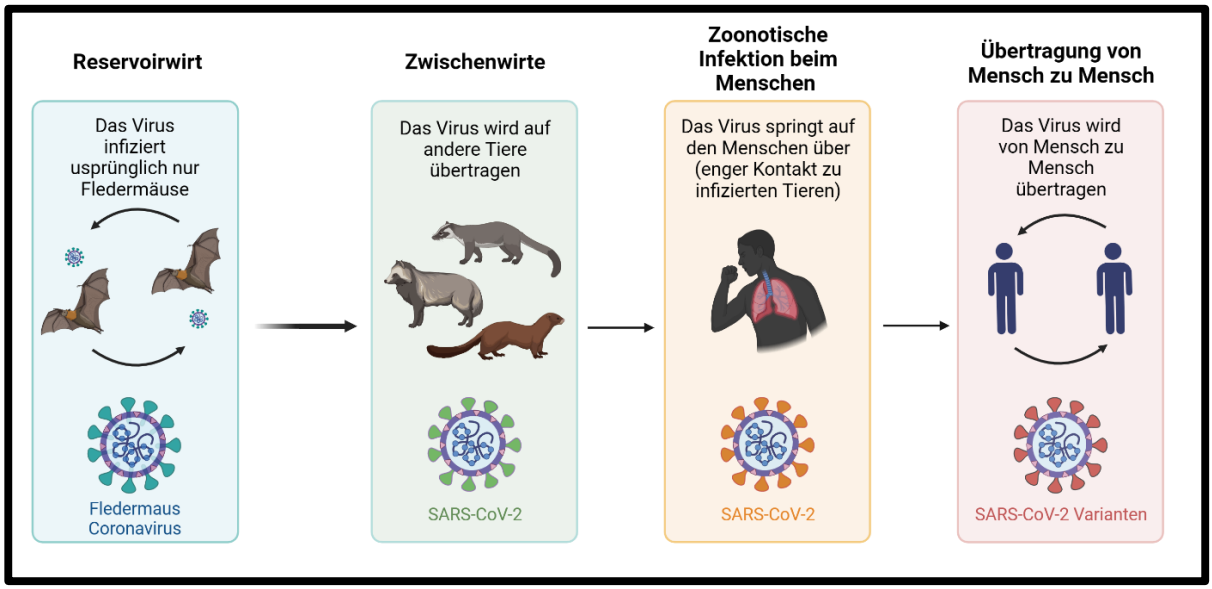

Abbildung 3: Epidemiologie von SARS-CoV-2. Die Fledermaus ist der Reservoirwirt von SARS-CoV-2. Aus dem ursprünglichen Fledermaus Coronavirus entsteht durch Mutationen SARS-CoV-2, welches auf Zwischenwirte wie Marderhunde, Pangoline oder Frettchen übertragen wird. Aufgrund engen Kontaktes des Menschen mit diesen Tieren kommt es zu zoonotischen Infektionen beim Menschen und das Virus wird von Mensch zu Mensch übertragen. (Modifizierte Abbildung von BioRender.com [https://app.biorender.com/biorender-templates] [2024]).

RNA-Viren haben eine hohe Mutationsrate von 10^{-6} bis 10^{-4} Substitution pro Nukleotid pro Zellinfektion (engl.: *substitutions per nucleotide per cell infection*, s/n/c) (Sanjuán *et al.*, 2010). Aufgrund dieser Mutationsrate ist es RNA-Viren möglich, sich besser an ihren Wirt anzupassen. Bei SARS-CoV-2 wurden Mutationen besonders im codierenden Bereich für das S-Protein entdeckt (Volz *et al.*, 2021). Hierdurch kommt es zu einer gesteigerten Infektiosität, ebenso kann die neu entstehende Variante der Immunantwort, vermittelt durch vorherige Infektion oder Impfung, entkommen (Peacock *et al.*, 2021). Bis heute sind verschiedene Varianten und Rekombinanten von SARS-CoV-2 bekannt, hiervon gelten einige als besorgniserregende Varianten (engl.: *variants of concern*, VOCs). Zu schwerwiegenden, großen Infektionswellen führten insbesondere die VOCs Alpha (B.1.1.7) und Beta (B1.351) ab September 2020, Delta (B1.617.2) ab Dezember 2020 und Omikron (B.1.529) ab November 2021. Insbesondere bei den VOCs Delta und Omikron gibt es mehrere Subvarianten (Hill *et al.*, 2022; Tegally *et al.*, 2022) (Abbildung 4).

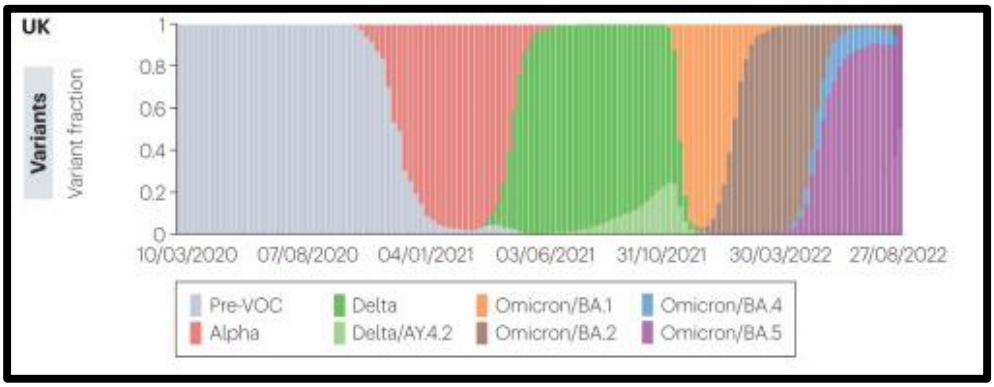

Abbildung 4: Varianten von SARS-CoV-2 im Vereinigten Königreich im Verlauf der Zeit. Bis Ende des Jahres 2020 war vornehmlich die ursprüngliche Variante von SARS-CoV-2 für Infektionen verantwortlich. Seit 2021 führen durch Mutationen entstandene Varianten und Sublinien von SARS-CoV-2 zu Infektionen. Einige Varianten sind dabei dominanter und führen über einen längeren Zeitraum zu Infektionen, wohingegen andere Varianten nur kurzweilig existieren (Carabelli *et al.*, 2023).

Zur Entstehung der Varianten gibt es verschiedene Theorien. Eine Möglichkeit wäre eine chronische Infektion immunsupprimierter Personen, wodurch es vermehrt zu Mutationen im Genom des Virus kommt und die neue Variante durch die infizierten Personen auf nicht-infizierte Personen übertragen wird (Hill *et al.*, 2022; Wilkinson *et al.*, 2022). Auch besteht die Möglichkeit einer Co-Infektion mit zwei aktuell vorherrschenden Varianten, was bei der Virusreplikation zu einer Rekombination aus beiden Genomen führen kann (Jackson *et al.*, 2021; Sekizuka *et al.*, 2022).
SARS-CoV-2 kann über infektiöse Tröpfchen übertragen werden. Diese werden insbesondere beim Husten freigesetzt (Pan *et al.*, 2020; Zou *et al.*, 2020). Die Tröpfchen können direkt von anderen Personen eingeatmet werden, sich allerdings auch auf Gegenständen absetzen oder als Aerosole in der Luft verbleiben und über eine größere Distanz im Luftzug transportiert werden (van Doremalen *et al.*, 2020). Infizierte Personen können SARS-CoV-2 im präsymptomatischen und symptomatischen Stadium der Infektion übertragen, allerdings auch bei asymptomatischem Verlauf (Arons *et al.*, 2020; Li *et al.*, 2020). Insbesondere die Übertragung von infizierten Personen im präsymptomatischen Stadium und Personen mit asymptomatischem Verlauf haben zur raschen Ausbreitung von SARS-CoV-2 geführt (Greenhalgh *et al.*, 2021; Mizumoto *et al.*, 2020; Rothe *et al.*, 2020).

2.5 Pathogenese

Die Inkubationszeit von SARS-CoV-2 beträgt durchschnittlich fünf bis sechs Tage, wobei auch bereits während dieser Zeit eine weitere Übertragung von Mensch zu Mensch möglich ist (Chan *et al.*, 2020b; Li *et al.*, 2020). Die Erkrankung beginnt mit

Symptomen ähnlich einer Erkältung. Die häufigsten Symptome stellen Fieber, Husten, Dyspnoe, Kopf- und Gliederschmerzen dar (Chen *et al.*, 2020). Aufgrund der Expression von ACE2 auf Zellen des Respirationstraktes, des Muskelgewebes, des Gehirns, des Gastrointestinaltraktes, des Endothels und der Kardiomyozyten kommt es zu pulmonalen Symptomen, wie Husten und Dyspnoe und zusätzlich zu extrapulmonalen Symptomen, wie Myalgie, Konzentrationsstörungen, Diarrhoe, Erbrechen und Herzmuskelentzündung (Chen *et al.*, 2020; Hikmet *et al.*, 2020; Puntmann *et al.*, 2020) (Abbildung 5).

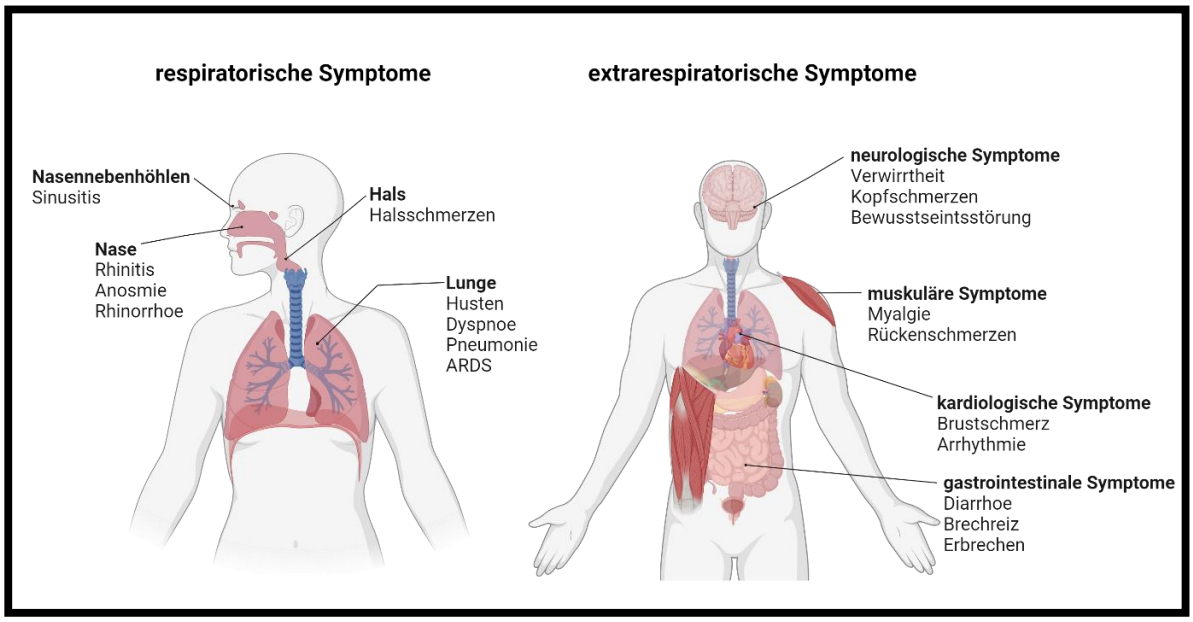

Abbildung 5: Symptomatik von COVID-19. Eine Infektion mit SARS-CoV-2 führt zu Symptomen im oberen und unteren Respirationstrakt wie Halsschmerzen, Sinusitis und Rhinitis. Zusätzlich klagen Patienten über Symptome außerhalb des Respirationstraktes. Diese umfassen neurologische, muskuläre, kardiologische und gastrointestinale Symptome. (Modifiziert mit BioRender.com [https://app.biorender.com/biorender-templates] [2024]) (Harrison *et al.*, 2020).

Bei COVID-19 werden unterschiedliche Verläufe der Erkrankung beobachtet. So gibt es asymptomatische Verläufe, bei denen Symptome ausbleiben, aber auch milde, moderate, schwere und kritische Verläufe (WHO, 2021b). Während der ersten Welle von COVID-19 zeigte sich bei 40 % der Infizierten ein milder Verlauf, bei 40 % ein moderater Verlauf, bei 15 % ein schwerer und bei 5 % ein kritischer Verlauf (WHO, 2021b). Die Zahlen unterliegen jedoch Schwankungen, da die unterschiedlichen Varianten von SARS-CoV-2 eine unterschiedliche Pathogenität aufweisen. Bei der Infektionswelle mit der Delta-Variante wurden mehr schwere Verläufe und eine höhere Mortalität im Vergleich zur Alpha-Variante beobachtet (Fisman & Tuite, 2021). Bei Omikron kommt es vermehrt zu milden und moderaten Verläufen, jedoch ist die

Übertragungsrate deutlich höher als bei der Delta-Variante (Ito *et al.*, 2022; Iuliano *et al.*, 2022; Pascall *et al.*, 2022).

Ein milder Verlauf ist gekennzeichnet durch grippeähnliche Symptome wie Fieber, Husten, Kurzatmigkeit und der Verlust von Geruchs- und Geschmackssinn (Chen *et al.*, 2020). Eine Hospitalisierung ist nicht erforderlich und es erfolgt zumeist eine symptomatische Behandlung (Siddiqi & Mehra, 2020). Bei einem moderaten Verlauf liegt zusätzlich zu den grippeähnlichen Symptomen eine Pneumonie vor. Aufgrund der Symptomatik im unteren Respirationstrakt kann es zu einer Hospitalisierung mit unterstützender Sauerstoffgabe kommen (Lucijanić *et al.*, 2023). Ein schwerer Verlauf ist definiert durch eine schwere Pneumonie sowie einer Atemfrequenz von mehr als 30 Atemzüge pro Minute oder einer Blutsauerstoffsättigung von unter 90 % (Verity *et al.*, 2020; WHO, 2021b). Patienten mit einem schweren Verlauf sind hospitalisiert und benötigen zumeist intensivmedizinische Versorgung und eine Sauerstoffgabe. Bei kritischen Verläufen kann es durch in die Lunge eingewanderte Makrophagen und Neutrophile zu Gewebeschäden der Lunge kommen, was zum akuten Atemnotsyndrom (engl.: *acute respiratory distress syndrome*, ARDS) führt. Des Weiteren wird durch die Immunzellen ein Zytokinsturm von pro-inflammatorischen Zytokinen verursacht (Harrison *et al.*, 2020). Die Zytokine führen zu einer Hyperinflammation mehrerer Organe, wodurch es zum multiplen Organversagen kommen kann (The Novel Coronavirus Pneumonia Emergency Response Epidemiology, 2020). Die Verläufe können fließend ineinander übergehen, sodass ein milder Verlauf rasch zu einem kritischen Verlauf mit möglicherweise tödlichem Ausgang werden kann. Dies hängt insbesondere von prädisponierenden Faktoren, wie Geschlecht, Alter, *Body Mass Index* (BMI) und bestehenden Vorerkrankungen, ab. Studien zeigen, dass das Risiko für einen schweren bis kritischen Verlauf bei Männern, älteren Personen (> 60 Jahre), Immunsupprimierten und Personen mit einem BMI >30 deutlich erhöht ist (Huang *et al.*, 2020; Zhang *et al.*, 2023). Kinder weisen zumeist einen milden Verlauf mit einer Mortalitätsrate < 1 % auf, wohingegen die Mortalitätsrate bei Personen >80 Jahren etwa 15 % beträgt (Dong *et al.*, 2020; Lu *et al.*, 2020; The Novel Coronavirus Pneumonia Emergency Response Epidemiology, 2020; Verity *et al.*, 2020). Ein weiterer Risikofaktor für einen schweren bis tödlichen Verlauf von COVID-19 sind Vorerkrankungen wie Bluthochdruck, Diabetes mellitus und koronare Herzkrankheit (Wu *et al.*, 2020; F. Zhou *et al.*, 2020). Zusätzlich zu den Verläufen zeigt sich bei COVID-19 ein neues Krankheitsbild, welches durch die Persistenz von

Symptomen oder das Auftreten neuer Symptome über einen langen Zeitraum gekennzeichnet ist.

2.6 Post-COVID-19

Die Weltgesundheitsorganisation (engl.: *World Health Organization*, WHO) hat im Oktober 2021 im Delphi-Konsens eine Falldefinition von Post-COVID-19 veröffentlicht. Diese Definition besagt, dass Post-COVID-19 bei Personen auftritt, die eine vermeintliche oder bestätigte SARS-CoV-2-Infektion durchgemacht haben und die in einem Abstand von drei Monaten zur Manifestation der Infektion langanhaltende, mindestens zwei Monate andauernde, oder neue Symptome entwickeln, welche nicht durch andere Diagnosen erklärbar sind (WHO, 2021a). Diese Definition ist Folge des zuvor weltweit beobachteten Phänomens, dass Personen nach einer Infektion mit SARS-CoV-2 weiterhin Symptome zeigen, ohne dass das Virus nachweisbar ist (Fernandes Valente Takeda *et al.*, 2020; Whitaker *et al.*, 2022). Als häufigste Symptome gelten Erschöpfung, Kopfschmerzen, Kurzatmigkeit, Muskelschmerzen und kognitive Störungen (WHO, 2021a). Die Symptome umfassen jedoch ein breites Spektrum und reichen von Husten, Mikrothromben, Brustschmerzen und Tachykardie bis hin zu Angst, Depression und Schlafstörungen (Dennis *et al.*, 2021; Soriano *et al.*, 2022). Diese post-viralen Symptome wurden auch nach Infektionen mit anderen Viren wie dem Epstein-Barr-Virus oder Herpesviren beobachtet, allerdings nicht in dieser Ausprägung und Dauer (Hotchin *et al.*, 1989; Shikova *et al.*, 2020).
Die Inzidenz von Post-COVID-19 ist sehr unterschiedlich. So entwickeln 10 – 30% der nicht-geimpften, nicht-hospitalisierten Personen Post-COVID-19, wohingegen die Inzidenz bei Geimpften auf 10 – 12% sinkt. Die größte Inzidenz zeigt sich bei Personen, die hospitalisiert waren, mit 50 – 70% (Hodgson & Broadley, 2023). Trotz der unterschiedlichen Inzidenz ist es bislang unklar, ob schwerere Verläufe öfter zu Post-COVID-19 führen als milde Verläufe. Forschende konnten einige Risikofaktoren für das Auftreten von Post-COVID-19 benennen, jedoch gibt es keine aussagkräftigen Parameter zur Vorhersage von Post-COVID-19. Zu den Risikofaktoren zählen das weibliche Geschlecht, ein höheres Alter, eine schlechtere soziodemografische Umgebung sowie Vorerkrankungen (Müller *et al.*, 2023; Thompson *et al.*, 2022). Weitere Untersuchungen sind notwendig, um geeignete Diagnose- und Therapiemöglichkeiten sowie ein besseres Verständnis von Post-COVID-19 zu erhalten.

2.7 Immunantwort auf SARS-CoV-2

Das humane Immunsystem gliedert sich in das unspezifische, angeborene und das spezifische, adaptive Immunsystem. Die Zellen des angeborenen Immunsystems, wie Granulozyten, Makrophagen, Natürliche Killerzellen (engl.: *natural killer cells*, NK), Monozyten und dendritische Zellen (engl.: *dendritic cells*, DC) reagieren innerhalb von Minuten nach Pathogenbefall aufgrund von Mustererkennungsrezeptoren (engl.: *pathogen recognition receptors*, PRR), welche konservierte Pathogen-assoziierte molekulare Muster (engl.: *pathogen-associated molecular patterns*, PAMP) erkennen (Akira *et al.*, 2006). Zu den PRR, die SARS-CoV-2 anhand von Oberflächenproteinen und viraler RNA erkennen, zählen die membranständigen oder zytosolischen Retinsäure-induzierbaren Gen-I-ähnliche Rezeptoren (engl.: *retinoic acid inducible gene I* (RIG-I)-*like receptor*, RLR) und Toll-ähnliche Rezeptoren (engl.: *toll-like-receptor*, TLR) (Akira *et al.*, 2006; Tan *et al.*, 2018). Die aktivierten antiviralen Signalwege resultieren in der Bildung von Typ-I und Typ-III Interferonen (IFN) sowie weiterer pro-inflammatorischen Zytokinen, wie Interleukinen (IL) und dem Tumornekrosefaktor-alpha (TNF-α) (Huang *et al.*, 2020; Ivashkiv & Donlin, 2014).
Stunden nach Beginn der Infektion werden die zum adaptiven Immunsystem gehörenden B- und T-Lymphozyten durch antigenpräsentierende Zellen (engl.: *antigen presenting cells*, APC) und Zytokine aktiviert. Die wichtigsten Merkmale der adaptiven Immunantwort sind ihre Spezifität und die Bildung eines immunologischen Gedächtnisses. Bei den T-Lymphozyten werden die *cluster of differentiation* (CD) 4^+ T-Helferzellen, welche wichtig für die Stimulation und Proliferation von B-Zellen sind, sowie die $CD8^+$ zytotoxischen T-Zellen, welche infizierte Zellen abtöten, unterschieden (Murphy, 2016). T-Lymphozyten produzieren weitere pro-inflammatorische Zytokine wie IFN-γ, IL-2 und TNF-α, wodurch sowohl Zellen der angeborenen als auch der adaptiven Immunantwort stimuliert werden (Seder *et al.*, 2008). B-Lymphozyten differenzieren zu Plasmazellen, welche Antikörper produzieren. Zunächst werden Antikörper der Klasse IgM sezerniert, nach erfolgtem Klassenwechsel, somatischer Hypermutation und Selektion im Keimzentrum werden hochaffine Antikörper der Klasse IgG sezerniert (De Silva & Klein, 2015). Während der Keimzentrumsreaktion differenziert ein Teil der B-Zellen zu Gedächtnis-B-Zellen (engl.: *memory B cells*), welche gemeinsam mit Gedächtnis-T-Zellen (engl.: *memory T cells*) das immunologische Gedächtnis bilden und bei einer Reinfektion mit demselben Pathogen zu einer schnelleren und spezifischeren Immunantwort führen (Murphy, 2016)

(Abbildung 6). Bei den meisten infizierten Personen können Antikörper der Klasse IgM fünf Tage nach Infektionsbeginn detektiert werden, die hochaffinen Antikörper der Klasse IgG können ein bis zwei Wochen nach Beginn der Infektion nachgewiesen werden (Guo *et al.*, 2020; Long *et al.*, 2020; Zhao *et al.*, 2020).

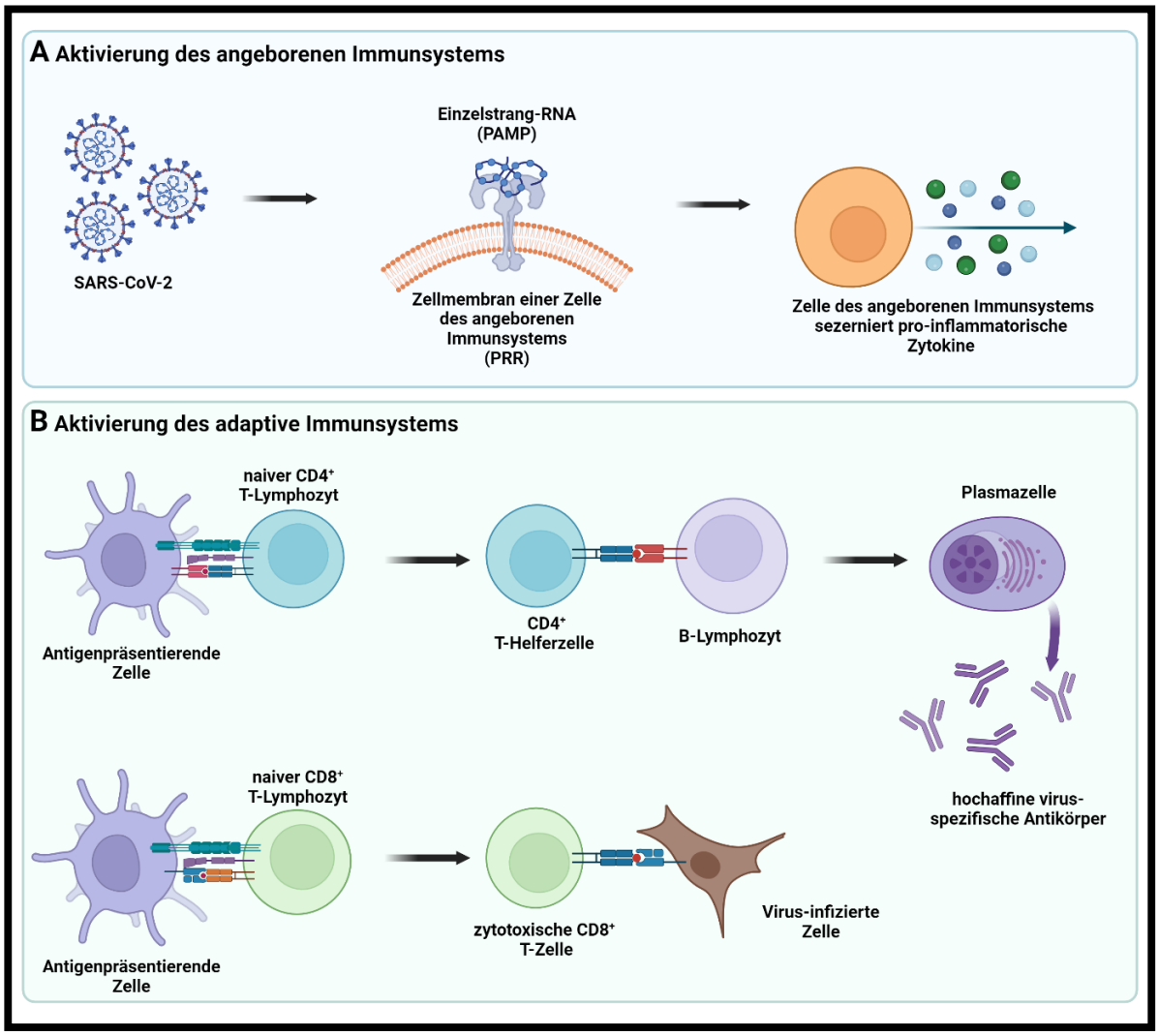

Abbildung 6: Angeborene und adaptive Immunantwort bei Virusinfektionen. (**A**) Zellen des angeborenen Immunsystems erkennen Pathogen-assoziierte molekulare Muster (engl.: *pathogen-associated molecular patterns*, PAMP) von Viren über Mustererkennungsrezeptoren (engl.: *pathogen recognition receptors*, PRR). Die Bindung von PAMP an PRR führt zur Bildung von pro-inflammatorischen Zytokinen wie Interferonen und Interleukinen. (**B**) Antigenpräsentierende Zellen prozessieren Antigene von Pathogenen und präsentieren diese im Haupthistokompatibilitätskomplex (engl.: *major histocompatibility complex*, MHC) naiven T-Lymphozyten. Die T-Lymphozyten differenzieren einerseits zu reifen $CD4^+$ T-Helferzellen, welche mit B-Lymphozyten interagieren und deren Differenzierung zu antikörperproduzierenden und -sezernierenden Plasmazellen unterstützen und andererseits zu reifen zytotoxischen $CD8^+$ T-Zellen, welche infizierte Zellen zerstören. (Modifiziert mit BioRender.com [2024]) (Mistry *et al.*, 2022).

Die Wichtigkeit der angeborenen und adaptiven Immunantwort für den Verlauf von COVID-19 konnte in mehreren Studien belegt werden. SARS-CoV-2 reagiert sensitiver auf Typ-I IFN als SARS-CoV und MERS-CoV, weist allerdings auch Proteine auf, welche die Bildung von IFN unterdrücken (Banerjee *et al.*, 2020; Lokugamage *et al.*,

2020). Hadjadj *et al.* zeigten, dass eine beeinträchtigte Aktivität von Typ-I Interferonen mit schweren und kritischen Verläufen von COVID-19 korreliert (Hadjadj *et al.*, 2020). Eine verspätet einsetzende adaptive Immunantwort, möglicherweise aufgrund einer reduzierten Freisetzung von Typ-I IFN, korreliert ebenfalls mit schweren Verläufen von COVID-19 (Magleby *et al.*, 2021). Bei Patienten mit schweren und kritischen Verläufen konnte zudem eine reduzierte Anzahl an Keimzentren sowie follikulären T-Helferzellen beobachtet werden, wodurch es zu einer unzureichenden adaptiven Immunantwort und Ausbildung des immunologischen Gedächtnisses kommt (Duan *et al.*, 2020; Kaneko *et al.*, 2020; Magleby *et al.*, 2021).

2.8 Therapiemöglichkeiten bei COVID-19

Die Entwicklung, Erforschung und Zulassung von Medikamenten ist ein sehr zeitintensiver Prozess. Insbesondere zu Beginn der Pandemie war diese Zeit nicht gegeben, sodass auf bereits für andere Erkrankungen zugelassene Medikamente zurückgegriffen und ihre Wirksamkeit gegenüber COVID-19 geprüft wurde. Im Verlauf der Pandemie wurden neue, zusätzliche Medikamente und Therapieansätze entwickelt.
Grundsätzlich wird zwischen der antiviralen, der immunmodulatorischen Therapie und alternativen Therapien unterschieden. Zu der antiviralen Therapie zählen Remdesevir, Nirmatrelvir/Ritonavir, Molnupiravir und neutralisierende monoklonale Antikörper (engl.: *monoclonal antibodies*, mAb). Januskinase-Inhibitoren (JAK-I) sowie Antagonisten des IL-6-Rezeptors werden bei der immunmodulatorischen Therapie angewendet. Alternative Therapien stellen die Gabe von Rekonvaleszenten-Plasmen (RKP) sowie Antidepressiva dar.

<u>Antivirale Therapeutika</u>
Remdesevir, ein Adenosin-Analogon Propharmakon, wird zur Behandlung von RNA-Viren wie MERS-CoV und dem Ebola-Virus eingesetzt und ist in der Lage ihre Replikation aufgrund frühzeitiger Kettenabbrüche zu inhibieren (Sheahan *et al.*, 2017; Warren *et al.*, 2016). Die Wirksamkeit von Remdesevir gegenüber SARS-CoV-2 konnte sowohl *in vitro* als auch im Tiermodell beschrieben werden (M. Wang *et al.*, 2020; Williamson *et al.*, 2020). Es wurde von der Europäischen Kommission im Jahr 2020 bedingt zur Behandlung von COVID-19 in der Frühphase zugelassen (Robert-Koch-Institut, 2023).

Paxlovid® ist ein Arzneimittel bestehend aus Nirmatrelvir und Ritonavir, welches im Jahr 2022 eine bedingte Zulassung in der Europäischen Union (EU) erhielt (Robert-Koch-Institut, 2023). Nirmatrelvir inhibiert die Protease M^{pro} von SARS-CoV-2, was zur Hemmung der Virusreplikation führt. Ritonavir ist ein Inhibitor des Enzyms Cytochrom P450 3A4 (CYP3A4), welches die pharmakokinetischen Eigenschaften von Nirmatrelvir verbessert (Dryden-Peterson *et al.*, 2023). Studien konnten die Wirksamkeit von Paxlovid® gegenüber verschiedenen VOCs zeigen, darunter auch die Delta- und Omikron-Varianten (Lin *et al.*, 2023; Najjar-Debbiny *et al.*, 2023; Vangeel *et al.*, 2022).

Das mutagene Ribonukleosid-Analogon Molnupiravir (Lageviro®) ist in Deutschland nicht zugelassen, wurde jedoch bis Februar 2023 vom Bundesministerium für Gesundheit (BMG) zentral beschafft und an Kliniken und Apotheken zur Behandlung von COVID-19-Patienten in der Frühphase abgegeben (BfArM, 2023). Sheahan *et al.* konnten die Wirksamkeit von Molnupiravir gegenüber SARS-CoV, MERS-CoV und SARS-CoV-2 *in vitro* zeigen, ein Jahr später wurde die Wirksamkeit gegenüber SARS-CoV-2 im Tiermodell bewiesen (Sheahan *et al.*, 2020; Wahl *et al.*, 2021). In einer klinischen Phase-3-Studie wurde eine Reduktion der Hospitalisierung oder tödlichen Ausgänge um 6,8 % beobachtet (Jayk Bernal *et al.*, 2022).

Immunmodulatorische Therapien

Eine deutlich spezifischere Therapie ist mit neutralisierenden mAb möglich. Sie verhindern die Bindung des Spike-Proteins an den ACE2-Rezeptor, indem sie sich gegen verschiedene Epitope des Spike-Proteins richten. In Deutschland zugelassen für die Therapie in der Frühphase von COVID-19 sind: Casirivmab/Imdevimab (Ronapreve®), Regdanvimab (Regkirona®) und Sotrovimab (Xevudy®). Tixagevimab/Cilgavimab (Evusheld®) wurde zur Prä-Expositionsprophylaxe zugelassen, aufgrund seiner langen Halbwertszeit (Levin *et al.*, 2022; Robert-Koch-Institut, 2023). In klinischen Phase-3-Studien konnte für alle Antikörper eine signifikante Reduktion der Progression von milden und moderaten COVID-19-Verläufen zu schweren und kritischen Verläufen beobachtet werden (Dougan *et al.*, 2021; Eom *et al.*, 2021; Gupta *et al.*, 2021; Montgomery *et al.*, 2022; Weinreich *et al.*, 2021). Neuere Studien zeigten jedoch, dass die verfügbaren mAb *in vitro* die nun vorherrschenden Omikron-Varianten nicht mehr oder nur mit sehr reduzierter Effizienz neutralisieren können (Cao *et al.*, 2022; Iketani *et al.*, 2022; Planas *et al.*, 2022; Wang *et al.*, 2023). Aufgrund dieser Daten wird die Behandlung mit neutralisierenden mAb

nur noch bei immunsupprimierten Patienten empfohlen, wenn Virostatika aufgrund von Kontraindikationen nicht verwendet werden können (Robert-Koch-Institut, 2023).
Ein schwerer bis kritischer Verlauf von COVID-19 ist unter anderem durch den Zytokinsturm gekennzeichnet, welcher zu schweren Gewebeschäden führt. Zur Vermeidung einer überschießenden Reaktion des Immunsystems können immunmodulatorische Medikamente wie Januskinase-Inhibitoren oder IL-6-Rezeptor-Antagonisten eingesetzt werden.
Der JAK-I Baricitinib verhindert die Produktion von IL-6, welches bei Personen mit einem schwerem bis kritischen Verlauf von COVID-19 vermehrt im Serum zu beobachten ist (Chen *et al.*, 2020; Liu *et al.*, 2020). Empfohlen ist die Gabe von Baricitinib bei Patienten, welche eine zusätzliche, nicht-invasive Sauerstoff-Supplementation benötigen. Bei dieser Kohorte konnten Kalil *et al.* zeigen, dass bei Gabe von Baricitinib sowohl die Zeit zur Besserung als auch die Mortalitätsrate deutlich reduziert werden (Kalil *et al.*, 2021). Dieses Ergebnis konnte in einer großen, randomisierten Studie bestätigt werden (RECOVERY, 2022).
Tocilizumab, ein IL-6-Rezeptor-Antagonist, ist ein rekombinant hergestellter, humanisierter mAb. Aufgrund der Bindung von Tocilizumab an membrangebundenes und lösliches IL-6, werden pro-inflammatorische Signale gehemmt. Die Gabe von Tocilizumab wird ab einem schweren bis kritischen Verlauf empfohlen. Studien konnten eine reduzierte Mortalitätsrate bei Gabe von Tocilizumab beobachten (Boretti & Banik, 2022; Flisiak *et al.*, 2023; Karampitsakos *et al.*, 2023).

Alternative Therapieansätze

Zusätzliche, weitere Therapieansätze stellen die Gabe von RKP und Antidepressiva dar. Die Gabe von RKP war zu Beginn der Pandemie die einzige verfügbare Therapie. Die Wirkung beruht auf der Virusneutralisation durch die im Plasma vorhandenen Antikörper. Aktuell ist die Transfusion von RKP umstritten und wird nicht empfohlen. Dies liegt insbesondere an dem Umstand, dass die Omikron-Varianten viele Fluchtmutationen (engl.: *escape mutations*) im Spike-Protein aufweisen, wodurch die neutralisierende Wirkung von Antikörpern aus zuvor gesammelten Plasmen herabgesetzt wird (Sullivan *et al.*, 2022). Ebenso kann es bei Transfusionen zu schweren Komplikationen kommen. Als individueller Heilversuch bei immunsupprimierten Personen, insbesondere bei nicht vorhandener eigener Produktion von Antikörpern, kann eine Transfusion von RKP erwogen werden. Studien zeigten divergente Ergebnisse beim Einsatz von RKP zur Behandlung von COVID-19.

Sowohl der Titer des Plasmas, als auch der Zeitpunkt der Gabe scheinen einen wesentlichen Einfluss auf den Effekt zu haben (Focosi & Franchini, 2022; Lindemann *et al.*, 2021). Die größten Vorteile konnten bei Immunsupprimierten gezeigt werden, wo sich eine Reduktion der Mortalität beobachten ließ (Senefeld *et al.*, 2023; Senefeld *et al.*, 2021).

Antidepressiva stellen eine kostengünstige, verfügbare und zugelassene Alternativbehandlung dar. Studien konnten bei Fluvoxamin eine anti-inflammatorische und antivirale Wirkung im Tiermodell nachweisen (Péricat *et al.*, 2022). Allerdings zeigten Phase-3-Studien und retrospektive Studien keinen signifikanten Unterschied bei der Hospitalisierungs- oder Mortalitätsrate bei Patienten mit COVID-19 im Vergleich zu Patienten ohne Behandlung mit Fluvoxamin (Bramante *et al.*, 2022; Zheng *et al.*, 2022). Eine Studie von Jittamala *et al.* konnte bei Gabe von Fluoxetin einen positiven Effekt bei Probanden mit leichten Symptomen von COVID-19 nachweisen (Jittamala *et al.*, 2024).

2.9 Prävention von COVID-19

Impfung

Eine der wichtigsten Präventionsmaßnahmen von Infektionskrankheiten stellen Impfungen dar. Grundsätzlich wird zwischen einer passiven und einer aktiven Immunisierung unterschieden (Andre *et al.*, 2008). Die passive Immunisierung ist die Verabreichung von Immunglobulinen gegen das jeweilige Pathogen. Die neutralisierenden Antikörper sollen eine Infektion mit dem Pathogen verhindern. Diese Art der Impfung wird zumeist als Postexpositionsprophylaxe genutzt, etwa nach einer Nadelstichverletzung oder einer Verletzung durch ein Tier, bei dem eine Infektion mit dem Tollwut-Virus nicht ausgeschlossen werden kann (Modrow *et al.*, 2010). Da das Immunsystem nicht selbst aktiviert wird und die Antikörper schnell abgebaut werden, hält der Schutz nur wenige Wochen an. Bei der aktiven Immunisierung werden Tot- und Lebendimpfstoffe unterschieden. Bei Lebendimpfstoffen werden avirulente, attenuierte Viren, rekombinante oder chimäre Viren verwendet. Impfstoffe mit avirulenten Viren führen zu einer humoralen und zellulären Immunantwort, da das Virus replizieren kann und neue virale Partikel entstehen (Modrow *et al.*, 2010). Bei Impfungen mit rekombinanten oder chimären Viren entsteht nicht nur eine Immunreaktion gegenüber dem gewünschten Pathogen, sondern darüber hinaus eine Immunantwort gegenüber dem Virus, welches geimpft wird und für die Proteine des

gewünschten Pathogens codiert (Bernstein *et al.*, 2020; Suárez-Pedroso *et al.*, 2021). Die Impfung mit Lebendimpfstoffen bietet aufgrund der Replikation der Viren einen Schutz vor vielen immundominanten viralen Proteinen und führt zu einer langanhaltenden humoralen und zellulären Immunantwort. Bei Immunsupprimierten besteht jedoch die Gefahr einer mitunter schweren Infektion, da die Viren voll replikationsfähig sind (Abbildung 7).

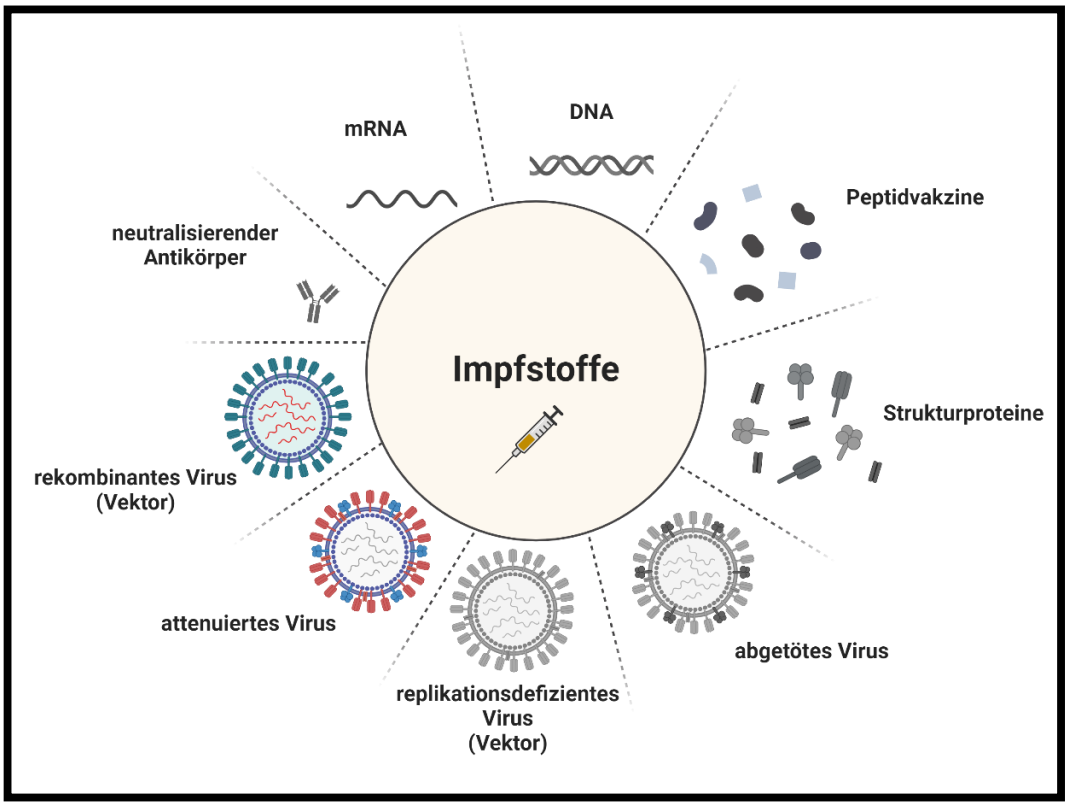

Abbildung 7: Arten von Impfstoffen. Es werden passive und aktive Immunisierungen unterschieden. Die passive Impfung besteht aus verabreichten Immunglobulinen, welche das Pathogen neutralisieren. Die aktive Immunisierung wird in Lebend- und Totimpfstoffe unterteilt. Zu den Lebendimpfstoffen zählen rekombinante, chimäre und attenuierte Viren. Bei Totimpfstoffen werden replikationsdefiziente oder abgetötete Viren verwendet, ebenso Strukturproteine oder Peptide des Pathogens. Seit einigen Jahren werden auch DNA- und mRNA-Impfstoffe genutzt, welche für bestimmte Gene des Pathogens codieren. (Modifizierte Abbildung von BioRender.com [https://app.biorender.com/biorender-templates] [2024]).

Als Totimpfstoffe werden abgetötete Pathogene, Strukturproteine oder Peptide des jeweiligen Pathogens verwendet. Da es zu keiner Replikation des Pathogens im zu immunisierenden Organismus kommt, wird zumeist nur eine Antikörperantwort hervorgerufen, jedoch nicht immer die Bildung von zytotoxischen T-Zellen (Altenburg *et al.*, 2014). Seit einigen Jahren werden Desoxyribonukleinsäure- (engl.: *deoxyribonucleic acid*, DNA) und mRNA-Impfstoffe verwendet, welche für einzelne virale Gene codieren (Fotin-Mleczek *et al.*, 2011). Der zu immunisierende Organismus transkribiert und translatiert diese Gene zu organismusfremden Proteinen, welche zu einer Immunreaktion führen. Hierdurch kommt es zur humoralen und zellulären

Immunantwort gegen dieses virale Protein, jedoch nicht gegen weitere virale Proteine (Hoerr *et al.*, 2000; Kramps & Probst, 2013).

In Deutschland kamen zu Beginn der Pandemie vor allem Vektor- und mRNA-Impfstoffe zum Einsatz gegen SARS-CoV-2. Zu den Vektor-Impfstoffen zählen Janssen Ad26.COV2.S der Firma Janssen-Cilag International und Vaxzevria® der Firma AstraZeneca. Beide Impfstoffe basieren auf Adenoviren, welche die genetische Information für das Spike-Protein von SARS-CoV-2 enthalten. Die mRNA-Impfstoffe Comirnaty® (BioNTech/Pfizer) und Spikevax® (Moderna) bestehen aus der für das Spike-Protein codierenden mRNA. Bei allen vier Impfstoffen konnten humorale und zelluläre Immunantworten nach Impfung festgestellt werden (Feikin *et al.*, 2022; Zhang *et al.*, 2022). Allerdings kam es bei den Vektorimpfstoffen zu fatalen Nebenwirkungen, wie zerebralen venösen Sinusthrombosen, sodass die Zulassung eingeschränkt wurde (Sharifian-Dorche *et al.*, 2021). Feikin *et al.* konnten zeigen, dass es insbesondere mit dem Auftreten der Delta-Varianten zu immer mehr Impfdurchbruchsinfektionen kam und die Wahrscheinlichkeit für eine solche Infektion steigt, je länger die Impfung gegen SARS-CoV-2 zurückliegt (Feikin *et al.*, 2022). Es wurden Auffrischungsimpfungen mit mRNA-Impfstoffen durchgeführt, welche zu einem Anstieg der humoralen und zellulären Immunantwort gegenüber SARS-CoV-2 führten, jedoch wirkten die Antikörper nur unzureichend gegen die Varianten Delta und Omikron (Tartof *et al.*, 2022).

Seit September 2023 sind an die SARS-CoV-2-Variante XBB.1 angepasste mRNA-Impfstoffe zugelassen (Comirnaty® XBB.1.5; Spikevax® XBB.1.5). Ein Protein-basierter Impfstoff von Novavax soll gemäß der ständigen Impfkommission (STIKO) zugelassen werden (STIKO, 2023). Eine erste Studie von Stankov *et al.* zeigte einen Anstieg der neutralisierenden Antikörper in den Seren der Probanden nach Impfung mit Comirnaty® XBB.1.5 (Stankov *et al.*, 2024).

Evusheld®

Insbesondere bei Immunsupprimierten wird durch eine Impfung nicht immer eine Immunantwort generiert. Eine Möglichkeit, diese Personen dennoch vor einer Infektion mit SARS-CoV-2 zu schützen, ist die Prä-Expositionsprophylaxe. In Deutschland zugelassen ist die Kombination der monoklonalen Antikörper Tixagevimab und Cilgavimab (Evusheld®) (Levin *et al.*, 2022; Robert-Koch-Institut, 2023). Studien konnten zeigen, dass Tixagevimab/Cilgavimab bei immunsupprimierten Personen, wie Patienten nach einer Stammzelltransplantation, einen Schutz vor einer SARS-CoV-2-

Infektion bietet, jedoch ist dies abhängig von der Variante (Focosi & Casadevall, 2022; Jondreville *et al.*, 2022; Nassar *et al.*, 2023).

Antidepressiva

Antidepressiva zeigen anti-inflammatorische und antivirale Wirkungen. Zusätzlich wirken einige Antidepressiva wie Fluoxetin, Sertralin und Amitriptylin als funktionelle Inhibitoren der sauren Sphingomyelinase-Aktivität (engl*.: functional inhibitors of acid sphingomyelinase activity*, FIASMA). Studienergebnisse legen nahe, dass SARS-CoV-2 an Ceramid-angereicherten Regionen in die Wirtszelle gelangt (Carpinteiro *et al.*, 2020). Diese Ceramide entstehen aufgrund der enzymatischen Reaktion der sauren Sphingomyelinase mit Sphingomyelinen zu Ceramiden (Gulbins *et al.*, 2013). Carpinteiro *et al.* konnten *in vitro* nachweisen, dass durch den Einsatz von FIASMA die Umsetzung von Sphingomyelin zu Ceramid und somit der Eintritt von SARS-CoV-2 in die Wirtszelle verhindert wird (Carpinteiro *et al.*, 2020). Dieser Mechanismus scheint unabhängig von den Varianten von SARS-CoV-2 zu sein. Dies bietet den Vorteil, dass ein Medikament über einen langen Zeitraum verabreicht werden könnte ohne nötige Anpassungen an die Varianten durchlaufen zu müssen. *In vivo* Studien konnten bisher die gute Verträglichkeit der Antidepressiva sowie ihre antivirale Wirkung gegenüber SARS-CoV-2 zeigen (Jittamala *et al.*, 2024). Insbesondere in Entwicklungsländern, in denen die finanziellen Mittel für gezielte präventive und therapeutische Medikamente fehlen, könnten Antidepressiva eine kostengünstige Alternative darstellen.

2.10 Zielsetzung der Arbeit

Ein wichtiger Meilenstein in der Eindämmung der COVID-19-Pandemie war die Entwicklung von Impfstoffen gegen SARS-CoV-2. Erkenntnisse über die humoralen und zellulären Immunantworten bei geimpften und nicht-geimpften Personen sind von besonderer Bedeutung für eine mögliche Vorhersage des Krankheitsverlaufes. In der vorliegenden Arbeit wurde die humorale und zelluläre Immunreaktion bei einer geriatrischen Kohorte untersucht. Ebenso wurden die Auswirkungen des Einzelnukleotid-Polymorphismus *GNB3* c.825C>T auf die zelluläre Immunantwort analysiert. Da immunsupprimierte Personen ein erhöhtes Risiko für einen schweren bis tödlichen Verlauf von COVID-19 haben und zudem nicht immer eine ausreichende Impfantwort generieren, ist die Untersuchung der Wirksamkeit der Impfungen bei dieser Personengruppe besonders wichtig. In der vorliegenden Arbeit haben wir die

humorale und zelluläre Immunantwort nach Impfung bei Personen nach Nierentransplantation und Stammzelltransplantation untersucht.

Die Entwicklung von Therapien zur Behandlung von COVID-19 und Vermeidung schwerer Verläufe war ebenfalls von großer Bedeutung. Ein Therapieansatz war die Transfusion von Plasma von Rekonvaleszenten. Ein weiterer Therapieansatz war der Einsatz neutralisierender, monoklonaler Antikörper zur Vermeidung schwerer Verläufe und Aufbau einer körpereigenen Immunität bei immungeschwächten Patienten. Wir haben die zelluläre Immunantwort bei geimpften und nicht-geimpften Immunsupprimierten fünf Monate nach Gabe von mAb analysiert.

In dieser Arbeit wurde die klinische Wirksamkeit einer monoklonalen Antikörpertherapie im Vergleich zur Gabe von Rekonvaleszenten-Plasmen und symptom-orientierter Behandlung bei vulnerablen, nicht-geimpften Patienten retrospektiv untersucht.

Im Verlauf der Pandemie schützten die Impfungen nur noch unzureichend gegen die durch Mutationen entstandenen neuen Varianten von SARS-CoV-2. Erkenntnisse über die durch Infektionen induzierte Immunantwort sind wichtig zum Verständnis, ob diese Immunantwort zu einem langanhaltenden Schutz vor weiteren Infektionen mit Varianten führen kann. Wir haben die humorale und zelluläre Immunantwort bei Durchbruchsinfektionen mit der Delta- und Omikron-Variante analysiert.

Aufgrund der neu auftretenden Varianten von SARS-CoV-2 bedarf es Anpassungen der Medikamente und Impfstoffe. Dies stellt insbesondere Entwicklungsländer vor eine große Herausforderung, da weder neue Wirkstoffe entwickelt noch importiert werden können. Bereits zugelassene Medikamente, welche auf ihre Wirksamkeit gegenüber anderen Krankheiten und Krankheitserregern hin erforscht werden, stellen eine mögliche Option zur Behandlung und Prävention von COVID-19 dar. Wir haben die Wirksamkeit der Antidepressiva Fluoxetin und Sertralin hinsichtlich ihrer inhibierenden Eigenschaften der Replikation von SARS-CoV-2 *in vitro* analysiert.

3. Publikationen

3.1 Cellular and Humoral Immunity after the Third Vaccination against SARS-CoV-2 in Hematopoietic Stem-Cell Transplant Recipients

Thümmler, L., Koldehoff, M., Fisenkci, N., Brochhagen, L., Horn, P. A., Krawczyk, A., Lindemann, M.

2022

Anteile:

- Durchführung der Experimente: 60 %
- Datenanalyse: 40 %
- Statistische Analyse: 50 %
- Manuskripterstellung: 50 %
- Überarbeitung des Manuskriptes: 20 %

Das Konzept dieser Studie wurde von Monika Lindemann erstellt. Laura Thümmler, Neslinur Fisenkci und Leonie Brochhagen haben die Experimente durchgeführt. Die Daten wurden durch Laura Thümmler, Michael Koldehoff, Monika Lindemann und Adalbert Krawczyk analysiert. Monika Lindemann und Laura Thümmler haben die statistische Analyse übernommen und das Manuskript erstellt. An der Überarbeitung des Manuskriptes waren Laura Thümmler, Monika Lindemann, Peter A. Horn und Adalbert Krawczyk beteiligt.

Laura Thümmler	Prof. Dr. med. Monika Lindemann

vaccines

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Article

Cellular and Humoral Immunity after the Third Vaccination against SARS-CoV-2 in Hematopoietic Stem-Cell Transplant Recipients

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Abstract: Protecting vulnerable groups from severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) infection is mandatory. Immune responses after a third vaccination against SARS-CoV-2 are insufficiently studied in patients after hematopoietic stem-cell transplantation (HSCT). We analyzed immune responses before and after a third vaccination in HSCT patients and healthy controls. Cellular immunity was assessed using interferon-gamma (IFN-γ) and interleukin-2 (IL-2) ELISpots. Furthermore, this is the first report on neutralizing antibodies against 11 variants of SARS-CoV-2, analyzed by competitive fluorescence assay. Humoral immunity was also measured by neutralization tests assessing cytopathic effects and by ELISA. Neither HSCT patients nor healthy controls displayed significantly higher SARS-CoV-2-specific IFN-γ or IL-2 responses after the third vaccination. However, after the third vaccination, cellular responses were 2.6-fold higher for IFN-γ and 3.2-fold higher for IL-2 in healthy subjects compared with HSCT patients. After the third vaccination, neutralizing antibodies were significantly higher ($p < 0.01$) in healthy controls, but not in HSCT patients. Healthy controls vs. HSCT patients had 1.5-fold higher concentrations of neutralizing antibodies against variants and 1.2-fold higher antibody concentrations against wildtype. However, half of the HSCT patients exhibited neutralizing antibodies to variants of SARS-CoV-2, which increased only slightly after a third vaccination.

Keywords: ELISpot; variants of concern; vaccination response; T cells

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1. Introduction

Since the beginning of the SARS-CoV-2 pandemic, over 500 million people have been infected, and more than six million people have died due to COVID-19. Stem-cell transplant recipients have increased mortality and morbidity from COVID-19 due to immunosuppression [1–4].

For individuals belonging to vulnerable groups, protection against SARS-CoV-2 infection through vaccination is of enormous importance [5]. It is equally important that people in their immediate environment are protected by vaccination, and that infection is avoided [6].

However, studies showed that immunocompromised patients developed no or only weak immune responses after SARS-CoV-2 infection and two vaccinations [2,7]. Simi-

larly, immunocompromised individuals suffer more frequently from vaccine breakthrough infection, i.e., COVID-19 despite two vaccinations [8,9].

Studies have shown that a third vaccination is needed, particularly in vulnerable groups, to provide a protective effect against SARS-CoV-2 or to boost the existing weak immune response [10–14]. Currently, there are limited data on whether a third vaccination can boost specific immunity in immunocompromised patients after hematopoietic stem-cell transplantation (HSCT). Our study focuses on an even more detailed investigation of cellular and humoral immunity, particularly with regard to the evolved variants of SARS-CoV-2, which now account for the majority of infections.

We examined the cellular and humoral immunity to SARS-CoV-2 in immunocompromised and non-immunocompromised vaccinated individuals before and after the third vaccination. Cellular immunity was tested by SARS-CoV-2-specific interferon (IFN)-γ and interleukin (IL)-2 ELISpot assay and a newly established fluorescence ELISpot assay which can detect IFN-γ and IL-2 simultaneously. Titers for neutralizing antibodies to wildtype SARS-CoV-2 were analyzed by neutralization test. In addition, we examined neutralizing antibodies to variants and mutations of SARS-CoV-2 by competitive fluorescence assay. SARS-CoV-2-specific IgG antibodies were measured by semi-quantitative and quantitative ELISA.

2. Materials and Methods

2.1. Volunteers

As a first group, we included 24 HSCT patients tested prior to the third vaccination and 18 HSCT patients tested after the third vaccination against SARS-CoV-2. HSCT was performed at a median of 4.1 years (range 0.5–24) prior to blood sampling. The subgroup tested prior to the third vaccination contained 10 males and 14 females; the median age was 62 years (range 39–73). In this group, the second vaccination took place at a median of 174 days (range 55–255) prior to testing. The subgroup tested after the third vaccination contained 10 males and eight females; the median age was 61 years (range 21–71). The group was examined at a median of 34 days (range 11–95) after the third vaccination.

As a second group, we included 18 healthy volunteers before and 19 healthy volunteers after the third vaccination. The subgroup before third vaccination was composed of seven males and 11 females, and the median age was 50 years (range 31v83). They were tested at a median of 187 days (range 60–273) after the second vaccination. The subgroup after the third vaccination contained 10 males and 19 females; the median age of the donors was 50 years (range 35–65). The group was examined at a median of 42 days (range 27–72) after the third vaccination. Both groups, the HSCT patients and the healthy controls prior to and post third vaccination, were unpaired.

The study was approved by the local ethics committee of the University Hospital Essen, Germany (20-9225-BO and 20-9254-BO), and all volunteers provided informed consent to participate in the study. The study was 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

For simultaneous staining of IFN-γ and IL-2, we used the CE-marked CoV-iSpot (AID, Strassberg, Germany) in 63 samples (22 HSCT patients, 41 healthy controls). This fluorescence ELISpot (Fluorospot) includes a peptide mix of the wildtype SARS-CoV-2 spike protein. In the negative controls, we could detect, on average, 0.9 spots (range 0–4) in the group of HSCT patients and 1.1 spots (range 0–10) in healthy controls. In positive controls, we saw, on average, 383 spots (range 105–768) in the group of HSCT patients and 508 spots (range 170–925) in healthy controls. The cutoff definition was described in a previous study [15]. We chose a spot increment of seven as a cutoff for the positivity for IFN-γ and a spot increment of three for IL-2.

2.3. In-House ELISpot Assay

To further assess cellular immunity to SARS-CoV-2, we separately performed IFN-γ and IL-2 ELISpot assays. The procedure for IFN-γ ELISpot assay was previously described [15,16]. Briefly, 60 µL of monoclonal antibodies against IFN-γ (10 µg/mL of clone 1-D1K, Mabtech, Nacka, Sweden) or IL-2 (10 µg/mL, clone MT2A91, Mabtech) were used for coating of plates containing polyvinylidene difluoride (PVDF) membranes (Millipore-Sigma™ MultiScreen™ HTS, Fisher Scientific, Schwerte, Germany) after activation with ethanol. Thereafter, ELISpot plates were washed and blocked for 30 min at 37 °C with 150 µL of AIM-V® (Thermo Fisher Scientific, Grand Island, NY, USA). After incubation, AIM-V® was discarded, and duplicates of 250,000 peripheral blood mononuclear cells (PBMCs) were cultured with or without of either PepTivator® SARS-CoV-2 protein S1/S2 or protein S1 (600 pmol/mL of each peptide, Miltenyi Biotec, Bergisch Gladbach, Germany) in 150 µL of AIM-V®. In parallel, cell cultures of 250,000 PBMC were grown in the presence or absence of a protein S1 (4 µg/mL, Sino Biological, Wayne, PA, USA) in 150 µL of AIM-V® for 19 h at 37 °C. After incubation, the ELISpot plates for IFN-γ were washed and incubated for 1 h with 50 µL of the alkaline phosphatase-conjugated monoclonal antibody against IFN-γ (clone 7-B6-1, Mabtech), diluted 1:200 with PBS plus 0.5% bovine serum albumin (BSA).The ELISpot plates for IL-2 were washed and incubated for 1 h with 50 µL of the biotinylated monoclonal antibody against IL-2 (clone MT2A91, Mabtech), diluted 1:100 with PBS plus 0.5% BSA. Thereafter, the plates were washed and incubated for 1 h with 100 µL of the alkaline phosphatase-conjugated streptavidin (Mabtech), diluted 1:1000 with PBS plus 0.5% BSA. After further washing of the IFN-γ and IL-2 ELISpot, 50 µL of NBT/BCIP was added, and purple spots appeared within 7 min. Spot numbers were analyzed by an ELISpot reader (AID Fluorospot, Autoimmun Diagnostika GmbH, Strassberg, Germany). Mean values of duplicate cell cultures were considered. SARS-CoV-2-specific spots were determined according to the spot increment as previously described [15]. The negative controls for IFN-γ had, on average, 0.5 spots (range 0–13) in the group of HSCT patients and 1.1 spots (range 0–10) in the group of healthy controls. The positive controls had, on average, 492 spots (range 91–600) in the group of HSCT patients and 512 spots (range 100–600) in the group of healthy controls. If the reader could not detect individual spots, we declared this as 600 spots. We chose five as cutoff for positivity for IFN-γ and seven for IL-2.

2.4. Assessment of Neutralizing Antibodies by Competitive Immunofluorescence

A commercial competitive immunofluorescence assay (Bio-Plex Human SARS-CoV-2 Variant Neutralization Antibody 11-Plex Panel, BIO-RAD) was used to detect neutralizing antibodies against wildtype SARS-CoV-2 and 11 mutations belonging to variants of SARS-CoV-2 (Table 1). In this commercial assay, neutralizing antibodies from serum compete with biotinylated ACE2 receptors. Detection is achieved by the addition of streptavidin–phycoerythrin (SA–PE), which binds to the biotinylated ACE2 receptor. A lower measured fluorescence indicates a higher concentration of neutralizing antibodies. Results $\geq$1000 ng/mL define the upper limit of the system.

2.5. Assessment of Neutralizating Antibodies by Cytopathic Effects

An endpoint dilution assay was used to determine the neutralization titer of sera from HSCT patients and healthy controls, as previously described [17]. Serial dilutions (1:20 to 1:2560) of the respective sera were incubated with 100 $TCID_{50}$ of SARS-CoV-2 for 1 h at 37 °C. After the incubation, the dilutions were added to confluent Vero-E6 cells in 96-well microtiter plates and incubated for 3 days. Thereafter, cells were stained with crystal violet (Roth, Karlsruhe, Germany) solved in 20% methanol (Merck, Darmstadt, Germany) and analyzed by light microscopy for the appearance of cytopathic effects (CPEs). The neutralization titer was defined as the reciprocal of the highest serum dilution at which no CPE breakthrough was observed in any of the three test wells.

Table 1. Overview of the mutations tested by the competitive immunofluorescence assay with assignment to the different variants of SARS-CoV-2.

Variant \ Mutation	HV69-70 del	Y144 del	K417N	K417T	L452R	E484K	E484Q	N501Y	A570D	D614G	P681H
Wildtype											
B 1.1.7 (alpha)	✓	✓						✓	✓	✓	✓
B 1.351 (beta)			✓			✓		✓		✓	
P.1 (gamma)			✓			✓		✓		✓	
B 1.617.2/AY.1/.2 (delta/delta plus)					✓					✓	
B 1.427/B 1.429 (epsilon)					✓					✓	
B 1.525 (eta)	✓					✓				✓	
B 1.526 (iota)						✓				✓	
B 1.617.1/B 1.17.3 (kappa)					✓		✓				
C.37 (lambda)										✓	
B 1.621 (mu)						✓		✓		✓	✓
B 1.1.529 (omicron)	✓		✓					✓		✓	✓

2.6. Antibody ELISA

For the detection of SARS-CoV-2-specific antibodies, we used a CE-marked anti-SARS-CoV-2 IgG semiquantitative ELISA (Euroimmun, Lübeck, Germany) and a quantitative ELISA (anti-SARS-CoV-2-QuantiVac-ELISA, Euroimmun, Lübeck, Germany). The ELISAs were performed according to the manufacturer's instructions automatically at a dilution of 1:100 of the sera, using the Immunomat (Virion\Serion, Würzburg, Germany). Plates were coated with wildtype recombinant SARS-CoV-2 spike protein (S1 domain). Results for the semiquantitative ELISA were given as the ratio of patient sample to control sample. An antibody ratio of >1.1 was considered positive, of $\geq$0.8 to <1.1 was considered borderline, and of <0.8 was considered negative. Results for the quantitative ELISA were given as BAU/mL.

2.7. Statistical Analysis

We used GraphPad Prism 9.3.1 (San Diego, CA, USA) software for statistical analysis. We used the Kolmogorov–Smirnov test to check for normality. Mann–Whitney test and Spearman analysis were used to correlate numerical variables (Tables S1–S4 and S7–S11). Two-sided p-values <0.05 were considered significant. To correlate the interval between HSCT and blood collection with SARS-CoV-2-specific immune responses, we used Spearman analysis (Tables S5 and S6).

3. Results

3.1. Comparison of Cellular Immunity in HSCT Patients and Healthy Controls before and after Third Vaccination

We analyzed the cellular immune response in HSCT patients and healthy controls before and after third vaccination. In the commercial CoV iSpot, there was no significant increase after the third vaccination in HSCT patients or in healthy controls, which was observed both for IFN-γ and IL-2, when cells were stimulated with the S pool of wildtype SARS-CoV-2 (Figure 1). Prior to the third vaccination, two patients out of 12 showed a positive response for IFN-γ, while three out of 12 showed a positive response for IL-2 and two out of 12 showed a positive response for both. After vaccination, the respective numbers in the HSCT patients were five out of 12 for IFN-γ, six out of 12 for IL-2, and two out of 12 for both. The spot increment for IL 2 after third vaccination differed significantly

between HSCT patients and healthy controls ($p = 0.007$) (Table S7). In addition, the spot increments for simultaneous secretion of IFN-γ and IL-2 were significantly lower in HSCT patients than in healthy controls after the third vaccination ($p = 0.02$).

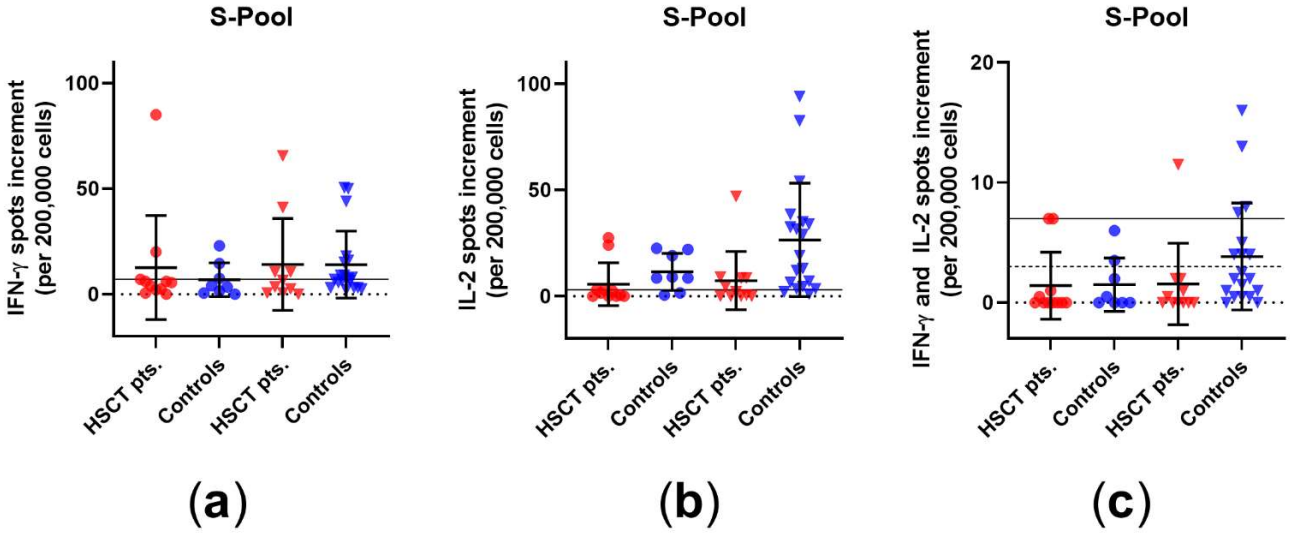

Figure 1. SARS-CoV-2-specific CoV-iSpot responses in HSCT patients and healthy controls before and after the third vaccination. Distribution of (**a**) IFN-γ, (**b**) IL-2, and (**c**) simultaneous IFN-γ and IL-2 CoV-iSpot responses upon stimulation with S pool of wildtype SARS-CoV-2. Please note that the scales differ. Circles indicate data before the third vaccination (red: HSCT, blue: healthy controls), while triangles indicate data after the third vaccination. Responses before and after the third vaccination were compared by two-tailed Mann–Whitney test. Horizontal lines indicate mean values, while error bars indicate the standard deviation. The dotted line represents the zero line. The black horizontal line indicates the cutoff. In panel (**c**), the continuous line indicates the cutoff for IFN-γ, while the dashed line indicates the cutoff for IL-2.

Using our *in-house* ELISpot, we observed after the third vaccination no significant increase in IFN-γ or IL-2 spots in one of the two groups after stimulation with a peptide mix of S1/S2 or S1 or with an S1 protein, which is recombinantly expressed in (human) HEK293 cells (called S1 Sino hereinafter) (Figure 2). Prior to the third vaccination, seven of the 24 patients showed a positive response to the S1/S2 peptide mix, six of the 24 showed a positive response to the S1 peptide mix, and two of the 24 showed a positive response to the S1 Sino. After vaccination, the respective numbers in the HSCT patients were five out of 18 for the S1/S2 peptide mix, six out of 18 for the S1 peptide mix, and two out of 18 for S1 Sino. For the S1/S2 peptide mix, there were no significant differences between healthy controls and HSCT patients before and after the third vaccination. For the S1 peptide mix, healthy controls displayed significantly higher IFN-γ spot increment than HSCT patients before and after third vaccination ($p = 0.01$ and $p = 0.006$). When stimulated with S1 Sino, healthy controls showed a significantly higher spot increment for IFN-γ after third vaccination than HSCT patients ($p = 0.001$) (Table S8). For IL-2, we detected no significant differences within the groups. However, healthy controls had a higher spot increment than HSCT patients after stimulation with the S1/S2 and S1 peptide mix and with S1 Sino, both before and after third vaccination (S1/S2: $p = 0.1$ and $p = 0.0005$; S1: $p = 0.04$ and $p = 0.01$; S1 Sino: $p = 0.02$ and $p = 0.02$) (Table S9).

Summarizing the cellular data, neither HSCT patients nor healthy controls displayed significantly higher SARS-CoV-2-specific responses after the third vaccination, which was detected by various assay formats and for the cytokines IFN-γ and IL-2. However, as expected, responses in healthy controls were overall higher than in HSCT patients.

3.2. Comparison of Humoral Immune Responses to Variants of SARS-CoV-2 in HSCT Patients and Healthy Controls

We examined using a competitive immunoassay whether vaccination also leads to a humoral immune response against different variants and mutations of SARS-CoV-2. In HSCT patients, there was no significant increase in neutralizing antibodies after third vaccination for any of the mutations tested, whereas, in healthy controls, there was a significant increase in neutralizing antibodies for each variant/mutation tested, i.e., alpha, beta, gamma, delta (plus), epsilon, eta, iota, kappa, lambda, mu, and omicron (Figure 3). In detail, prior to the third vaccination, 15 of the 24 patients showed a positive response to the D614G mutation, found in the delta and omicron variant (Figure 3i), 15 of the 24 showed a positive response to the K417N mutation (omicron variant, Figure 3j), and 13 of the 24 showed a positive response to the N501Y mutation (omicron, Figure 3k). After vaccination, the respective numbers in the HSCT patients were 11 out of 18 for D614G, 12 out of 18 for K417N, and 11 out of 18 for N501Y. Thus, about half of the patients responded to mutations found in the delta (plus) and omicron variant. Prior to the third vaccination, eight of the nine healthy controls responded to the D614G mutation, seven of nine responded to K417N, and seven of nine responded to N501Y. After vaccination, the numbers in healthy controls were 19 out of 19 for D614G, 19 out of 19 for K417N, and 19 out of 19 for N501Y. The comparison between HSCT patients and the healthy control group after third vaccination revealed significant differences in neutralizing antibodies against all variants/mutations tested (Table S10).

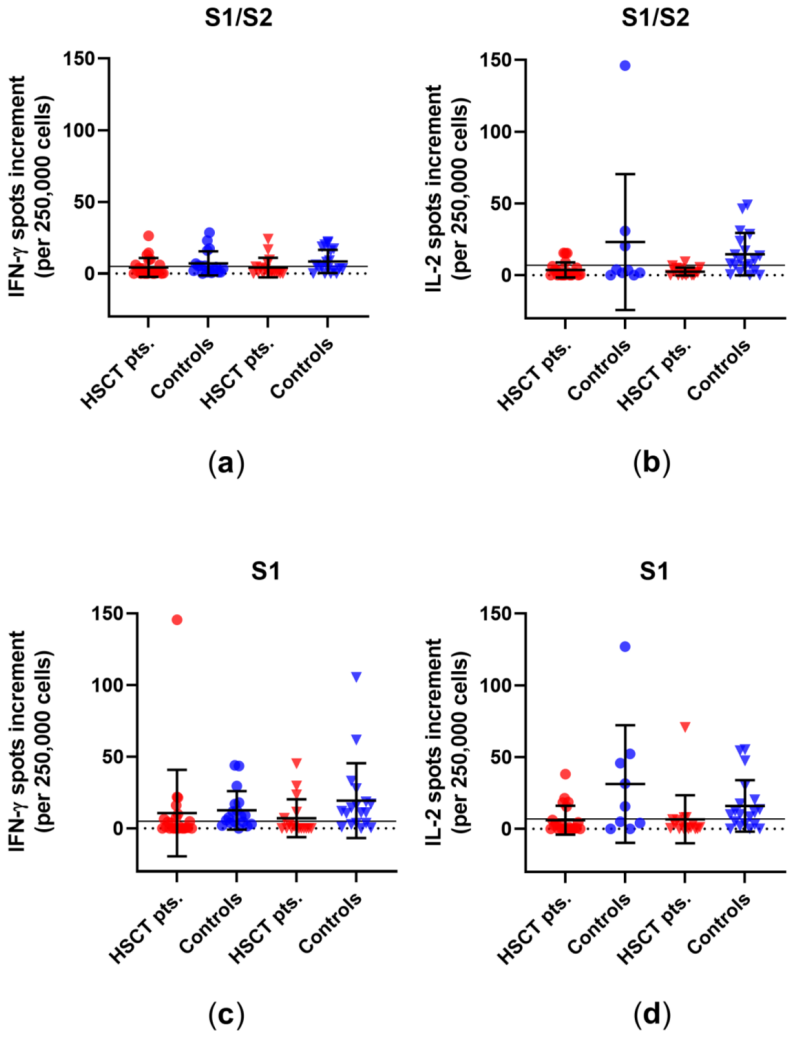

Figure 2. *Cont.*

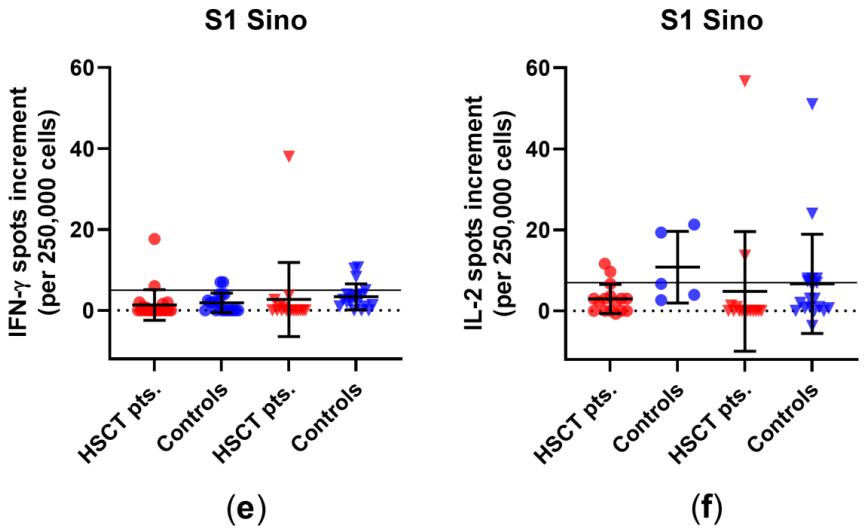

Figure 2. SARS-CoV-2-specific ELISpot responses in HSCT patients and healthy controls before and after third vaccination. Distribution of (**a**) IFN-γ and (**b**) IL-2 ELISpot responses upon stimulation with an S1/S2 peptide mix. Distribution of (**c**) IFN-γ and (**d**) IL-2 ELISpot responses upon stimulation with an S1 peptide mix. Distribution of (**e**) IFN-γ and (**f**) IL-2 ELISpot responses upon stimulation with S1 Sino. Please note that the scales differ. Circles indicate data before the third vaccination (red—HSCT, blue—healthy controls), while triangles indicate data after the third vaccination. Responses were compared by two-tailed Mann–Whitney test. Horizontal lines indicate mean values, while error bars indicate the standard deviation. The dotted line represents the zero line. The black horizontal line indicates the cutoff.

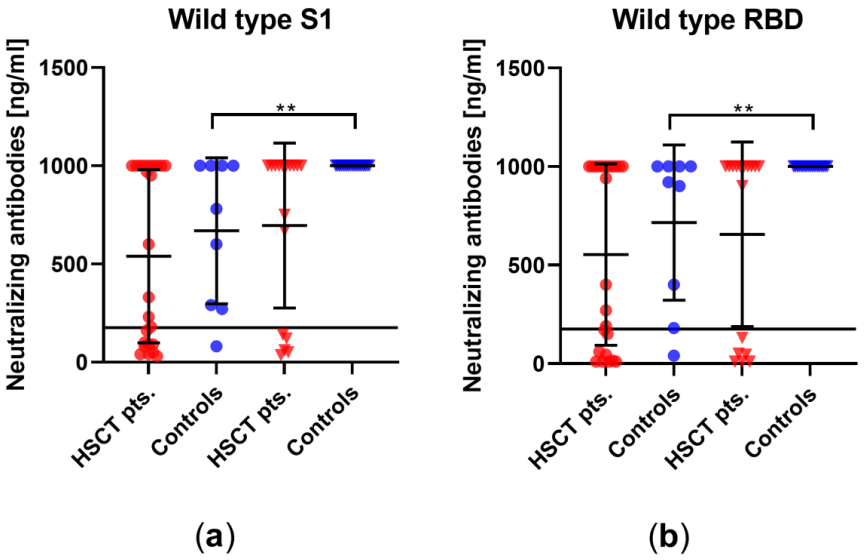

Figure 3. *Cont.*

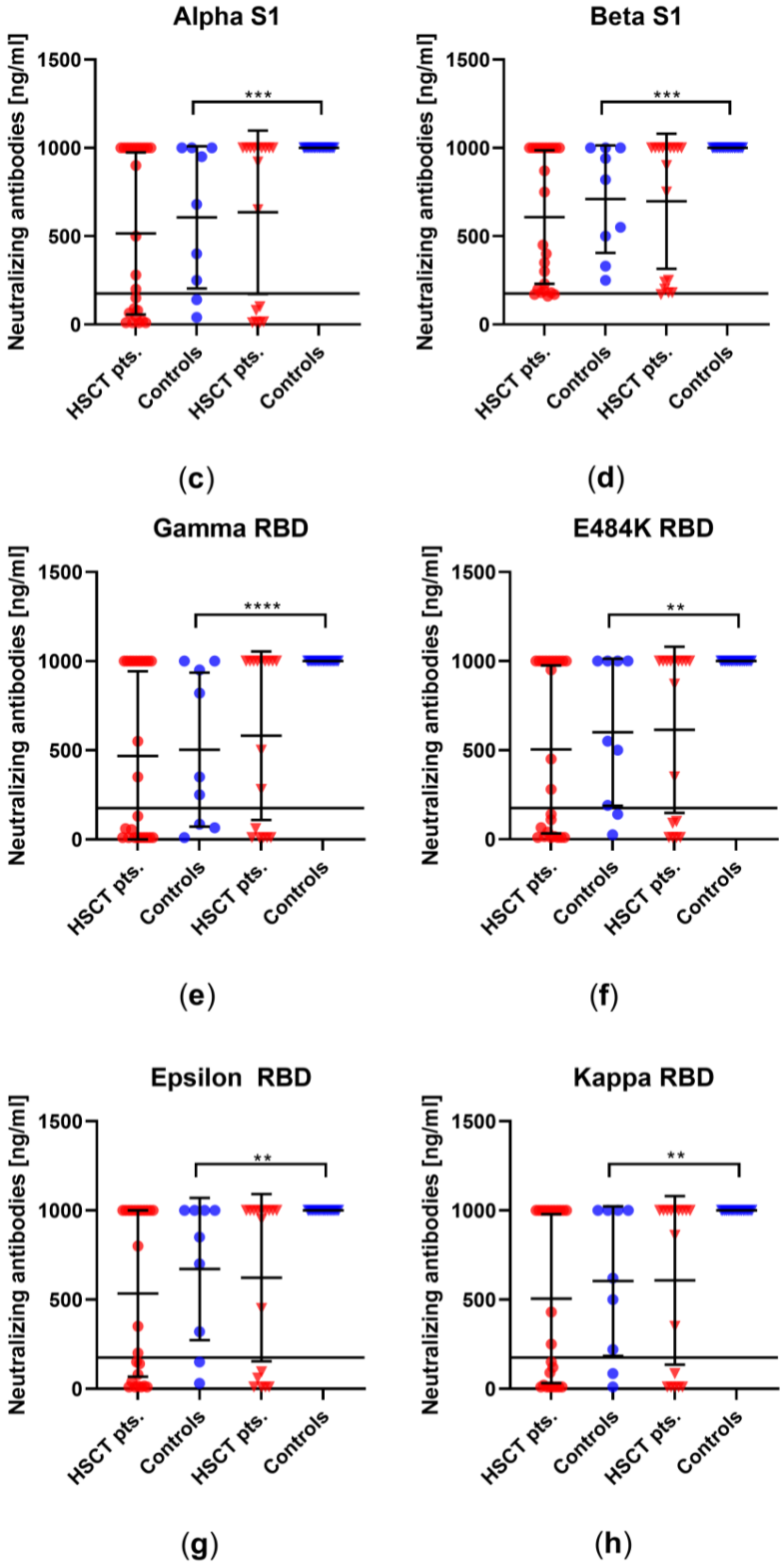

Figure 3. *Cont.*

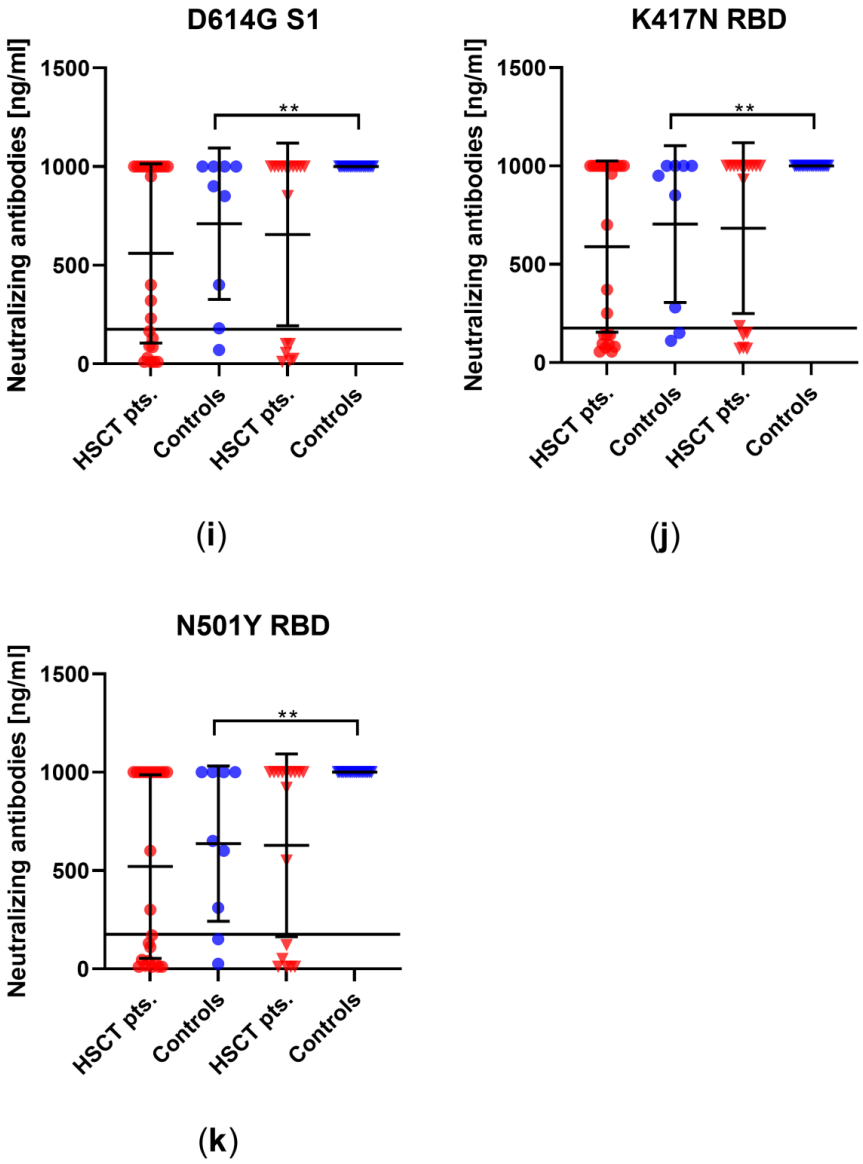

Figure 3. Concentration of neutralizing antibodies against different variants of the subunit 1 of spike protein (S1) or the receptor-binding domain (RBD) of SARS-CoV-2 in HSCT patients and healthy controls before and after third vaccination. Humoral responses before and after third vaccination against (**a**) wildtype S1, (**b**) wildtype RBD, (**c**) alpha, (**d**) beta, (**e**) gamma, (**f**) E484K, (**g**) epsilon, (**h**) kappa, (**i**) D614G, (**j**) K417N, and (**k**) N501Y. D614G is a mutation found in delta and omicron variants, while K417N and N501Y are mutations in the omicron variant. Circles indicate data before the third vaccination (red—HSCT, blue—healthy controls), while triangles indicate data after the third vaccination. Responses were compared by two-tailed Mann–Whitney test (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Horizontal lines indicate mean values, while error bars indicate the standard deviation.

3.3. Comparison of Humoral Vaccination Responses to Wildtype Virus in Patients after Hematopoietic Stem-Cell Transplantation and Healthy Controls

We evaluated neutralizing antibodies against wildtype SARS-CoV-2 in the sera to determine if the immunocompromised individuals might build up a similar level of neutralizing antibodies to healthy controls. In HSCT patients, no significant increase in neutralizing antibodies after the third vaccination could be detected (mean titer^{-1} of 432 vs. 686, $p = 0.4$), whereas there was a significant increase in neutralizing antibodies in healthy controls

(mean titer^{-1} of 140 vs. 884, $p < 0.0001$) (Figure 4). A significantly lower titer was observed in HSCT patients than in healthy controls after the third vaccination ($p = 0.05$).

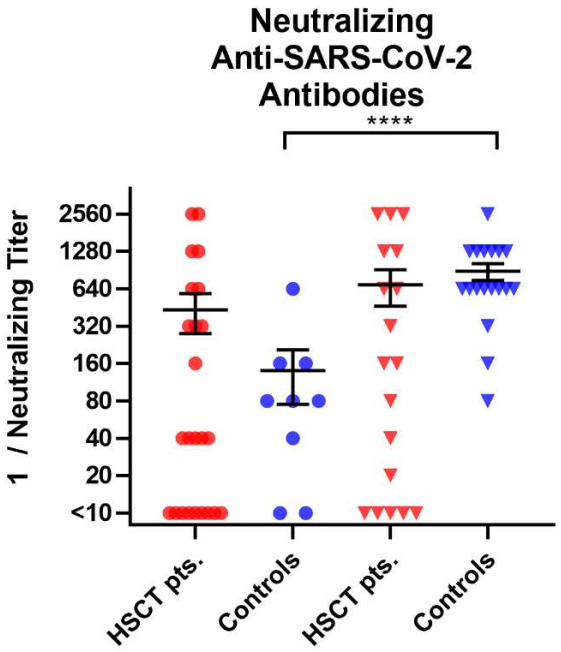

Figure 4. Titer of SARS-CoV-2-specific neutralizing antibodies in HSCT patients and healthy controls before and after third vaccination. The *y*-axis shows the reciprocal of the titer of neutralizing anti-SARS-CoV-2 antibodies. Circles indicate data before the third vaccination (red—HSCT, blue—healthy controls), while triangles indicate data after the third vaccination. Responses were compared by two-tailed Mann–Whitney test (**** $p < 0.0001$). Horizontal lines indicate mean values, while error bars indicate the standard deviation.

In addition, we measured the antibody ratio and the antibody concentration in 24 HSCT patients and 18 healthy controls before the third vaccination against SARS-CoV-2, as well as in 18 HSCT patients and 19 healthy controls after the third vaccination. There was no significant difference in antibody ratios before and after the third vaccination in HSCT patients (mean ratio of 5.4 vs. 6.5, $p = 0.6$, geometric mean concentration of 195 vs. 350 BAU/mL, $p = 0.4$); however, there was in healthy controls (mean ratio of 4.8 vs. 10.6, $p < 0.0001$, geometric mean concentration of 353 vs. 2065 BAU/mL, $p < 0.0001$) (Figure 5). The antibody ratio showed a significant difference between healthy controls and HSCT patients after the third vaccination ($p = 0.002$, Table S11).

3.4. Correlation of SARS-CoV-2-Specific Immune Responses with Age and Interval between HSCT and Blood Collection

We analyzed the correlation of the results of cellular and humoral immunity with age of HSCT patients and healthy controls by Spearman analyses. For the cellular immunity, we observed in the CoV iSpot significant correlations between the age and the spot increment only for IL-2, with simultaneous staining of IFN-γ and IL-2 in HSCT patients after the third vaccination (IL-2: $r = -0.7$, $p = 0.008$, IFN-γ and IL-2: $r = -0.6$, $p = 0.03$). In our *in-house* ELISpot, we detected a significant correlation between age and spot increment for IFN-γ in HSCT patients after the third vaccination after stimulation with S1 Sino ($r = -0.5$, $p = 0.03$). After stimulation with S1/S2 peptide mix and S1 peptide mix, we observed a significant correlation between age and spot increment for IFN-γ in healthy controls after the third vaccination (S1/S2: $r = -0.8$, $p < 0.0001$, S1: $r = -0.6$, $p = 0.01$) (Tables S1 and S2). For humoral immunity, we could not detect a significant correlation with age for titers of neutralizing antibodies or for antibody ratio or concentration (Tables S3 and S4).

We also performed Spearman analyses to correlate the interval between HSCT and blood collection with cellular or humoral immunity. For cellular immunity, we detected a significant correlation only for IFN-γ after stimulation with S1 Sino after the third vaccination ($r = -0.5$, $p = 0.03$) (Table S5). For humoral immunity, we observed no significant correlation (Table S6).

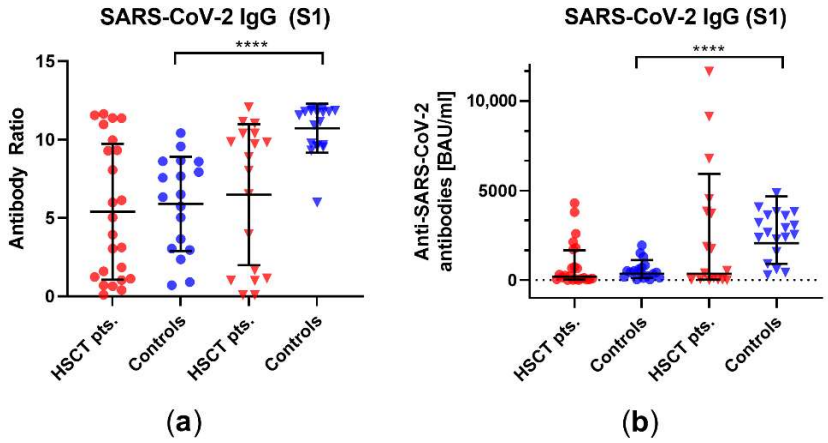

Figure 5. SARS-CoV-2-specific IgG antibody responses in HSCT patients and healthy controls before and after the third vaccination. SARS-CoV-2-specific IgG antibody responses are shown (**a**) as antibody ratios, which determines a quotient of antibodies in the patient samples and in a control sample, and (**b**) as SARS-CoV-2-specific IgG antibody concentrations in units of BAU/mL. Circles indicate data before the third vaccination (red—HSCT, blue—healthy controls), while triangles indicate data after the third vaccination. Responses were compared by two-tailed Mann–Whitney test (**** $p < 0.0001$). The dotted line represents the zero line. For the antibody ratio, horizontal lines indicate mean values, while error bars indicate the standard deviation. For the antibody concentration, the geometric mean and geometric standard deviation factor are indicated.

4. Discussion

We could not observe significant increases in cellular immunity after the third vaccination in both groups. However, healthy controls showed higher mean values for IFN-γ and IL-2 spot increments before and after the third vaccination than HSCT patients. A limitation of our study is the low frequency of SARS-CoV-2-specific cells in HSCT patients. Nevertheless, as indicated by the negative and positive controls, all assays included into our study were valid.

Our study indicates that HSCT patients and healthy controls both displayed neutralizing antibodies against variants and mutations of SARS-CoV-2 before the third vaccination against SARS-CoV-2. However, these antibodies increased only slightly after vaccination in HSCT patients, whereas healthy controls showed a significant increase. When looking at the individual values, an increase in humoral immunity was observed in about 15% of the HSCT patients. However, it should be noted that a protective effect can hardly be assumed due to the detection of specific antibodies. Dhakal et al. previously reported that only one-third of HSCT patients generated a humoral immune response against SARS-CoV-2 after second vaccination [18]. One reason for this finding is immunosuppressive therapy after stem-cell transplantation [19]. Other hematological malignancies, such as chronic lymphocytic leukemia (CLL), also led to impaired vaccination responses to SARS-CoV-2 [20]. Similarly, the timing of vaccination, immune status prior to HSCT, and type of HSCT may also play a role, as shown for other vaccines [21,22].

The results of the antibody ELISA and neutralization assay indicate that, after the third vaccination, antibody concentrations and the distribution of titers remained similar in immunosuppressed individuals, whereas a significant increase was seen in healthy controls. Other studies have also previously shown that the humoral immune response to

SARS-CoV-2 vaccination is reduced in 30–60% of HSCT patients [13,14,23]. Currently, there are very few studies on immune responses after third vaccination in HSCT patients. Most studies focus on the humoral immune response, but point to the important role of cellular immunity, which we investigated [24–26]. Einarsdottir et al. were the only investigators of the cellular immune response. They examined 37 HSCT patients for humoral and cellular immune response 4 weeks after the third vaccination against SARS-CoV-2 by ELISA. T cells were stimulated with the N-terminus of the spike protein of SARS-CoV-2 and were measured after 48 h of incubation. Einarsdottir et al. demonstrated that 49% of tested volunteers lacked a cellular immune response after the third vaccination against SARS-CoV-2 [27]. However, they used a different method from the current study and measured the released IFN-γ in plasma following stimulation of whole-blood samples, but not PBMCs at a defined cell number. Furthermore, they did not compare responses after the second and third vacciantion in that study. In another study, they reported on T-cell responses in 50 HSCT recipients after the second vaccine dose and unexpectedly showed that 28% failed to achieve detectable T-cell responses [23]. Presumably, there were slight differences in the experiemntal settings of their two studies, because the data would otherwise imply that the fraction of patients with positive T-cell responses even declined after the third vaccination.

In a previous study on immunity toward wildtype SARS-CoV-2 [28], we investigated the humoral and cellular immune responses of HSCT patients and healthy controls before the first vaccination, after the first vaccination, after the second vaccination, and after infection. After the second vaccination, the humoral immune responses in HSCT patients increased by about a factor of 15 compared to the immune response after the first vaccination. In the current study, the humoral responses increased by a maximum factor of 1.1 in HSCT patients. In terms of cellular immune responses, the comparison showed stronger responses before the third vaccination than could be detected in the previous study at a median of 30 days after the second vaccination. It could, thus, be assumed that there may be a delayed cellular vaccination response. However, after the third vaccination, the increase in spot increment was very small. In the present study, in contrast to the previous one, no correlation was found between the strength of immune responses and the interval between HSCT and blood collection. However, because of a median interval between HSCT and blood collection of 4.1 years, a correlation was not expected. There was a negative correlation between age and immune responses. This was to be expected due to the median age of the tested groups, as immune responses decrease with age.

In conclusion, about half of the HSCT patients exhibited low levels of neutralizing antibodies to variants of SARS-CoV-2, which could not be increased significantly by a third vaccination. Moreover, cellular immunity against SARS-CoV-2, as observed in about 20% of the patients, did not increase after the third vaccination in HSCT patients. Of note, previous data indicate that vaccinated individuals retain T-cell immunity to the SARS-CoV-2 omicron variant and, thus, showed a minimal escape at the T-cell level [29]. Nevertheless, the HSCT patients should be partly protected against SARS-CoV-2, either by neutralizing antibodies to variants of SARS-CoV-2 or by cross-reactive T cells. A third dose of the vaccine should be given to HSCT patients, especially because we observed by trend an increase in neutralizing antibodies against variants of SARS-CoV-2.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/vaccines10060972/s1: Table S1. Spearman analysis of cellular immune response in HSCT patients before and after the third vaccination; Table S2. Spearman analysis of cellular immune response in healthy controls before and after the third vaccination; Table S3. Spearman analysis of humoral immune response in HSCT patients before and after the third vaccination; Table S4. Spearman analysis of humoral immune response in healthy controls before and after the third vaccination; Table S5. Spearman analysis of spots increment in HSCT patients before and after the third vaccination; Table S6. Spearman analysis of antibodies tested in HSCT patients before and after the third vaccination; Table S7. *p*-Values of two-tailed Mann–Whitney tests comparing the results of HSCT patients and healthy controls before and after the third vaccination; Table S8. *p*-Values of two-tailed Mann–Whitney tests comparing the results for IFN-γ of HSCT patients and healthy

controls before and after the third vaccination; Table S9. *p*-Values of two-tailed Mann–Whitney tests comparing the results for IL-2 of HSCT patients and healthy controls before and after the third vaccination; Table S10. *p*-Values of two-tailed Mann–Whitney tests comparing the results of HSCT patients and healthy controls before and after the third vaccination; Table S11. *p*-Values of two-tailed Mann–Whitney tests comparing the results of HSCT patients and healthy controls before and after the third vaccination.

Author Contributions: Conceptualization, M.L.; methodology, L.T., N.F., and L.B.; software, L.T.; validation, M.L., M.K., and A.K.; formal analysis, L.T. and M.L.; investigation, M.L.; resources, M.L., P.A.H., M.K., 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., P.A.H., and A.K., visualization, L.T.; supervision, M.L. and P.A.H.; project administration, M.L. and P.A.H. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital Essen, Germany (20-9225-BO and 20-9254-BO, 02.04.2020).

Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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Conflicts of Interest: The authors declare no conflict of interest.

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

Thümmler, L., Gäckler, A., Bormann, M., Ciesek, S., Widera, M., Rohn, H., Fisenkci, N., Otte, M., Alt, M., Dittmer, U., Horn, P. A., Witzke, O., Krawczyk, A., Lindemann, M.

2022

Anteile:

- Durchführung der Experimente: 70 %
- Datenanalyse: 40 %
- Statistische Analyse: 50 %
- Manuskripterstellung: 50 %
- Überarbeitung des Manuskripts: 20 %

Monika Lindemann hat das Konzept der vorliegenden Studie entwickelt. Laura Thümmler, Neslinur Fisenkci, Mona Otte, Mira Alt und Maren Bormann führten die Experimente durch. Die Daten wurden von Monika Lindemann, Anja Gäckler und Laura Thümmler analysiert. Monika Lindemann und Laura Thümmler führten die statistische Analyse durch und erstellten das Manuskript. Die Überarbeitung des Manuskripts erfolgte durch Monika Lindemann, Laura Thümmler, Ulf Dittmer, Peter A. Horn, Oliver Witzke und Adalbert Krawczyk.

Laura Thümmler	Prof. Dr. med. Monika Lindemann

vaccines

MDPI

Article

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-γ and IL-2 simultaneously, as well as by colorimetric SARS-CoV-2-specific IFN-γ 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 20 females	5 males 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: $p = 0.001$).

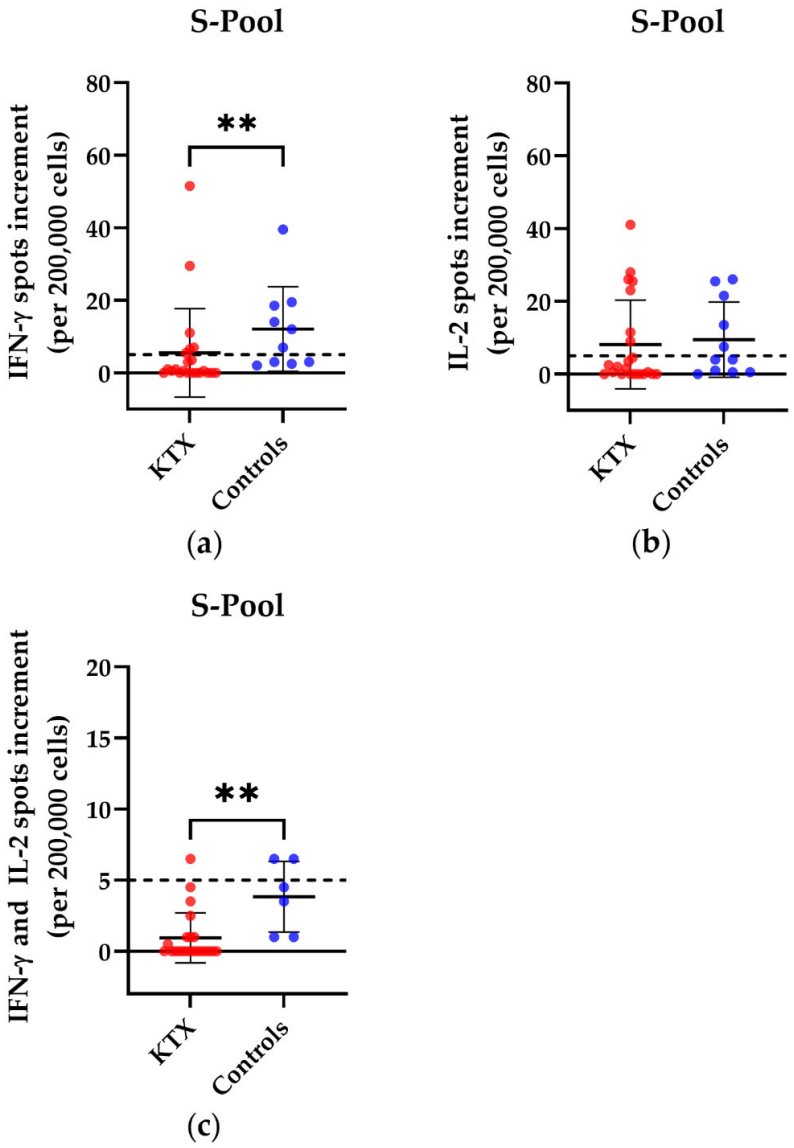

Figure 1. SARS-CoV-2-specific CoV-iSpot responses in kidney transplant (KTX) patients and healthy volunteers after booster vaccination. Distribution of (**a**) IFN-γ, (**b**) IL-2, and (**c**) simultaneous IFN-γ and IL-2 CoV-iSpot responses after stimulation with the S pool of the wild type SARS-CoV-2. 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.

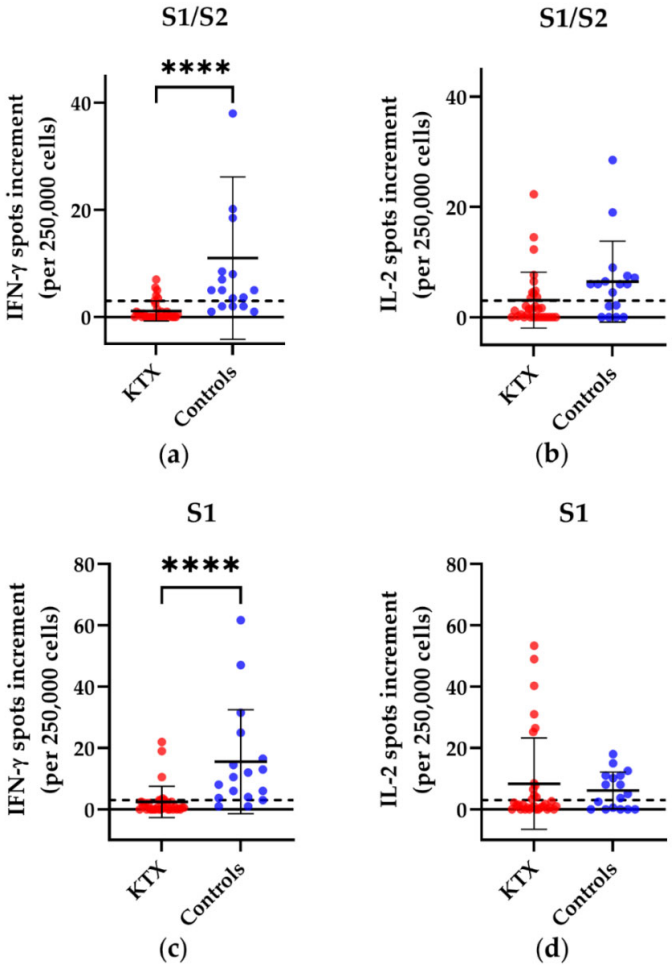

Figure 2. *Cont.*

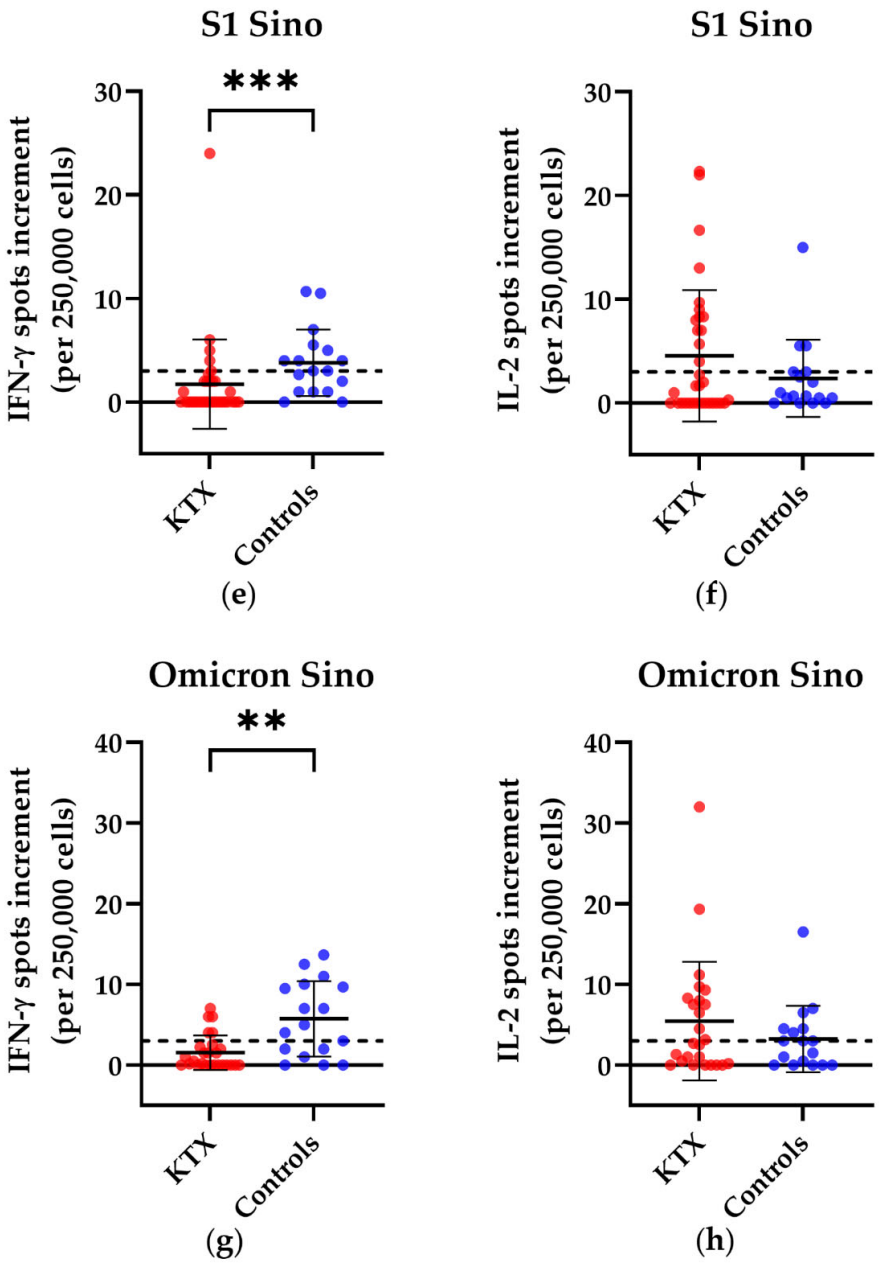

Figure 2. SARS-CoV-2-specific responses in kidney transplant (KTX) patients and healthy controls after booster vaccination, using our in-house ELISpot assay. Distribution of (**a**) IFN-γ and (**b**) IL-2 ELISpot responses after stimulation with an S1/S2 peptide mix, with an S1 peptide mix (**c**,**d**), 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: $p = 0.6$).

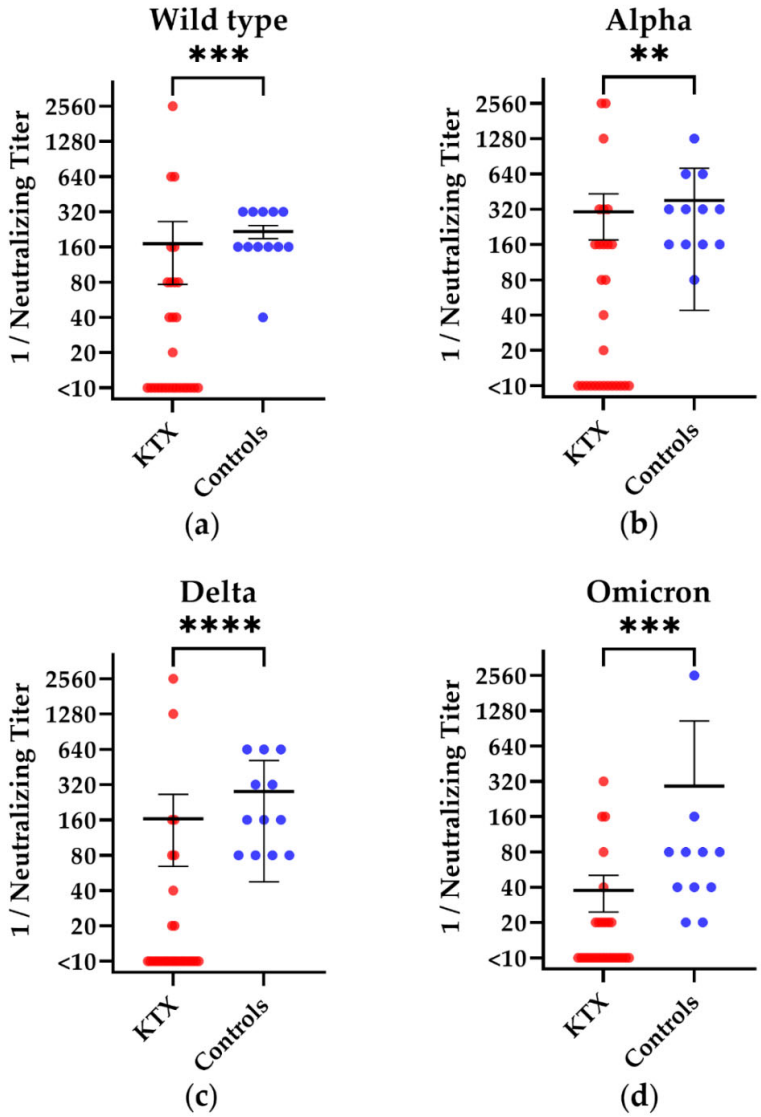

Figure 3. Titer of SARS-CoV-2-specific neutralizing antibodies in kidney transplant (KTX) patients and healthy volunteers. The reciprocal of the titer of neutralizing anti-SARS-CoV-2 (**a**)D614G (wild type), (**b**) alpha, (**c**) delta, and (**d**) omicron (BA.1) antibodies is shown on the *y*-axis. 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.

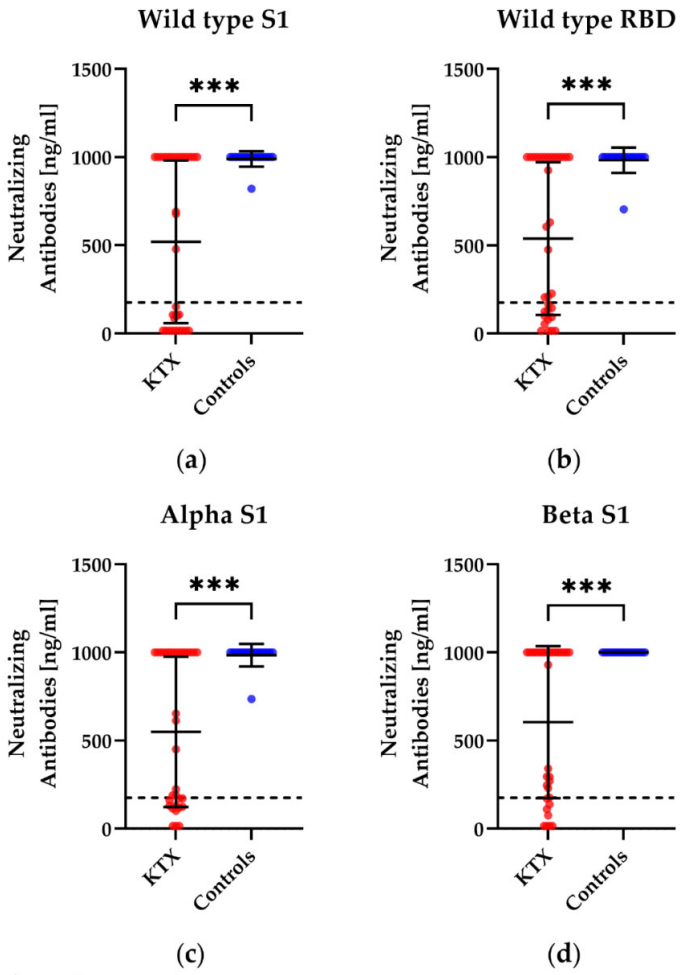

Figure 4. *Cont.*

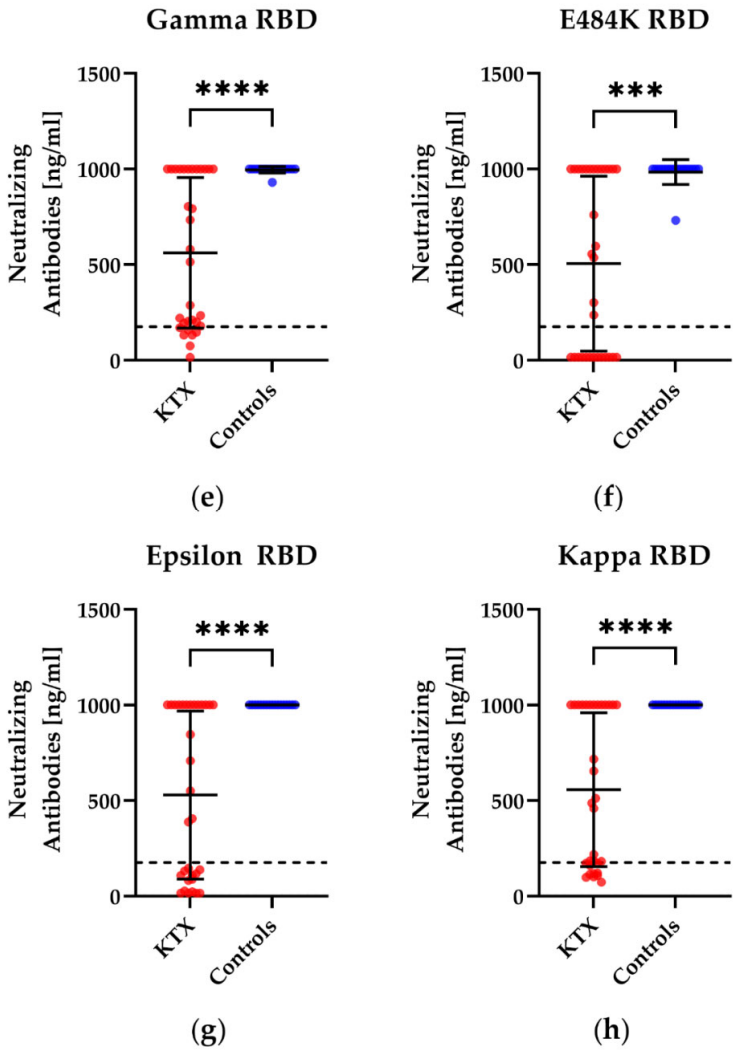

Figure 4. *Cont*.

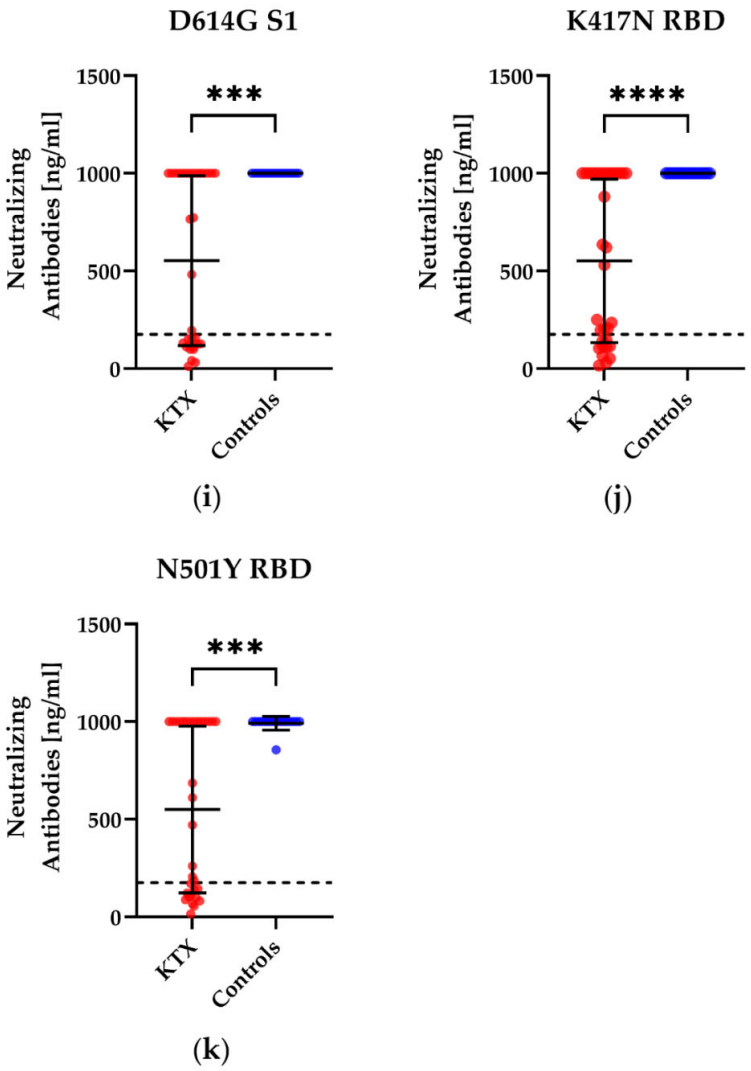

Figure 4. Concentration of potential neutralizing antibodies towards different variants of the subunit 1 of spike protein (S1) or the receptor-binding domain (RBD) of SARS-CoV-2 in kidney transplant (KTX) patients and healthy volunteers (controls) after booster vaccination. Humoral responses after booster 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 ($p = 0.7$).

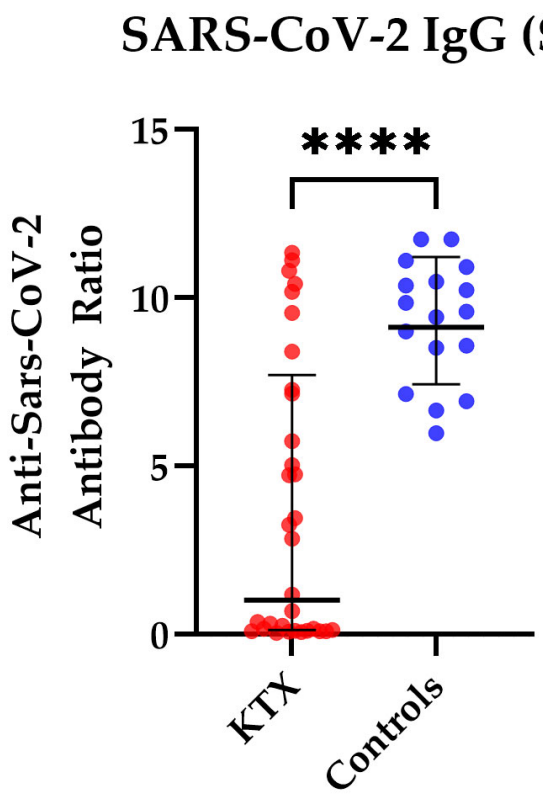

Figure 5. SARS-CoV-2-specific IgG antibody responses in kidney transplant (KTX) patients and healthy controls. SARS-CoV-2-specific IgG antibody responses are shown as antibody ratios, which determines a quotient of antibodies in the patient samples and in a control sample. Red circles show data of the 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-γ. 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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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital Essen, Germany (20-9753-BO).

Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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Conflicts of Interest: The authors declare no conflict of interest.

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3.3 Long-term cellular immune response in immunocompromised unvaccinated COVID-19 patients undergoing monoclonal antibody treatment

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Long-term cellular immune response in immunocompromised unvaccinated COVID-19 patients undergoing monoclonal antibody treatment

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Immunocompromised patients are at increased risk for a severe course of COVID-19. Treatment of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection with anti-SARS-CoV-2 monoclonal antibodies (mAbs) has become widely accepted. However, the effects of mAb treatment on the long-term primary cellular response to SARS-CoV-2 are unknown. In the following study, we investigated the long-term cellular immune responses to SARS-CoV-2 Spike S1, Membrane (M) and Nucleocapsid (N) antigens using the ELISpot assay in unvaccinated, mAb-treated immunocompromised high-risk patients. Anti-SARS-CoV-2 mAb untreated though vaccinated COVID-19 immunocompromised patients, vaccinated SARS-CoV-2 immunocompromised patients without COVID-19 and vaccinated healthy control subjects served as control groups. The cellular immune response was determined at a median of 5 months after SARS-CoV-2 infection. Our data

suggest that immunocompromised patients develop an endogenous long-term cellular immune response after COVID-19, although at low levels. A better understanding of the cellular immune response will help guide clinical decision making for these vulnerable patient cohorts.

KEYWORDS

COVID-19, immunosuppression, SARS-CoV-2, monoclonal antibody treatment, cellular immune response

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a pandemic of coronavirus disease 2019 (COVID-19), resulting in more than 530 million infected people and 6.3 million deaths (June 2022). Despite the availability of vaccines, the pandemic remains a global health burden (1). Many risk factors for the progression of COVID-19 to a severe and critical stage have been identified, including age, underlying comorbidities such as diabetes, obesity, chronic lung diseases, and immunodeficiency (2–4). Primary SARS-CoV-2 infections as well as breakthrough infections represent a potential risk for these vulnerable groups (5) resulting in a high burden of morbidity and mortality (6). During the early pandemic, COVID-19 patients were treated with SARS-CoV-2 convalescent plasma. Numerous studies were conducted, indicating that early onset of antiviral treatment is necessary to improve the course of disease and protect against a severe outcome (7–10). Later, monoclonal antibodies (mAb) against SARS-CoV-2 became available (11). Early treatment of SARS-CoV-2 infections with mAbs such as bamlanivimab (12) or a combination of the monoclonal antibodies casirivimab and imdevimab (11) has been shown to markedly reduce the risk of hospitalization or death among high-risk patients with COVID-19 (11, 12). However, the occurrence of novel SARS-CoV-2 variants of concern (VOCs) such as Alpha (Pango nomenclature B.1.1.7), Beta (B.1.351), Gamma (P1, B.1.1.28), Delta (B.1.617.2) and Omicron (B.1.1.529) led to an increase in the frequency of reinfection and vaccination breakthrough SARS-CoV-2 infections (3, 13, 14). Some of the mutations within the SARS-CoV-2 spike antigen are associated with immune escape, and thus a reduced effectivity of monoclonal antibodies against SARS-CoV-2 spike protein variants (15, 16). However, a recent study suggests that monoclonal antibody treatment, with respect to available antibody formulations and circulating viral variants, may provide favorable outcomes for mild to moderate COVID-19 in vulnerable patients, such as solid organ recipients (17).

Although the role of antibodies induced by immunization or additionally administrated early upon infection in those patients was already described, less is known about the cellular immune response in immunocompromised patients with primary or breakthrough infections and antibody treatment (18).

In the present study, we investigated the long-term cellular immune response in severely immunocompromised unvaccinated patients suffering from a SARS-CoV-2 infection and treated with the mAb bamlanivimab or a combination of the mAbs casirivimab and imdevimab in the early phase of infection. We compared the cellular immune response of these patients with those of vaccinated immunocompromised patients with a SARS-CoV-2 infection but without antibody treatment as well as vaccinated immunocompromised patients and immunocompetent volunteers without SARS-CoV-2 infection.

Methods

Study subjects and sampling

In the present study, we investigate the long-term cellular immune response against SARS-CoV-2 spike (S), Membrane (M) and Nucleocapsid (N) antigens in immunocompromised patients with primary SARS-CoV-2 infection after early mAb treatment (group 1) up to 5 months after COVID-19. Vaccinated immunocompromised patients with COVID-19 (group 2), as well as vaccinated immunocompromised patients (group 3) and vaccinated healthy volunteers (group 4) without COVID-19 served as controls. All immunocompromised patients (group 1-3) had a medical condition associated with secondary severe immunodeficiency. Patients suffering from a primary SARS-CoV-2 infection (group 1) were treated early with monoclonal antibodies (mAbs) bamlanivimab (LY-CoV555, Eli Lilly) or casirivimab/imdevimab (Ronapreve, Roche and Regeneron), which both bind to the SARS-CoV-2 spike protein. Group 1 included 12 non-vaccinated patients. Of the 12 patients, 8 were treated with 700 mg bamlanivimab (concentration 35 mg/ml) and 4 with a combination of 1200 mg casirivimab/imdevimab (concentration of 120 mg/ml each). Antibodies were administrated intravenously. The group consisted of 2 men and 10 women, and the median age was 57 years (range 31-78). The cellular immune response in the first

group was examined at a median of 146 days (range 74-182) after mAb therapy. One of the patients had breast cancer, three had a kidney transplantation (median since transplantation 5.6 years, range 4 months – 10 years), seven had a lung transplantation (median since transplantation 10.2 months, range 4 - 25 months), one prostate cancer and one cachexia. In this first group, the three kidney transplant patients had concomitant arterial hypertension and an impaired renal function. In the lung transplant recipient group, one patient had coronary artery disease as an additional risk factor for a severe COVID-19 course. Except for one patient, all solid organ transplant patients had triple immunosuppressive therapy containing prednisone, the calcineurin inhibitor (CNI) tacrolimus, and mycophenolic acid (MMF) or the mTOR inhibitor everolimus. One renal transplant patient had triple immunosuppressive therapy containing prednisolone, MMF, and belatacept.

Group 2 included 10 immunocompromised, vaccinated patients with a SARS-CoV-2 infection. All patients were vaccinated with an mRNA vaccine (BioNTech or Moderna). The group was composed of seven men and three women and the median age was 59 years (range 20-69) after hematopoietic stem cell transplantation (HSCT). HSCT took place at a median of 2.9 years (range 0.9-17) prior to blood collection and all patients achieve complete remission of their underlying hematologic malignancy. Three patients had coronary artery disease as additional COVID-19 risk factor, one patient had grade I obesity, and one had a history of chronic obstructive pulmonary disease and splenectomy. 7 patients had developed graft versus host disease (GVHD) after HSCT and were treated immunosuppressive with steroids with or without CNI for this purpose. One patient received an mTor inhibitor plus steroids. The cellular immune response was explored at a median of 145 days (range 61-230) after infection.

Group 3 included 14 immunocompromised, vaccinated patients without SARS-CoV-2 infection. All patients were vaccinated with an mRNA vaccine (n=13 Comirnaty, BioNTech/Pfizer; n=1 Spikevax, Moderna). The group contained six men and eight women with a median age of 55 years (range 21-64). Of the 14 patients, four had a HSCT a median of 4.3 years (range 1.3-16.1) prior to testing and ten had a kidney transplant at a median of 3.1 years (range 0.09-10.5) prior to blood collection. The cellular immune response in this group was examined at a median of 87 days (range 16-238) after vaccination. 3 HSCT patients were on dual immunosuppressive therapy (steroid, with CNI or MMF) because of persistent GVHD; one patient had additional arterial hypertension and diabetes mellitus as risk factors for severe COVID-19 progression. All except one kidney transplant recipients had concomitant arterial hypertension and impaired renal function and received triple immunosuppressive therapy with prednisolone, MMF, and CNI. One patient received triple immunosuppressive therapy with prednisolone, MMF, and belatacept.

Group 4 included 14 healthy volunteers after the third SARS-CoV-2 vaccination with an mRNA SARS-CoV-2 vaccine (n=3 Comirnaty, BioNTech/Pfizer; n=11 Spikevax, Moderna). The group was composed of six men and eight women and the median age was 50 years (range 29-65). They were tested at a median of 47 days (range 30-72) after the third vaccination.

There were no significant differences between the different cohorts with respect to the known COVID-19 related risk factors being sex, age, and lymphocyte count (Table 1).

The study was conducted according to the Helsinki principles and was approved by the local ethics committee of the University Hospital Essen, Germany (20-9225-BO, 20-9254-BO, and 20-9374-BO). All subjects provided informed consent to participate in the study.

T cell ELISpot assays for S1-, M-, and N-derived SARS-CoV-2 peptides

To analyze SARS-CoV-2 specific cellular immune responses, we performed interferon gamma (IFN-γ) enzyme-linked immunospot (ELISpot) assays as previously described (19). 250,000 peripheral blood mononuclear cells (PBMC) were cultured in the presence or absence of the PepTivator® SARS-CoV-2 membrane (M) protein, nucleocapsid (N) protein (600 pmol/mL of each peptide, Miltenyi Biotec, Bergisch Gladbach, Germany) or the S1 protein (4 µg/ml, Sino Biological, Wayne, PA, USA) (each in single cell culture) in 150 µL of AIM-V®. The peptide mixes corresponding to the M and N proteins cover the complete sequence of the glycoproteins. The S1 protein is a recombinant protein expressed in HEK293 cells and covers the sequence of aa 1 to aa 692 of the spike protein subunit 1. The peptide pools consisted of 15-mer sequences with an overlap of 11 amino acids. After 19 h of incubation at 37°C, IFN-γ production was measured as previously described (19). The spot numbers were evaluated by an ELISpot reader (AID Fluorospot; Autoimmun Diagnostika GmbH, Strassberg, Germany). From duplicate cell cultures, the mean value was considered. SARS-CoV-2 specific spots were determined as stimulated minus non-stimulated values (spot increment). The negative controls had an average of 0.21 spots (range 0-3) and their three-fold standard deviation was 3 x 0.67 spots = 2.01 spots (which we considered as background for the negative controls). As we used increment values, a three-fold higher value versus background means 3 x 2.01 spots minus 1 x 2.01 spots, which is 4.02 spots increment. We therefore chose a cut-off point of 4 as positivity.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.3.1 software (San Diego, CA, USA). We used the Kruskal-

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TABLE 1 Overview of the study cohort.

group	1	2	3	4	p-value
Antibody therapy? (Y: yes, N: no)	Y	N	N	N	
Infected? (Y: yes, N: no)	Y	Y	N	N	
Vaccinated? (Y: yes, N: no)	N	Y	Y	Y	
Severely Immunocompromised? (Y: yes, N: no)	Y	Y	Y	N	
Total number	12	10	14	14	
number of women	10	3	8	8	$p = 0.09$
number of men	2	7	6	6	
median age [years] (range)	57 (31-78)	59 (20-69)	55 (21-64)	50 (29-65)	$p = 0.23$
Count of lymphocytes [x103/µl] Mean (SD)	8.8 (6.4)	9.6 (13.0)	14.1 (5.8)	12.1 (5.0)	$p = 0.29$
Interval infection/vaccination – blood collection Mean (range)	146 days (74-182)	145 days (61-230)	87 days (16-238)	47 days (30-72)	*p< 0.001*
SARS-CoV-2 vairant or SARS-CoV-2 vaccine	SARS-CoV- 2 D614G (wild type)	SARS-CoV- 2 D614G (wild type) 8x Comirnaty® (BioNTech/ Pfizer) 2x Spikevax® (Moderna)	13x Comirnaty® (BioNTech/ Pfizer) 1x Spikevax® (Moderna)	3x Comirnaty® (BioNTech/ Pfizer) 11x Spikevax® (Moderna)	
Number of SARS-CoV-2 vaccinations	0	2	2	3	

Characteristics of the four different study groups. Groups 1-3 include the different cohorts of patients in terms of monoclonal antibody treatment, SARS-CoV-2 infection, and SARS-CoV-2 vaccination; group 4 represents the healthy control group (SARS-CoV-2 vaccination, without SARS-CoV-2 infection). Comparison between COVID-19 related risk factors sex, age and lymphocyte counts was performed using the Kruskal Wallis test. Statistical significance was set at the level of $p < 0.05$.

Wallis-test and the Mann-Whitney U test for numerical variables. Fisher's exact test was used for categorical variables. Two-sided p values < 0.05 were considered significant.

Results

In the present study, we examined the long-term cellular immune response unvaccinated severely immunocompromised patients suffering from a SARS-CoV-2 infection after treatment with bamlanivimab or a combination of the monoclonal antibodies casirivimab and imdevimab early after infection using ELISpot assay. We also investigated the cellular immune response in vaccinated immunocompromised patients after SARS-CoV-2 infection as well as in vaccinated immunocompromised patients and immunocompetent volunteers without a history of SARS-CoV-2 infection.

Unvaccinated immunocompromised patients with SARS-CoV-2 infection and early mAb treatment (group 1) showed a similar cellular immune response to all stimuli in the ELISpot assay to vaccinated immunocompromised patients with a SARS-CoV-2 infection (group 2) (Figures 1A–C). The measured mean values of spots increment after stimulation with spike S1 protein were 2.1 in group 1 and 2.8 in group 2 ($p = 0.2$) (Figure 1A), after stimulation with M protein 2.0 in group 1 and 7.9 in group 2 ($p = 0.09$) (Figure 1B) and after stimulation with N protein 6.1 in group 1 and 6.2 in group 2 ($p = 0.2$) (Figure 1C). In particular, the cellular immune response in group 1 and 2 was higher than in vaccinated immunocompromised patients (group 3) (Figures 1A–C). Significant differences in spots increment were observed between group 2 and 3 after stimulation with S1 protein (2.8 in group 2 and 1.1 in group 3 ($p = 0.04$)) (Figure 1A) and N protein (6.2 in group 2 and 0.6 in group 3 ($p = 0.002$)) (Figure 1C). As expected, healthy immunocompetent vaccinated volunteers (group 4) showed a higher cellular immune response than vaccinated immunocompromised patients (group 3) (Figure 1A). The spots increment after stimulation with the spike S1 protein were 1.1 in group 3 and 3.8 in group 4 ($p = 0.01$) (Figure 1A). No significant differences between groups 3 and 4 could be observed after stimulation with M protein (3.7 in group 3 and 1.1 in group 4 ($p = 0.5$) or N protein (0.6 in group 3 and 0.9 in group 4 ($p = 0.6$) (Figures 1B, C).

Interestingly, after mAb treatment (group 1), the frequency of single and combined positive cellular response to S1, M, or N proteins was lower than in the vaccinated cohort with SARS-CoV-2 infection (group2) (S1: 14% vs. 30%; M: 21% vs. 50%; N: 14% vs. 30%, combined: 33% vs. 60% statistically not significant as calculated with the Fischer´s exact test). In the groups

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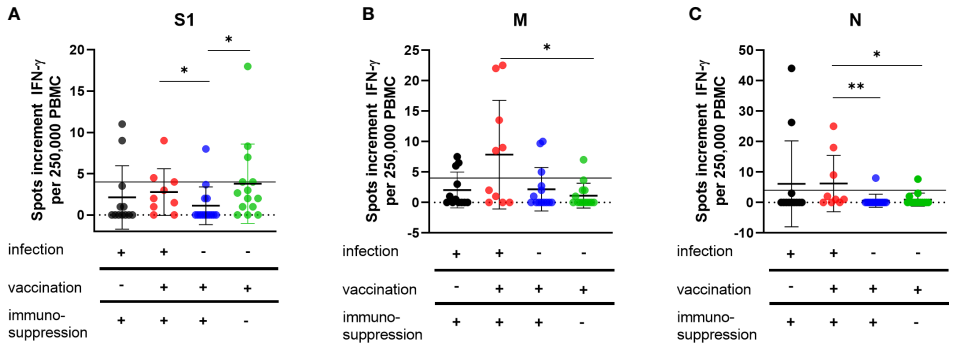

FIGURE 1
Cellular immunity to SARS-CoV-2 antigens in vaccinated and/or recovered immunosuppressed individuals. Cellular immune responses towards SARS-CoV-2 S1 **(A)**, M **(B)** and N **(C)** antigens were determined by IFN-γ ELISpot assay. Please note that the scales differ. Horizontal lines indicate mean values, error bars indicate the standard deviation (SD). The dotted gray line represents the assay cut-off. Differences between the groups were compared by Mann-Whitney U test (*p < 0.05).

vaccinated against SARS-CoV-2 without COVID-19, only one of 14 immunocompromised patients (group 3) developed a positive response to S1 (7%), compared with 5 of 14 (35%) in the volunteer group (group 4). Immune responses against the M antigen could be detected in three volunteers from group 3 and two from group 4 and against the N antigen in one volunteer from group 3 and 4, respectively. All of these volunteers had no documented SARS-CoV-2 infection.

Discussion

In this paper, we present the profiling of cellular immunity in a cohort of immunocompromised, unvaccinated patients who developed COVID-19 and thus were treated with bamlanivimab or with the combination therapy casirivimab and indevimab. A cohort of immunocompromised patients vaccinated against SARS-CoV-2 who developed COVID-19, as well as immunized immunocompromised patients or healthy participants without COVID-19, served as controls. Our data suggest that immunocompromised patients may develop an endogenous long-term cellular immune response after COVID-19. The observed T cell-mediated immunity against the spike protein in unvaccinated immunocompromised patients after mAb therapy, seems to be blunted compared to vaccinated and mAb untreated immunocompromised patients with COVID-19. Consistent with this finding, cellular immune responses in our patient cohort were lower after mAb treatment compared with previously published results from immunosuppressed cohorts after COVID-19 but without mAb treatment (20–24).

Early treatment of SARS-CoV-2 infection in high-risk cohorts with mAbs is widely accepted, and mAbs clinical trials have reported overall reduced hospitalization rates in patients with mild to moderate COVID-19 (25–29). While many studies focused on the clinical efficacy of the treatment, its effects on long-term immunologic responses to the virus are largely unknown (12, 17, 30–33). The optimal use of these therapeutic options requires a sophisticated understanding of their effects on both the virus and the host immune system. For a long time, anti-SARS-CoV-2 T cell immunity was considered less important than specific antibodies as a parameter for immune protection in patients at risk of severe COVID-19 (34). However, the humoral anti-SARS-CoV-2 response declines over time, whereas SARS-CoV-2-specific T cell immunity appears to persist longer, even in seronegative convalescents (35–38). To our knowledge, no study has explored the effect of mAb treatment on cellular immunity in severely immunocompromised patients at risk for severe COVID-19 and only two studies explored the effect of treatment on humoral immunity (39, 40). Both studies demonstrated that passive immunization of COVID-19 patients with anti-S monoclonal IgG preparations profoundly suppressed the induction of the endogenous anti-S IgM response and, to a lesser extent, the anti-N IgG response. It is noteworthy that not only immunosuppressed patients were included in those analyses, as these patients would likely exhibit a reduced immune response due to immunosuppression (41–45). Anti-SARS-CoV-2 mAb preparations are reportedly able to reduce viral load (46, 47). Reduction of viral load in the early stage of infection might be expected to result in reduced immune responses. Additionally, stronger SARS-CoV-2 specific T cell responses are well documented in patients who had recovered from more severe symptoms of COVID-19 (48–51). Therefore, it seems possible that after mAbs treatment, the improvement in COVID-19 course is causative of the decreased immunologic response.

Conclusion

Most of the immunosuppressed, non-vaccinated COVID-19 patients treated with monoclonal antibodies within the present study developed no SARS-CoV-2 specific T-cell response. The adaptive immune response is an important factor in the clinical course after SARS-CoV-2 infection and may protect from reinfections. Deeper immunophenotyping of immunocompromised patients after mAb therapy will be important in expanding knowledge about long-term immunity to SARS-CoV-2. Its understanding is not only essential to evaluate the potential effect of COVID-19 treatment on future reinfection but also crucial for further risk assessment especially in the high-risk cohort of immunocompromised patients.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The study was conducted according to the Helsinki principles and was approved by the local ethics committee of the University Hospital Essen, Germany (20-9225-BO, 20-9254-BO, and 20-9374-BO). All subjects provided informed consent to participate in the study.

Author contributions

Conceptualization, MaK, ML, HR, OW, PAH, and AK; methodology, LT, MaK, AG, FT, and CT; validation, AK, HR, and OW; investigation, LT, NF, PB, and SJ; resources, MaK, MiK, AG, CT, and MZ; data curation, LT, MZ, OA, and OW; writing—original draft preparation, LT, HR, and AK; writing—review and editing, LT, HR, ML, and AK; visualization, LT, HR, and AK; supervision, HR and AK; funding acquisition, OW, HR, and AK. All authors have read and agreed to the published version of the manuscript.

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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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3.4 Early Treatment with Monoclonal Antibodies or Convalescent Plasma Reduces Mortality in Non-Vaccinated COVID-19 High-Risk Patients

Thümmler, L., Lindemann, M., Horn, P. A., Lenz, V., Konik, M., Gäckler, A., Boss, K., Theodoropoulos, F., Besa, V., Taube, C., Brenner, T., Witzke, O., Krawczyk, A., Rohn, H.

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viruses

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Communication

Early Treatment with Monoclonal Antibodies or Convalescent Plasma Reduces Mortality in Non-Vaccinated COVID-19 High-Risk Patients

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Abstract: Vulnerable patients such as immunosuppressed or elderly patients are at high risk for a severe course of COVID-19 upon SARS-CoV-2 infection. Immunotherapy with SARS-CoV-2 specific monoclonal antibodies (mAb) or convalescent plasma represents a considerable treatment option to protect these patients from a severe or lethal course of infection. However, monoclonal antibodies are not always available or less effective against emerging SARS-CoV-2 variants. Convalescent plasma is more commonly available and may represent a good treatment alternative in low-income countries. We retrospectively evaluated outcomes in individuals treated with mAbs or convalescent plasma and compared the 30-day overall survival with a patient cohort that received supportive care due to a lack of SARS-CoV-2 specific therapies between March 2020 and April 2021. Our data demonstrate that mAb treatment is highly effective in preventing severe courses of SARS-CoV-2 infection. All patients treated with mAb survived. Treatment with convalescent plasma improved overall survival to 82% compared with 61% in patients without SARS-CoV-2 targeted therapy. Our data indicate that early convalescent plasma treatment may be an option to improve the overall survival of high-risk COVID-19 patients. This is especially true when other antiviral drugs are not available or their efficacy is significantly reduced, which may be the case with emerging SARS-CoV-2 variants.

Keywords: SARS-CoV-2; convalescent plasma; monoclonal antibody treatment; Coronavirus Disease 19

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1. Introduction

Since its initial description in late 2019, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spread over the world and caused a pandemic causing enormous health and socioeconomic implications. The symptoms of a SARS-CoV-2 infection range from cold-like symptoms accompanied by cough and fever to severe pneumonia or disseminated infection that may be fatal [1]. Patients with pre-existing chronic conditions such as cardiovascular disease, obesity, diabetes, cancer, chronic kidney or lung disease, and compromised immune status are at particularly high risk for COVID-19-related morbidity and mortality [2–5]. During the early pandemic, COVID-19 patients received supportive medical care because antiviral treatment was not yet available. Convalescent plasma

from recovered patients contains neutralizing antibodies and thus may represent a beneficial treatment option for viral infections. Immunotherapy of potentially lethal infections, lacking specific preventive and therapeutic options, with passive immunization with convalescent plasma was successfully used in other viral diseases such as SARS, Ebola or influenza A (H5N1) [6–9]. The experimental treatment of SARS-CoV-2 infection with convalescent plasma has therefore been proposed particularly in the vulnerable cohort of high-risk patients [10–15]. Initial studies reported an improvement in the clinical condition and survival in patients undergoing plasma treatment [16–19]. However, recent studies with a large number of patients with varying underlying health conditions have failed to demonstrate the significant clinical benefit of convalescent plasma in COVID-19 patients [20,21]. Notably, two large randomized, double-blind, placebo-controlled trials studies examined the efficacy of early-administered convalescent plasma in elderly patients and reported a reduced progression of COVID-19 [22,23]. Within the progress of the pandemic, major advances have been achieved in understanding, prevention, and treatment of the disease. COVID-19 can be subdivided in three different phases: An early phase with viremia, fever and cough (phase I), a pulmonary vascular disease (phase II) and a hyperinflammatory syndrome (phase III) [13]. Notably, the viral replication plays an important role in the early stage of infection. Obviously, the onset of passive antibody treatment must occur in a timely manner after infection to be effective [24]. Consequently, antiviral interventions are most effective during the early phase of the disease, whereas immunoregulatory treatment occurs effective during the hyperinflammatory stage [13]. The direct antiviral drugs remdesivir, molnupinavir and nirmatrelvir/ritonavir have been shown to be effective for preventing severe forms of COVID-19 if administered early [25]. The approval of neutralizing monoclonal antibodies (mAb) against the spike protein of SARS-CoV-2 was a landmark in the treatment of COVID-19 in high-risk patients [26]. In several large studies, anti-SARS-CoV-2 mAb have been shown to be highly effective in preventing the progression of COVID-19, especially when administered in the early stages of the disease [27,28]. However, despite the availability and efficacy of novel vaccines and COVID-19-targeted therapeutics, the emergence of new variants of SARS-CoV-2 continues to be a major challenge [29]. Antiviral monoclonal antibodies showed reduced efficacy against recently circulating SARS-CoV-2 variants of concern such as the Omicron B.1.1.529/BA.2 [30–33]. In consequence, in the European Union most available mAb are no longer recommended and in the United States no longer authorized to treat COVID-19. Of note, while the presence of the Omicron variant has been associated with a lower severity of COVID-19 in the general population, recent data indicate that pre-diseased and immune-compromised patients remain at high risk for morbidity and mortality from COVID-19 [34,35]. Thus, rapidly available and affordable treatment options are still urgently needed. Since the development of novel antivirals or monoclonal antibodies against emerging variants may take months to years, treatment with convalescent plasma from patients that recovered from infection with the respective variants of concern may still represent a therapeutic option especially for vulnerable high-risk patients.

Thus, we evaluated the clinical efficacy of monoclonal antibody in comparison to convalescent plasma and supportive care treatment in highly vulnerable non-vaccinated COVID-19 patients.

2. Materials and Methods

2.1. Study Population and Study Design

We conducted a monocentric retrospective observational study at our university hospital to evaluate the clinical efficacy of early monoclonal antibodies and convalescent plasma treatment in a non-vaccinated cohort at high-risk for severe COVID-19. The patients were selected in the frame of a retrospective study at different phases of the pandemic. The first antiviral treatment that was available was convalescent plasma (CP) derived from people who recovered from COVID-19. Accordingly, high-risk patients were treated with CP where indicated. Subsequently, monoclonal antibodies became available and replaced

convalescent plasma therapy. The control group includes patients that were hospitalized at the early stage of the pandemic, where no antiviral treatment (such as remdesivir, convalescent plasma or monoclonal antibodies) was available or patients who refused antiviral treatment. Inclusion criteria were belonging to a high-risk cohort, WHO stage at primary presentation < 6 and giving informed consent to the study. Exclusion criteria were the following: previous immunization against COVID-19 or history of virologically confirmed COVID-19, participation in a COVID-19-treatment trial or unwilling to participate in the study. The follow-up period was 30 days.

According to WHO COVID-19 clinical progression scale the majority of patients initially presented with mild to moderated disease, none of the patients initially presented with severe disease (Table 1) [36].

Table 1. Demographic and clinical overview of the study cohort.

Group	Treated with mAB (1)	Treated with Plasma (2)	Supportive Care (3)
Total number	26	11	18
Sex (w/m)	17/9	5/6	8/10
Median age [years]	58 (17–78)	63 (44–84)	75 (35–93)
Mean Charlson Comorbidity Index Score, ±SD	3.23 ± 2.0	6.54 ± 2.7	5.0 ± 2.12
Severely immunocompromised Patients, *n* (%)	17 (65%)	7 (63%)	12 (66%)
History of pulmonary disease, *n* (%)	19 (73%)	3 (27%)	5 (27%)
Predominant circulating variant?	Alpha B.1.1.7	Alpha B.1.1.7	D614G and Alpha B.1.1.7
Range of admission to hospital	02.2021 to 04.2021	07.2020 to 11.2020	03.2020 to 02.2021
Number of Patient with moderate disease	5 (19%)	3 (27%)	3 (16%)
Progression to severe COVID-19 defined by WHO score ≥ 6, *n* (%)	1 (4%)	6 (54%)	12 (66%)

In total, 55 patients with PCR-confirmed COVID-19 hospitalized between March 2020 and April 2021 at the University Medicine Essen, Germany were enrolled in this study (Table 1).

COVID-19 treatment was administered according to international recommended treatment protocols, patient risk factors, treatment availability and circulating variants at time point of infection.

The first group (group 1; n = 26 patients) received monoclonal antibody therapy according to expanded Emergency Use Authorization (EUA) criteria of the European Medical Agency (EMA) in February 2021 [37]. The group consisted of 17 women and nine men with a median age of 58 years (range 17–78) (Table 1). 16 patients had a solid organ transplant (n = 3 kidney; n = 13 lung), six patients had a history of lung disease (n = 1 non-small lung cancer, n = 1 asthma, n = 1 lung fibrosis and n = 4 stage 4 chronic obstructive pulmonary disease (COPD)), one patient had breast cancer, one cachexia and one prostate cancer.

The second group (group 2; n = 11 patients) received high-titer convalescent plasma from COVID-19 recovered individuals. This group was composed of five females and six males, and the median age was 63 years (range 44–84) (Table 1). The main indication for early convalescent plasma treatment was the risk of a fulminant COVID-19 course due to immunodeficiency (n = 5 with solid organ transplantation: two patients with kidney transplantation, one with liver transplantation and two with lung transplantation), a severely impaired renal function (n = 4), a severe lung disease (stage 4 COPD (n = 1)) or cancer (n = 1). The median neutralizing antibody titer was determined with a cell culture-based neutralization test. Only convalescent plasmas with a neutralizing antibody titer ratio of at least 1:160 were administered. The decision for convalescent plasma therapy was

based solely on clinical indication and the therapy was initiated within the first 48 h after a PCR confirmed SARS-CoV-2 infection.

The third group of 18 patients (group 3) received the best supportive care at the early beginning of the pandemic when targeted antiviral therapy against SARS-CoV-2 was not available and immunomodulatory therapy was not proven for COVID-19. The group included eight women and ten men, and the median age was 75 years (range 35–93) (Table 1). Two had lung transplantation, four had kidney transplantation, four had stage 4 COPD, one had rheumatoid arthritis, one had a hematopoietic stem cell transplantation, one had esophagus cancer, one had non-small lung cancer, one had Hodgkin lymphoma, one had non-Hodgkin lymphoma, one had follicular lymphoma, and one had hepatocellular cancer. Severe COVID-19 was defined according to WHO progression scale as pneumonia with oxygen requirement. The primary outcome was all-cause in-hospital mortality within 30 days of admission, the secondary outcome was the progression of COVID-19.

2.2. Therapy Regiments

In the present study, we focused on the 30-day survival of COVID-19 patients that were treated with SARS-CoV-2 neutralizing monoclonal antibodies or convalescent plasma and compared it with survival rates of patients receiving supportive medical care due to the lack of specific antiviral treatment in the early phase of the pandemic (Figure 1).

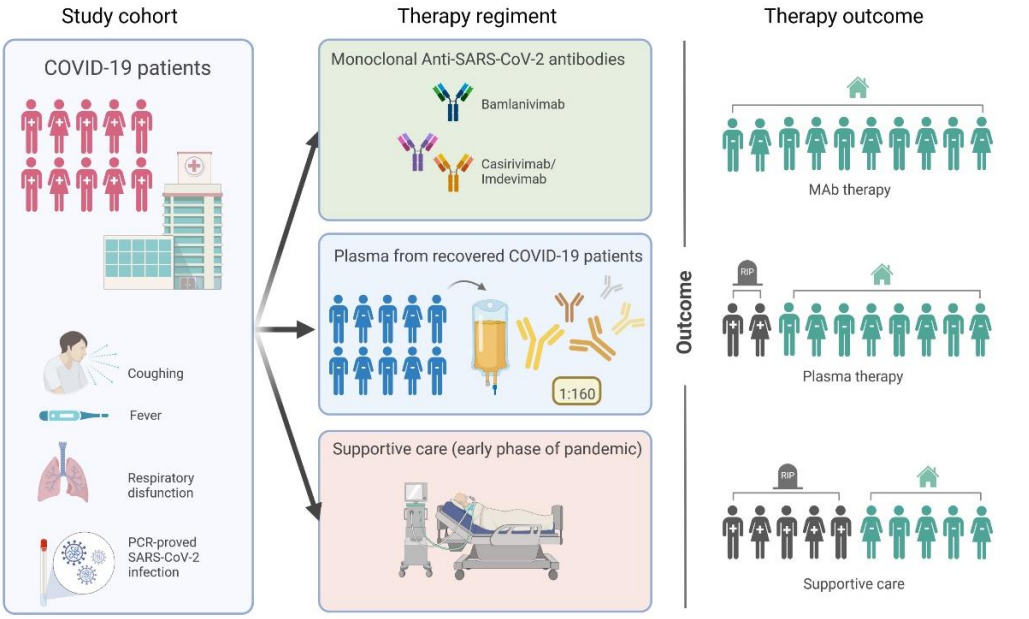

Figure 1. Study overview. Vulnerable patients at high risk for a severe course of COVID-19 were enrolled in the present study. The efficacy of monoclonal antibody treatment and convalescent plasma treatment was investigated and compared with best supportive care. As outcome parameter, 30-day mortality was assessed.

2.2.1. Monoclonal Antibody Therapy

Group 1, consisting of 26 patients, was treated with monoclonal anti-SARS-CoV-2 antibodies according to expanded EUA criteria of the EMA [37]. Antibody therapy was adjusted depending on the susceptibility of the circulating variants towards the respective antibodies. 23 patients received 700 mg bamlanivimab at a concentration of 35 mg/mL. Due to the increase in the locally circulating variant B.1.5 which was resistant to monotherapy with 700 mg bamlanivimab at a concentration of 35 mg/mL, three patients received 1200 mg casirivimab/imdevimab at a concentration of 120 mg/mL each. The therapy was administered according to the manufacturer's recommendation.

2.2.2. Treatment with High-Titer Convalescent Plasma

The 11 patients from group 2 were treated with plasma from COVID-19-recovered individuals. COVID-19 convalescent plasma collection and transfusion were performed according to EU guidance [38]. The acquisition of potential plasma donors and plasmapheresis were described previously [39,40].

The plasma units that had a neutralization titer of at least 1:160 were administered to patients in an AB0-compatible manner. Neutralizing antibody titers were determined as previously described [34]. In 10 of 11 patients, therapy was performed with two plasma units of 200–280 mL each on day 1 and 3; in one patient, a third unit was applied on day 5.

2.2.3. Best-Supportive Care

The 18 patients from group 3 became SARS-CoV-2 infected early at the beginning of the pandemic. At that time, no approved therapies against SARS-CoV-2 infection existed.

2.3. Ethics

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). The production of the convalescent plasma was conducted under the permission of the county government (AZ24.05.05.02). All volunteers and patients/their legal guardians provided informed consent to participate in the study.

2.4. Statistics

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) software. 30-day mortality was estimated by the Kaplan–Meier method and compared with the Chi-square test. Multivariate linear models were used to determine the effects of the three different treatments and the previously described risk factors at baseline on the severity of COVID-19 outcomes. A two-sided p-value of 0.05 or lower was considered statistically significant.

3. Results

3.1. Study Overview

In the present study, we investigated the 30-day survival of COVID-19 patients that were treated with SARS-CoV-2 neutralizing antibodies or convalescent plasma and compared it with survival rates of patients receiving supportive medical care due to the lack of specific antiviral treatment in the early phase of the pandemic. In total, 55 patients with a PCR-confirmed SARS-CoV-2 infection were included in the study. Of them, 26 were treated with SARS-CoV-2 neutralizing antibodies bamlanivimab or casirivimab/imdevimab, 11 with convalescent plasma, and 18 received the best supportive medical care (Figure 1). Monoclonal antibodies were applied according to manufacturer instructions. Convalescent plasma treatment was conducted with two plasma units of 200–280 mL each on day 1 and 3 in 10 of 11 patients; in one patient, a third unit was given on day 5. The average neutralizing titer of convalescent plasmas used was 1:160. The survival rate was analyzed for a period of 30 days after hospitalization.

3.2. Antiviral Immunotherapy with Monoclonal Antibodies or Convalescent Plasma Reduced the 30-Day Mortality in High-Risk COVID-19 Patients

The efficacy of monoclonal antibody treatment with bamlanivimab or casirivimab/imdevimab and convalescent plasma treatment was investigated and compared with best supportive care. Patient outcome was observed for a period of 30 days upon hospitalization. All patients that were treated with monoclonal antibodies survived. Nine out of 11 patients (82%) receiving convalescent plasma treatment and eleven out of 18 (61%) of patients that received the best supportive care survived (Figure 2). Overall, the survival

was significantly higher in patients who received monoclonal antibodies when compared to convalescent plasma treatment ($p = 0.03$) or best supportive care ($p < 0.0001$) (Figure 2).

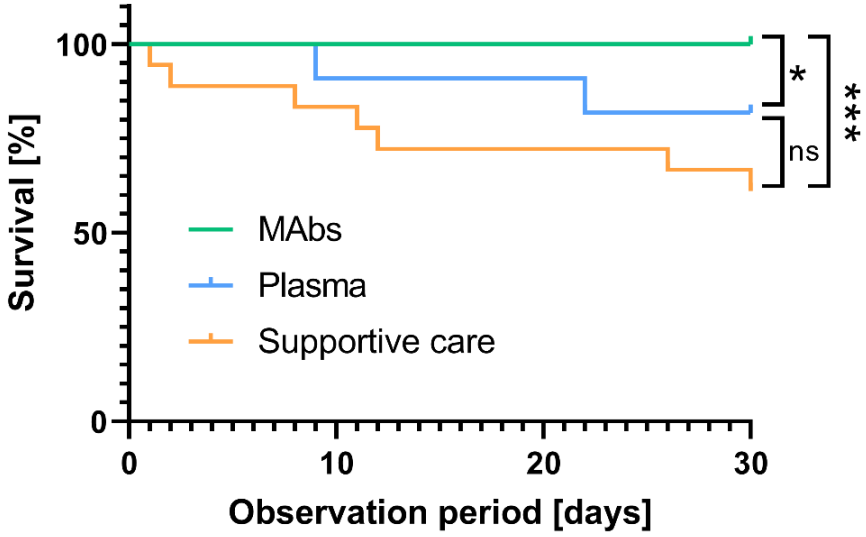

Figure 2. Overall survival of COVID-19 patients received either mAbs, convalescent plasma or supportive care. The survival of the patients was monitored for 30 days after initiating the treatment. Differences in the overall survival at day 30 were calculated using the Chi-square test. Comparisons were considered significant at * $p < 0.05$ and *** $p < 0.001$.

Notably, the survival was 21% higher in patients treated with convalescent plasma compared to the best supportive care group ($p = 0.2$) (Figure 2). In the mAb group severe respiratory disease developed in 1 of 26 patients (3.8%), in the convalescent plasma group in 6 of 11 (54.5%) and in the best supportive care group in 12 of 18 patients (66.7%) ($p < 0.0001$). To assess the impact on the severity of COVID-19, as defined by the most severe WHO COVID-19 score, in our cohort by the known risk factor being age, sex, Charlson Comorbidity Index (CCI), WHO COVID-19 severity on presentation and treatment group 1–3 membership, multivariable linear regression analysis was performed. In this analysis for COVID-19 severity outcome, patients' membership in treatment group 1 (mAb, reference group) significantly influenced the COVID-19 severity score compared with the convalescent plasma group and supportive care group. In addition, only the COVID-19 severity score at presentation was associated with a more severe COVID-19 score (Table 2). The R^2 value for this model was 0.42.

Table 2. Results of the regression model indicating the determinants of the COVID-19 severity defined by WHO score. Comparisons were considered significant at * $p < 0.05$ and ** $p < 0.01$. CCI = Charlson index; G1–3 = group 1–3; mAb = monoclonal antibody therapy; SC = supportive care.

Parameter	Standardized Coefficients ß	95% CI	*p* Value
Sex	0.371	−0.8074 to 1.550	0.53
Age	−0.0027	−0.07792 to 0.02377	0.29
CCI	0.139	−0.1187 to 0.3968	0.28
WHO COVID-19 severity score at presentation	0.81	0.01247 to 1.605	0.04 *
G1 mAb vs. G2 Plasma	2.002	0.3113 to 3.693	0.021 *
G1 mAb vs. G3 SC	3.318	1.754 to 4.881	<0.0001 **

4. Discussion

In the present study, we evaluated 30-day survival in differently treated unvaccinated COVID-19 patients at high risk for severe disease outcome. Depending on the availability of therapeutic options, three treatment groups could be defined: one cohort treated with monoclonal antibodies (bamlanivimab or a combination of casirivimab and imdevimab), a second cohort received convalescent plasma treatment from recovered COVID-19 individuals and a third cohort received best supportive care at the early beginning of the pandemic when no antiviral treatment against SARS-CoV-2 was available.

We demonstrated that early antiviral treatment with monoclonal antibodies is associated with a better survival rate in high-risk patients in the real-world setting compared with convalescent plasma treatment or best supportive care. Our data are in line with prior studies. Meanwhile, the therapy with mAbs in early-stage SARS-CoV-2 infections in high-risk groups is widely accepted, and clinical trials with mAbs have reported overall lower hospitalization rates in patients with mild-to-moderate COVID-19 [41–43]. However, previous studies have demonstrated that not all approved mAbs are suitable for treating the different variants of SARS-CoV-2 such as Omicron [44,45]. It became apparent that the development of new monoclonal antibodies is in a permanent race with the emergence of new SARS-CoV-2 variants [30,46].

In the present study, convalescent plasma treatment was associated with a considerably higher survival rate of 82%, compared with the best supportive care cohort (61%). Many studies have demonstrated the benefits of treatment with convalescent plasma such as reduced mortality and shorter hospitalization time [47,48]. However, it also became evident that both the timing of plasma administration as well as the neutralizing antibody titer of the plasma administered are critical for patient outcome [49–51]. Studies have shown that the administration of plasma in very severe courses of COVID-19 is no longer beneficial and does not reduce mortality [51–53].

The higher average age of patients from group 3 (supportive care) is a limitation of the study, since higher age (>60) is commonly known as a risk factor for increased mortality in COVID-19. Accordingly, older patients were the first who became vaccinated in Germany, and thus were protected from a lethal and severe course of disease. As a consequence, predominantly people around 60 were treated for COVID-19 when convalescent plasma and monoclonal antibodies became available.

Besides age, both primary and secondary immunosuppression due to inborn errors of immunity, organ transplantation, cancer treatment or other immunomodulatory treatment represents a risk factor for a severe course of COVID-19 [2].

Early treatment with convalescent plasma or monoclonal antibodies as well as antivirals may improve the course of disease and clinical outcome in those patients [14,54,55].

Both mAbs and convalescent plasma therapy show a significantly higher survival than best supportive care alone in high-risk patients. Monoclonal antibodies are not effective against every variant of SARS-CoV-2, which is a major drawback. However, timing and titer are very critical in the administration of plasma. Our data points out, however, that in the absence of approved antibodies for emerging variants, early administration of convalescent plasma may make a critical difference in the survival probability of high-risk individuals.

5. Conclusions

Our data highlight that early targeted passive immunization with mAbs or high-titer convalescent plasma can significantly reduce 30-day mortality in high-risk patients. Clinicians should consider early treatment with high-titer convalescent plasma as an alternative option for COVID-19-specific therapy for high-risk patients if other therapeutic options are unavailable. In addition, convalescent plasma may be a therapeutic option for infections with newly emerging SARS-CoV-2 variants that are less susceptible to approved monoclonal antibodies.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee of the University Hospital Essen (approval no. 20-9665-BO).

Informed Consent Statement: All volunteers and patients/their legal guardians provided informed consent to participate in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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3.5 Fluoxetine and sertraline potently neutralize the replication of distinct SARS-CoV-2 variants

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Laura Thümmler	Prof. Dr. med. Monika Lindemann

viruses

MDPI

Article

Fluoxetine and Sertraline Potently Neutralize the Replication of Distinct SARS-CoV-2 Variants

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Abstract: The pandemic caused by SARS-CoV-2 is still a major health problem. Newly emerging variants and long-COVID-19 represent a challenge for the global health system. In particular, individuals in developing countries with insufficient health care need easily accessible, affordable and effective treatments of COVID-19. Previous studies have demonstrated the efficacy of functional inhibitors of acid sphingomyelinase against infections with various viruses, including early variants of SARS-CoV-2. This work investigated whether the acid sphingomyelinase inhibitors fluoxetine and sertraline, usually used as antidepressant molecules in clinical practice, can inhibit the replication of the former and recently emerged SARS-CoV-2 variants in vitro. Fluoxetine and sertraline potently inhibited the infection with pseudotyped virus-like particles and SARS-CoV-2 variants D614G, alpha, delta, omicron BA.1 and omicron BA.5. These results highlight fluoxetine and sertraline as priority candidates for large-scale phase 3 clinical trials at different stages of SARS-CoV-2 infections, either alone or in combination with other medications.

Keywords: SARS-CoV-2; antidepressants; COVID-19

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1. Introduction

Infections with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) have declined because of successful vaccination campaigns and the lower lethality of omicron variants compared to the former ones, and hospitalization rates have dropped significantly. Nevertheless, the virus is still responsible for more than 20,000,000 current infections, including severe and life-threatening diseases [1]. In low- and middle-income countries worldwide, coronavirus disease 2019 (COVID-19) remains a serious health problem [2]. Although several vaccines have been approved within an impressively short time, public opposition against vaccination and an increasing vaccine fatigue are major obstacles in maintaining immunity in the worldwide population [3]. Furthermore, newly emerging SARS-CoV-2 variants accompanied by an increasing number of breakthrough infections require developing prophylactic and therapeutic treatment options against SARS-CoV-2 infections [4–6]. Antidepressants represent a promising class of cheap and globally available drugs with proven antiviral activity against SARS-CoV-2 [7].

Several pre-clinical and clinical studies demonstrated that antidepressants belonging to the group of functional inhibitors of the acid sphingomyelinase (FIASMA) showed a broad antiviral activity against viruses such as enteroviruses [8–10], coxsackieviruses [11], hepatitis C [12], arenaviruses [13] and SARS-CoV-2 [7,14–19]. Notably, patients treated with such antidepressants showed less severe COVID-19 symptoms, a less frequent requirement of ventilation and most importantly showed a less often lethal course of disease [20–23].

We have previously demonstrated that pre-treatment of cultured Vero E6 epithelial cells or freshly isolated human nasal epithelial cells with antidepressants such as amitriptyline, desipramine, imipramine, fluoxetine, sertraline, maprotiline or escitalopram prevented infections with SARS-CoV-2 spike pseudotyped vesicular stomatitis virus (VSV) particles and an early isolate of SARS-CoV-2 D614G [24].

Mechanistically, we demonstrated that binding of SARS-CoV-2 spike to ACE2, the cellular receptor of SARS-CoV-2, induces activation of the acid sphingomyelinase, the subsequent conversion of sphingomyelin to ceramide and the formation of ceramide-enriched membrane platforms at the outer leaflet of the plasma membrane [24,25]. Inhibition of the acid sphingomyelinase by antidepressants prevented these biochemical changes and therefore blocked the infection of target cells with SARS-CoV-2 [24].

Thus, antidepressants targeting the acid sphingomyelinase represent a promising strategy for the treatment and prevention of severe COVID-19 [26,27]. Although initial studies demonstrated potent antiviral activity against SARS-CoV-2 D614G, it remains unclear if antidepressants such as fluoxetine and sertraline can interfere with the replication of newly emerged SARS-CoV-2 variants of concern.

In the present study, we investigated the antiviral activity of fluoxetine and sertraline against the SARS-CoV-2 variants D614G, alpha, delta, omicron BA.1 and omicron BA.5.

2. Materials and Methods

2.1. Cells and Viruses

All cell lines were incubated at 37 °C and 5% CO_2 in a humidified atmosphere in a standard tissue incubator unless specifically indicated otherwise. HEK293T (human kidney cell line) cells were obtained from Merck (Darmstadt, Germany) via the lab of Ulf Brockmeier (Department of Neurology and Center for Translational Neuro- and Behavioral Sciences (C-TNBS), University Hospital Essen, Essen, Germany) and maintained in Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (GE Healthcare, Chicago, IL, USA), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Thermo Fisher Scientific). HEK293T cells were used for the production of VSV*ΔG-Fluc spike pseudotype virus-like particles. Vero E6 (African green monkey kidney cell line) cells were obtained from ATCC and maintained in DMEM supplemented with 10% FBS (GE Healthcare), 100 U/mL penicillin, 0.1 mg/mL streptomycin, 100 µM non-essential amino acids, 1 mM sodium pyruvate (each Thermo Fisher Scientific) and 10 mM HEPES, pH 7.3 (Carl Roth, Karlsruhe, Germany). Vero cells

were utilized in the preliminary infection experiments with pseudotype virus-like particles. Human lung A549-AT cells stably transfected with ACE2 and TMPRSS2 [28] were cultured in minimal essential medium (MEM, Thermo Fisher Scientific) supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 100 µM non-essential amino acids, 1 mM sodium pyruvate and 10 mM HEPES, pH 7.3 (each Thermo Fisher Scientific). The enhanced expression of ACE2 and TMPRSS2 contributed to a robust infection rate of cells with various SARS-CoV-2 variants. This justified the utilization of these cells for evaluating the antiviral efficacy of fluoxetine and sertraline against different clinical SARS-CoV-2 isolates.

The clinical SARS-CoV-2 isolates of wild type (D614G), alpha, delta, omicron BA.1 and BA.5 were obtained from nasopharyngeal swabs of COVID-19 patients hospitalized at the University Hospital Essen, Germany, as previously described [4,29]. The SARS-CoV-2 variants were identified after sequencing the spike gene and correlating with the corresponding variants according to the list of variants of concern from the WHO [World Health Organization Tracking SARS-CoV-2 Variants [30]]. The viruses were propagated on A549-AT cells and stored at −80 °C. Viral titers were determined by a standard end-point dilution assay and calculated as 50% tissue culture infective dose ($TCID_{50}$)/mL, as previously described [31]. The clinical SARS-CoV-2 isolates used here were sequenced in a previous work [4]. The datasets presented in this study can be found online (ENA; https://www.ebi.ac.uk/ena/browser/view/PRJEB59607 (accessed on 9 January 2024)).

2.2. Plasmids

The initial neutralization experiments were performed using pp-VSV-SARS-CoV-2 spike pseudotyped virus-like particles. These were generated by using pCG1_SARS-2-S-del18 plasmid, which was kindly provided by Markus Hoffmann and Stefan Pöhlmann (Infection Biology Unit, German Primate Centre–Leibniz Institute for Primate Research, Göttingen, Germany) and previously described [32]. SARS-CoV-2 spike variants with point mutations in the receptor-binding domain (RBD) were generated sequentially by using the Q5 site-directed mutagenesis kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions and confirmed by sequencing (Table S1).

Expression plasmids carrying all mutations of the respective variants of concern were a gift from David Nemazee (Wt: pCDNA3.3_CoV2_D18, Addgene plasmid #170442; alpha: pCDNA3.3_CoV2_B.1.1.7, Addgene plasmid #170451; beta: pCDNA3.3_CoV2_501V2, Addgene plasmid #170449; gamma: pCDNA3.3_CoV2_P1, Addgene plasmid number #170450; delta: pCDNA3.3-SARS2-B.1.617.2, Addgene plasmid #172320) or obtained from GenScript (omicron: SARS-CoV-2 Omicron Strain S gene Human codon_pcDNA3.1(+), #MC_0101274) (Table S2). Plasmids were amplified in *E. coli* DH5α competent cells (New England Biolabs) and isolated and purified using Maxiprep Kits according to the manufacturer's instructions (Qiagen, Hilden, Germany and Macherey-Nagel, Düren, Germany).

*2.3. Preparation of VSV*ΔG-Fluc Spike Pseudotype Virus-like Particles*

The replication-restricted VSV system for pseudoviral particles with enhanced green fluorescent protein (eGFP) and firefly luciferase (Fluc) reporters (VSV*ΔG-FLuc) was kindly provided by Gert Zimmer (Institute of Virology and Immunology, Mittelhäusern/Switzerland), passed on by the lab of Stefan Pöhlman and previously described by Berger Rensch and Zimmer [33]. We followed the protocol for pseudoviral particle generation described by Becker et al. [34]. VSV*ΔG-Fluc (VSV-G) stocks and anti-VSV-G antibody supernatants were prepared and titrated as described. The SARS-CoV-2 pseudotyped VSV particles were prepared according to the described protocol, but with slight modifications; low-passage HEK293T cells were seeded into 10 cm cell culture dishes (at 1.8×10^6 cells/dish) and grown for 24 h at 37 °C with 5% CO_2 in a standard cell culture incubator. On the next day, the cells received fresh medium (9 mL/dish), and the transfection mixtures were prepared by mixing 42 µg of the respective plasmid DNA per dish and sterile ultrapure water to a final volume of 400 µL. A total of 100 µL sterile-filtered $CaCl_2$ (stock conc. 2.5 M, Thermo Fisher Scientific) was added and mixed. A total of 500 µL 2× HBS buffer (280 mM NaCl,

50 mM HEPES, 20 mM KCl, 1.5 mM Na_2HPO_4, pH 7.1, each Thermo Fisher Scientific) was added dropwise while bubbling the solution with an electronic pipettor. The mixture was then immediately vortexed and incubated for 20 min at room temperature. The transfection complexes were added dropwise to the cells and incubated for 28-30 h at 33 °C with 5% CO_2. Cells were inoculated with VSV*ΔG-FLuc at a multiplicity of infection of five for one hour at 33 °C and 5% CO_2 in a standard tissue incubator. The supernatant was then removed and cells washed two times with PBS (Thermo Fisher Scientific). Fresh medium containing anti-VSV-G antibodies was added, and cells were incubated overnight (18 h) at 33 °C with 5% CO_2 in a standard tissue incubator. The supernatant containing the pseudoviral particles was then harvested, cellular debris was removed by centrifugation at 2000× *g* for 10 min and the clarified supernatant was used immediately for experiments.

2.4. Transduction In Vitro Experiments

Vero E6 cells were seeded at 1×10^4 cells/well in 48-well plates and grown for 48 h. Cells were then pre-treated for 4 h with antidepressants in DMEM (Thermo Fisher Scientific) with only 1% FBS (Thermo Fisher Scientific) prior to infection. Fluoxetine hydrochloride and sertraline hydrochloride (each Sigma Aldrich, St. Louis, MO, USA) were dissolved in dimethylsulfoxide (DMSO, Carl Roth) and diluted to a final concentration of 25 μM fluoxetine or 10 μM sertraline. Mock treated cells were assessed as controls. The medium was removed, and the cells were incubated with a medium containing VSV*ΔG-Fluc spike and 25 μM fluoxetine or 10 μM sertraline. The medium containing VSV*ΔG-Fluc spike and corresponding DMSO concentrations without the addition of antidepressants served as the control. Cells were infected for 1 h at 33 °C with 5% CO_2 in a standard tissue culture incubator. Pseudoviral supernatants were discarded, and the cells overlaid with fresh medium and incubated for 18 h at 33 °C with 5% CO_2. The effect of antidepressants on the infection of the cells was assessed by qualitative and quantitative analysis of the eGFP expression. The cell cultures were scanned for eGFP fluorescence on a Typhoon FLA biomolecular imager (GE Healthcare) and microscopic fluorescence images were obtained (EVOS, Thermo Fisher Scientific). For quantitative analysis, cells were harvested for flow cytometric analysis (Attune NxT, Thermo Fisher Scientific). The infection efficacy in the mock controls was set as 100%, and the effect of the antidepressants was reported relative to these controls.

2.5. Neutralization Assay

The antiviral activity of fluoxetine and sertraline was determined by a cell-culture-based endpoint dilution assay. A549-AT cells were seeded in a 24-well plate (at 1×10^4 cells/well) and grown for 24 h at 37 °C with 5% CO_2 in a standard cell culture incubator. The confluent A549-AT cells were pre-incubated with different concentrations of fluoxetine (0 μM, 5 μM, 10 μM, 20 μM, 25 μM or 30 μM) or sertraline (0 μM, 5 μM or 10 μM) with MEM (Thermo Fisher Scientific) supplemented with 2% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 100 μM non-essential amino acids, 1 mM sodium pyruvate and 10 mM HEPES, pH 7.3 (each Thermo Fisher Scientific) for two hours at 37 °C with 5% CO_2. Subsequently, the A549-AT cells were infected by adding 100 $TCID_{50}$ of SARS-CoV-2 into the medium, and the cells were then incubated for three days at 37 °C with 5% CO_2. Untreated A549-AT cells served as a negative control. Thereafter, the supernatants were harvested and the viral titers were determined by endpoint dilution. Therefore, serial dilutions of the cell culture media (1:10–1:10^8) were incubated on A549-AT cells grown on a 96-well microtiter plate for three days at 37 °C with 5% CO_2. The inoculation medium was then removed, the cells stained with 0.5% crystal violet (Roth), solved in 20% methanol (Merck) and evaluated for cytopathic effects (CPEs) using light microscopy. The experiment was performed in triplicate.

2.6. Quantification of SARS-CoV-2 RNA

Cell culture supernatants were centrifuged to remove cell debris and stored at −80 °C before further processing. Total RNA was purified from cell culture supernatants using the QIAmp viral RNA Mini Kit (Qiagen) according to manufacturer's instructions with preceding DNase I digestion with the RNase-Free DNase Set (Qiagen). A total of 250 ng of total RNA was reverse transcribed using the PrimeScript RT Master Mix (Takara, Kusatsu, Japan) for relative determination of SARS-CoV-2 M- or N-gene expression. For RT-qPCR, GoTaq Probe qPCR Master Mix (Promega, Madison, WI, USA) was used according to the manufacturer's instructions, with gene-specific primers and probes (Table S3). RT-qPCR was performed on a LightCycler 480 II (Roche, Basel, Switzerland) instrument, with the following conditions: initial denaturation for 2 min at 95 °C with a ramp rate of 4.4 °C/s, followed by 40 cycles of denaturation for 15 s at 95 °C with a ramp rate of 4.4 °C/s and amplification for 60 s at 60 °C with a ramp rate of 2.2 °C/s. To assess M- and N-gene copy numbers, the M- and N-genes were partially cloned into pCR2.1 (Thermo Fisher Scientific) or pMiniT 2.0 (NEB, Ipswich, MA, USA), respectively. A 1:10 plasmid dilution series was used as a reference.

2.7. Cytotoxicity Assay

Potential cytotoxicity of various concentrations of fluoxetine and sertraline towards A549-AT cells was determined using the Orangu™ cell counting solution (Cell guidance systems, Cambridge, UK) according to the manufacturer's protocol. Briefly, increasing fluoxetine or sertraline concentrations (0 µM, 5 µM, 10 µM, 20 µM or 30 µM) in MEM (Thermo Fisher Scientific) were supplemented with 2% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 100 µM non-essential amino acids, 1 mM sodium pyruvate and 10 mM HEPES, pH 7.3 (each Thermo Fisher Scientific) and incubated with 1×10^4 A549-AT cells per well of a 96-well-plate at 37 °C, 5% CO_2. At four distinct time points (2 h, 24 h, 48 h and 72 h), the inoculation medium was removed and replaced by a medium containing 10% of Orangu™ cell counting solution. After 60 min of incubation (37 °C, 5% CO_2), cell viability was measured at an absorbance of 450 nm using Tristar 3 (Berthold Technologies, Oak Ridge, TN, USA) and normalized to untreated control cells. The experiment was performed in triplicate.

2.8. Statistics

Statistical analysis was performed using GraphPad Prism 10.0 software (San Diego, CA, USA). The analysis of categorical variables was performed by one-way ANOVA (Friedman test) with Dunnett's correction for multiple comparisons, as appropriate. Two-sided p values < 0.05 were considered significant.

3. Results

3.1. Initial Investigation of the Antiviral Activity of Fluoxetine and Sertraline against SARS-CoV-2

As the first step, the ability of fluoxetine and sertraline to inhibit the SARS-CoV-2 infection of cells was evaluated with VSV*ΔG-Fluc spike pseudotyped virus-like particles. Therefore, VeroE6 cells were pre-treated for 4 h with 25 µM fluoxetine or 10 µM sertraline and subsequently infected with different VSV*ΔG-Fluc spike variant pseudotyped virus-like particles for 1 h, and the antiviral efficiency was assessed by fluorescence microscopy as well as quantitatively by flow cytometry the next day.

Initially, VSV*ΔG-Fluc spike pseudotyped virus-like particles with mutations only in the spike RBD region were used. Fluoxetine treatment led to significantly lower infection rates with wild type ($p < 0.0001$), alpha ($p < 0.0001$), beta ($p < 0.0001$), gamma ($p < 0.0001$), delta ($p < 0.0001$), lambda ($p < 0.0001$) and mu ($p < 0.0001$) compared to the DMSO control. The mean percentage infection of cells treated with fluoxetine was 48% for wild type, 43% for alpha, 35% for beta, 42% for delta, 35% for gamma, 58% for lambda and 66% for mu.

Accordingly, treatment with sertraline led to significantly lower infection rates with wild type ($p < 0.0001$), alpha ($p < 0.0001$), beta ($p < 0.0001$), gamma ($p < 0.0001$), delta

($p < 0.0001$), lambda ($p < 0.0001$) and mu ($p < 0.0001$). In the case of sertraline treatment, the mean percentage infection of cells observed was 37% for wild type, 31% for alpha, 21% for beta, 29% for gamma, 23% for delta, 32% for lambda and 41% for mu. Overall, sertraline treatment resulted in a greater reduction of infection compared to fluoxetine (Figure 1).

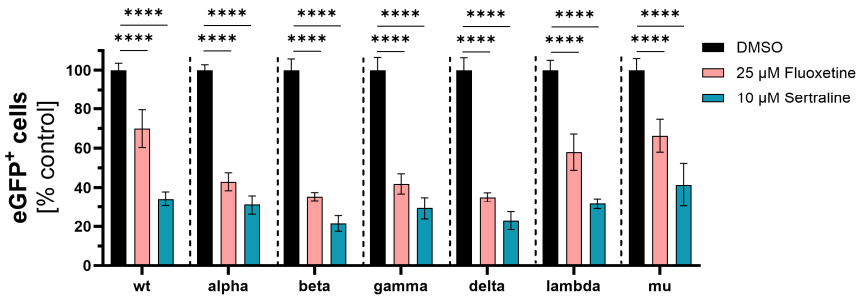

Figure 1. Inhibition of SARS-CoV-2 infection with distinct VSV*ΔG-Fluc spike pseudotyped virus-like particles. Vero E6 cells were pre-treated with either DMSO, 25 µM fluoxetine or 10 µM sertraline and subsequently infected with VSV*ΔG-Fluc spike pseudotyped virus-like particles with RBD mutations of different SARS-CoV-2 variants. The effect of antidepressants on the infection of the cells was assessed by quantitative analysis of the eGFP expression. Data are shown as mean and standard deviation (SD). Differences between fluoxetine/sertraline and the DMSO control were compared by one-way ANOVA (**** $p < 0.0001$).

Next, expression plasmids coding for spike proteins containing all mutations of the respective variants of concern were used to generate VSV*ΔG-Fluc pseudotyped virus-like particles, and the inhibitory effect of fluoxetine and sertraline against the infection of cells with SARS-CoV-2 was analyzed. Fluoxetine significantly reduced the infection of the cells with VSV*ΔG-Fluc spike pseudotyped virus-like particles of wild type D614G to 64% ($p < 0.0001$), of alpha to 55% ($p < 0.0001$), of beta to 65% ($p < 0.0001$), of gamma to 68% ($p = 0.0025$), of delta to 59% ($p < 0.0001$) and of omicron to 84% ($p < 0.0001$) compared to the DMSO control. Sertraline also led to a significant reduction of the infection with VSV*ΔG-Fluc spike pseudotyped virus-like particles of wild type D614G to 38% ($p < 0.0001$), alpha to 42% ($p < 0.0001$), beta to 43% ($p < 0.0001$), gamma to 52% ($p < 0.0001$), delta to 45% ($p < 0.0001$) and omicron to 48% ($p < 0.0001$) compared to the DMSO control. Fluoxetine and sertraline showed broad antiviral activity against VSV*ΔG-Fluc spike pseudotyped virus-like particles displaying distinct SARS-CoV-2 spike protein variants. Interestingly, sertraline more potently inhibited the infection with VSV*ΔG-Fluc spike pseudotyped virus-like particles compared to fluoxetine (Figure 2).

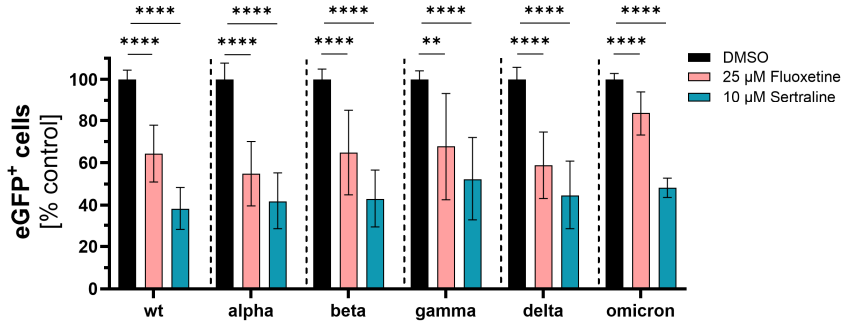

Figure 2. Inhibition of SARS-CoV-2 infection with distinct commercial VSV*ΔG-Fluc spike pseudotype virus like particles. Vero E6 cells were pre-treated with either DMSO, 25 µM fluoxetine or 10 µM sertraline and subsequently infected with VSV*ΔG-Fluc spike pseudotyped virus-like particles with mutations of different SARS-CoV-2 variants. The effect of antidepressants on the infection of the cells was assessed by quantitative analysis of the eGFP expression. Data are shown as mean and standard deviation (SD). Differences between fluoxetine/sertraline and the DMSO control were compared by one-way ANOVA (** $p < 0.01$; **** $p < 0.0001$).

The inhibitory effect of fluoxetine and sertraline on the SARS-CoV-2 infection of Vero E6 cells with different VSV*ΔG-Fluc spike pseudotype virus-like particles was confirmed by fluorescence microscopy (Figure 3).

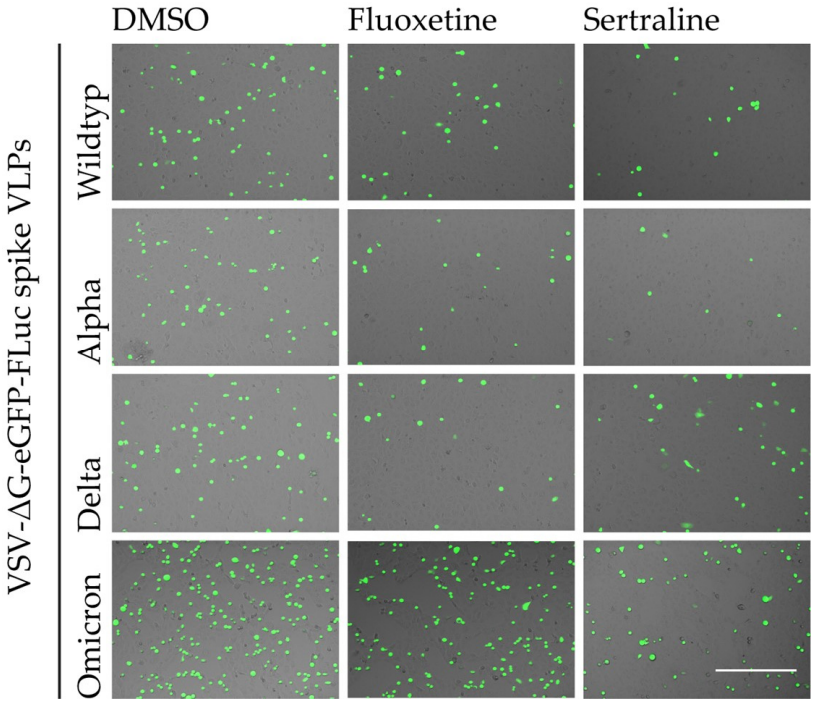

Figure 3. Qualitative assessment of the antiviral activity of fluoxetine and sertraline against SARS-CoV-2. Vero E6 cells were pre-treated with either DMSO, 25 µM fluoxetine or 10 µM sertraline and subsequently infected with VSV*ΔG-Fluc spike pseudotyped virus-like particles with mutations of different SARS-CoV-2 variants. The effect of antidepressants on the infection of the cells was assessed by fluorescence microscopy. Scale bar represents 400 µm.

3.2. Fluoxetine and Sertraline Effectively Blocks SARS-CoV-2 Infection

To examine whether the antidepressants fluoxetine and sertraline can inhibit infection with a real virus, the antiviral efficacy of fluoxetine (0–30 µM) or sertraline (0–10 µM) was investigated against clinical isolates SARS-CoV-2 D614G, alpha, delta, omicron BA.1 and omicron BA.5.

Fluoxetine inhibited the replication of SARS-CoV-2 D614G, alpha, delta, omicron BA.1 and omicron BA.5 in a dose-dependent manner. Complete inhibition of all SARS-CoV-2 variants was reached at a concentration of 30 µM. Significant reduction of the viral load was found for SARS-CoV-2 D614G (5 µM $p = 0.0097$; 10 µM $p = 0.0003$; 20 µM $p < 0.0001$; 30 µM $p < 0.0001$) and omicron BA.5 (10 µM $p = 0.0073$; 20 µM $p = 0.0037$; 30 µM $p = 0.0036$) compared to the medium control (Figure 4a).

Accordingly, sertraline inhibited the replication of SARS-CoV-2 D614G, alpha, delta, omicron BA.1 and omicron BA5 in a dose-dependent manner, where all SARS-CoV-2 variants were completely neutralized at a concentration of 10 µM sertraline. The incubation with sertraline significantly decreased the viral replication of SARS-CoV-2 alpha (5 µM $p = 0.0006$; 10 µM $p = 0.0005$), delta (5 µM $p < 0.0001$; 10 µM $p < 0.0001$), omicron BA.1 (5 µM $p = 0.0012$; 10 µM $p = 0.0012$) and omicron BA.5 (5 µM $p = 0.0017$; 10 µM $p = 0.0016$) at both tested concentrations compared to the control (Figure 4b).

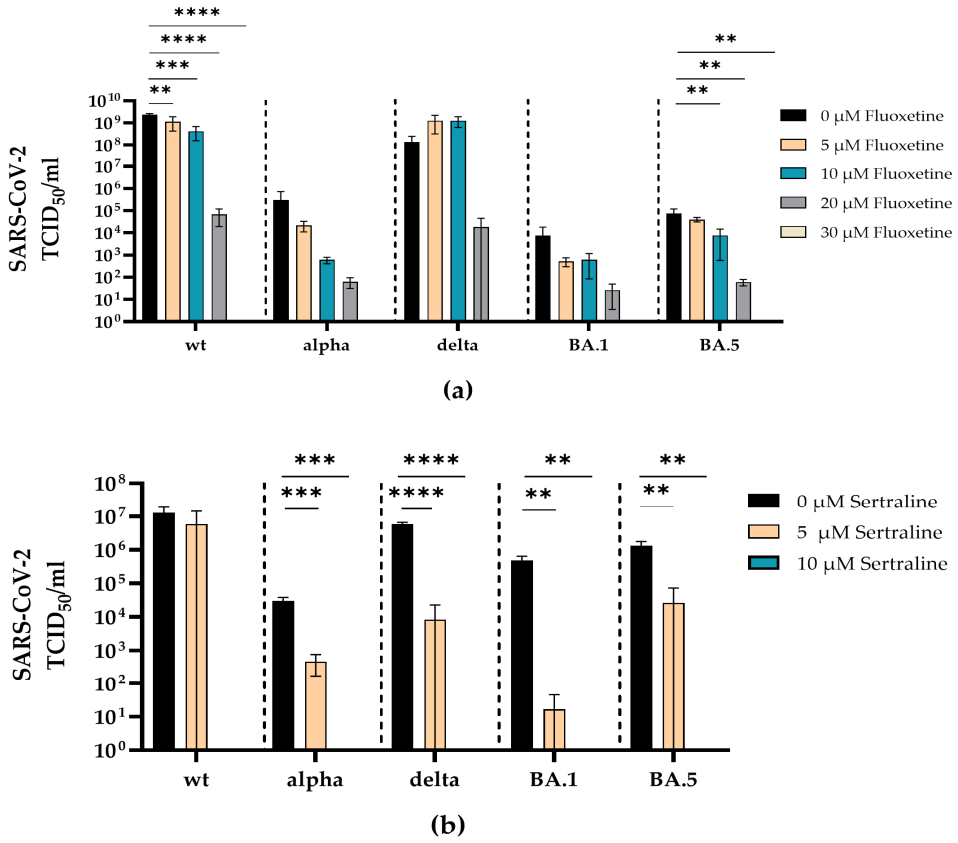

Figure 4. Fluoxetine and sertraline completely inhibit SARS-CoV-2 infection. A549-AT cells were infected with 100 $TCID_{50}$ of different clinical SARS-CoV-2 isolates (D614G, alpha, delta, omicron BA.1 and omicron BA.5) in the presence of different concentrations of fluoxetine (0–30 µM) (**a**) or sertraline (0–10 µM) (**b**). After three days of incubation, the viral loads in the respective cell culture supernatants were determined by endpoint dilution. The experiments were performed in triplicate. Data are shown as mean and standard deviation (SD). Differences between fluoxetine/sertraline and the control were compared by one-way ANOVA (** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

To additionally validate the antiviral effect of fluoxetine and sertraline against SARS-CoV-2 variants, SARS-CoV-2 RNA from cell culture supernatants of infected and fluoxetine- or sertraline-treated cells was quantified. Therefore, the RNA of the respective supernatant was isolated and reverse transcribed, and the M- and N-gene of SARS-CoV-2 were quantified by RT-qPCR. Fluoxetine treatment reduced the copy-number of SARS-CoV-2 M- and N-gene RNA in a dose-dependent manner. There was a significant reduction of M-gene RNA of SARS-CoV-2 D614G (10 µM $p = 0.0039$; 20 µM $p = 0.0006$; 30 µM $p = 0.0006$), alpha (5 µM $p = 0.0107$; 10 µM $p = 0.0096$; 20 µM $p = 0.0078$; 30 µM $p = 0.0076$), delta (10 µM $p = 0.037$; 20 µM $p = 0.011$; 30 µM $p = 0.011$), omicron BA.1 (5 µM $p = 0.029$; 10 µM $p = 0.0099$; 20 µM $p = 0.0079$; 30 µM $p = 0.0077$) and omicron BA.5 (10 µM $p = 0.0032$; 20 µM $p = 0.0022$; 30 µM $p = 0.0022$) at different fluoxetine concentrations (Figure 5a). Furthermore, a significant reduction of N-gene RNA was found in cell culture supernatants from SARS-CoV-2 D614G (10 µM $p = 0.0085$; 20 µM $p = 0.0006$; 30 µM $p = 0.0006$), alpha (5 µM $p = 0.010$; 10 µM $p = 0.0096$; 20 µM $p = 0.0078$; 30 µM $p = 0.0076$), delta (10 µM $p = 0.024$; 20 µM $p = 0.0056$; 30 µM $p = 0.0056$), omicron BA.1 (5 µM $p = 0.0043$; 10 µM $p = 0.0030$; 20 µM $p = 0.0026$; 30 µM $p = 0.0027$) and omicron BA.5 (10 µM $p = 0.0005$; 20 µM $p = 0.0003$; 30 µM $p = 0.0003$) infected cells treated with fluoxetine (Figure 5b). The M-gene RNA decreased slightly more than the N-gene RNA after treatment with fluoxetine.

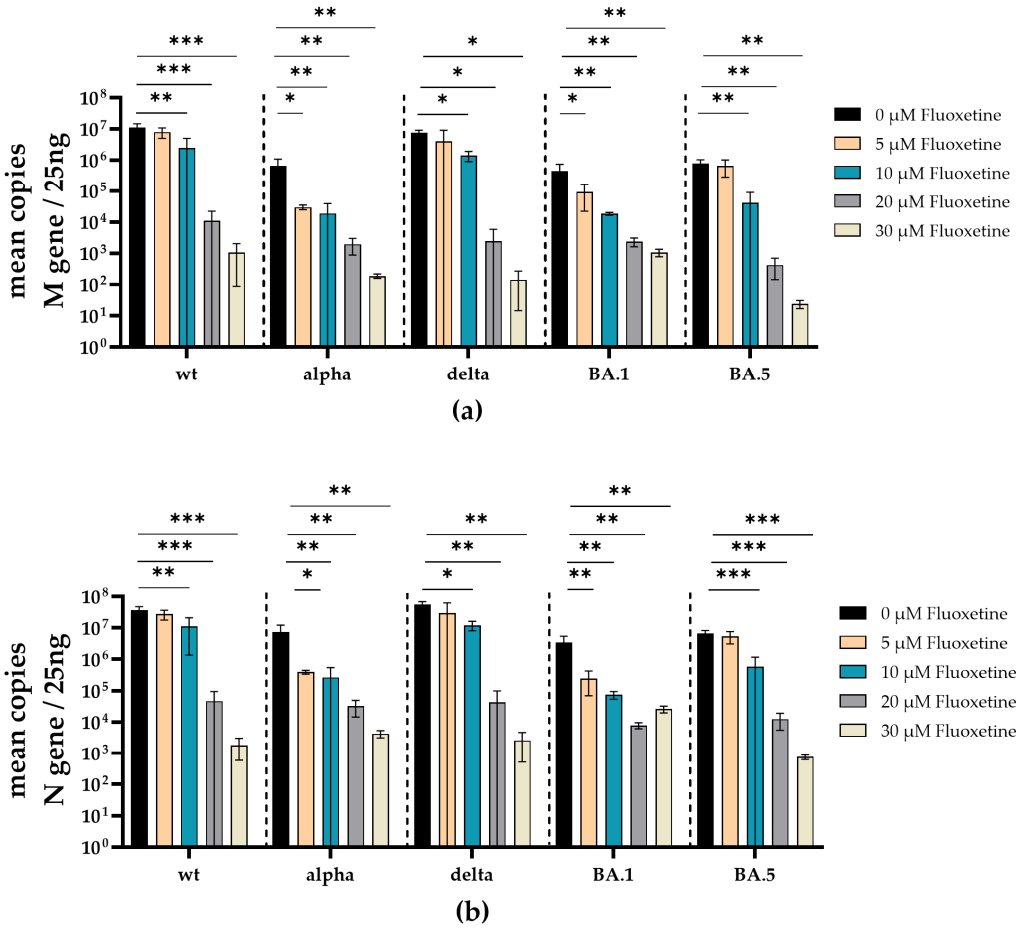

Figure 5. Treatment with fluoxetine significantly reduces SARS-CoV-2 M- and N-gene levels. A549-AT cells were infected with 100 $TCID_{50}$ of different SARS-CoV-2 variants in the presence of different concentrations of fluoxetine (0–30 µM). After incubation for 72 h, supernatants were harvested. Total RNA was isolated and reverse transcribed, and the SARS-CoV-2 M- (**a**) and N-gene (**b**) were quantified by RT-qPCR. Data are shown as mean and standard deviation (SD). Differences between fluoxetine/sertraline and the control were compared by one-way ANOVA (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Similar to the treatment with fluoxetine, the total amounts of SARS-CoV-2 M- and N-gene RNA were substantially decreased under sertraline treatment. Significant reduction of M-gene RNA was found in the supernatants of SARS-CoV-2 D614G (5 µM $p = 0.021$; 10 µM $p = 0.017$), alpha (5 µM $p = 0.0052$; 10 µM $p = 0.0030$), delta (5 µM $p = 0.0028$; 10 µM $p = 0.0028$) and omicron BA.5 (5 µM $p = 0.0173$; 10 µM $p = 0.0170$) infected and sertraline-treated cells. SARS-CoV-2 N-gene RNA levels of omicron BA.1 were reduced by several log levels but did not reach a significant level (Figure 6a). Furthermore, a significant decrease in N-gene RNA was found in cell culture supernatants from SARS-CoV-2 D614G (5 µM $p = 0.027$; 10 µM $p = 0.022$), alpha (5 µM $p = 0.0040$; 10 µM $p = 0.0022$), delta (5 µM $p = 0.0016$; 10 µM $p = 0.0016$) and omicron BA.5 (5 µM $p = 0.0209$; 10 µM $p = 0.0205$) infected cells treated with sertraline. Moreover, SARS-CoV-2 N-gene RNA levels of omicron BA.1 were reduced by several log levels but did not reach a significant level (Figure 6b).

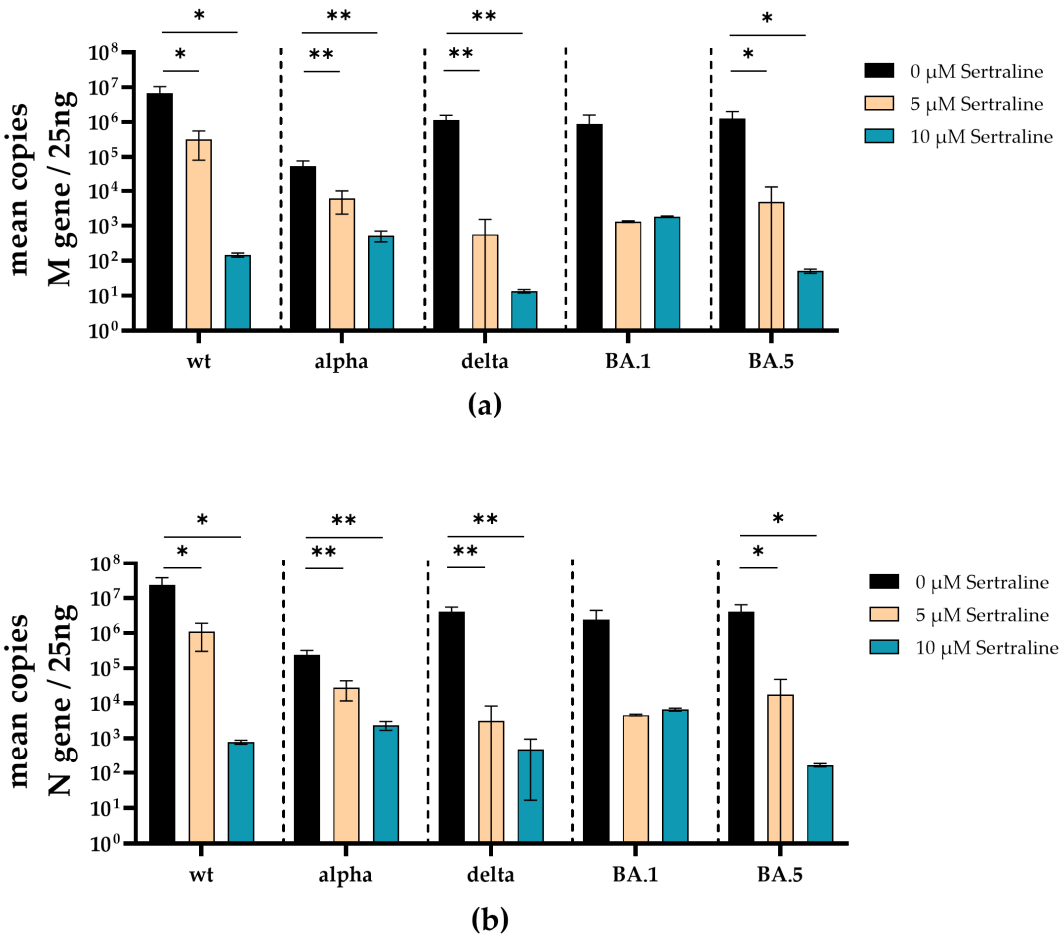

Figure 6. Treatment with sertraline significantly reduces SARS-CoV-2 M- and N-gene levels. A549-AT cells were infected with 100 $TCID_{50}$ of different SARS-CoV-2 variants in the presence of different sertraline concentrations (0–10 µM). After 72 h of incubation, the supernatants were harvested and the total RNA was isolated and reverse transcribed, and the M- (**a**) and N-gene (**b**) of SARS-CoV-2 were quantified by RT-qPCR. Data are shown as mean and standard deviation (SD). Differences between fluoxetine/sertraline and the control were compared by one-way ANOVA (* $p < 0.05$; ** $p < 0.01$).

3.3. Fluoxetine and Sertraline Showed No Toxic Effects at SARS-CoV-2 Neutralizing Concentrations

Potential cytotoxic effects of the antidepressants fluoxetine and sertraline were analyzed by determining cell viability. Therefore, A549-AT cells were incubated with different concentrations of fluoxetine (0–30 µM) or sertraline (0–10 µM) and tested for viability after 2, 24, 48 and 72 h of incubation at 37 °C and 5% CO_2. After 72 h, no substantial toxicity could be detected even if the cells were exposed to the highest fluoxetine (30 µM) or sertraline (10 µM) concentrations. Thus, fluoxetine and sertraline potently neutralize SARS-CoV-2 D614G and the variants alpha, delta, omicron BA.1 and omicron BA5 at subtoxic concentrations (Figure 7).

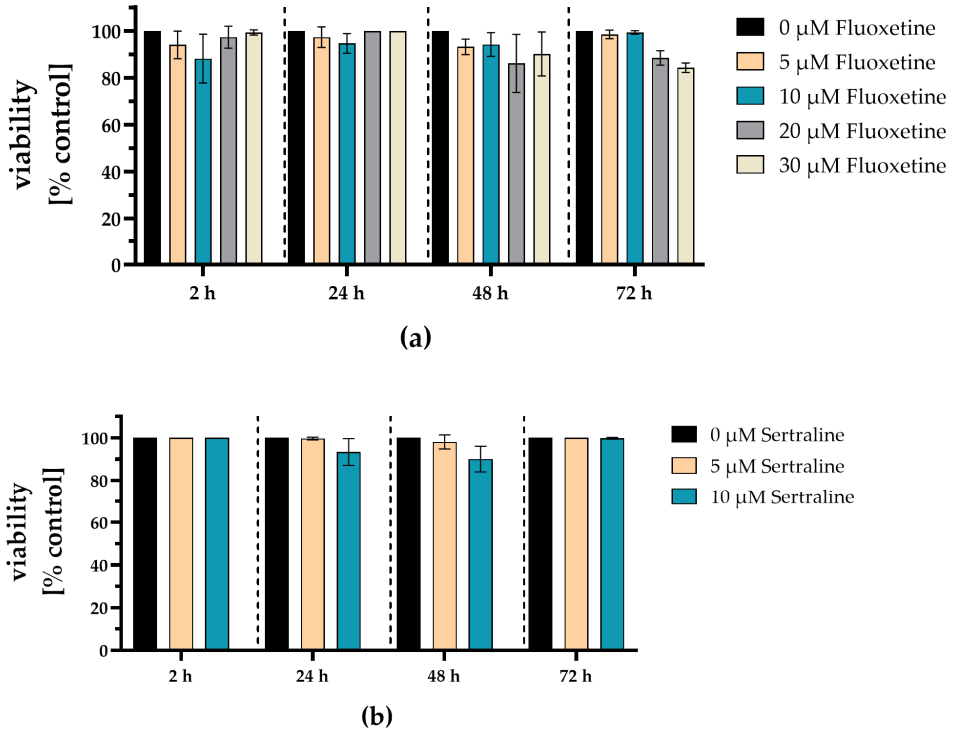

Figure 7. Fluoxetine and sertraline showed no toxic effects on A549-AT cells. Potential cytotoxic effects of fluoxetine or sertraline were tested towards A549-AT cells using "Orangu cell counting solution". Different concentrations of (**a**) fluoxetine or (**b**) sertraline were incubated with a confluent layer of A549-AT cells and the cell viability was evaluated after 2, 24, 48 and 72 h. All experiments were performed as triplicate. Data are shown as mean and standard deviation (SD). Differences between fluoxetine/sertraline and the control were compared by one-way ANOVA.

4. Discussion

After three years of the pandemic, SARS-CoV-2 remains a global health burden marked by the persistence of severe courses of disease with substantial numbers of hospitalizations and deaths. Furthermore, in many cases, there are long-lasting symptoms, known as long-COVID-19. With increasing vaccination fatigue and a growing number of vaccine-breakthrough infections, there is an urgent need for rapidly available drugs to treat or prevent SARS-CoV-2 infections, in particular such with newly emerging variants. Various antidepressants were described as potent entry inhibitors of early-emerged SARS-CoV-2 variants and other coronaviruses [17,18]. However, it remained unclear whether antidepressants that target the SARS-CoV-2 spike S1 domain may inhibit newly emerging SARS-CoV-2 variants despite mutations that additionally occur during SARS-CoV-2 evolution.

In the present study, we investigated the antiviral efficacy of fluoxetine and sertraline against distinct SARS-CoV-2 variants, including D614G, alpha, delta, omicron BA.1 and omicron BA.5. As a first step, the ability of fluoxetine and sertraline to inhibit SARS-CoV-2 infection was examined by using VSV*ΔG-Fluc spike pseudotyped virus-like particles with mutations within the RBD or the full spike S1 domain. This method offers the advantage of rapid testing of active substances against different SARS-CoV-2 variants without the need for an S3 laboratory [35,36]. In line with previous studies, fluoxetine and sertraline effectively blocked the infection with VSV*ΔG-Fluc spike pseudotyped virus-like particles [7,37]. The antiviral activity of fluoxetine and sertraline was shown for the first time on this broad variety of variants.

Notably, fluoxetine and sertraline completely neutralized the replication of all used clinical SARS-CoV-2 isolates (D614G, alpha, delta, omicron BA.1 and omicron BA.5) at a subtoxic concentration of 30 µM (fluoxetine) or 10 µM (sertraline). The neutralizing effect of these antidepressants was unaffected by the respective mutations within the SARS-CoV-2

spike protein. This is an interesting finding, as fluoxetine and sertraline were described to interfere with SARS-CoV-2 entry, and at least sertraline directly targeted the SARS-CoV-2 spike protein [7]. In addition to targeting the spike protein, fluoxetine and sertraline were described as functional inhibitors of the acid sphingomyelinase (ASM), which plays an important role during SARS-CoV-2 entry into the host cell [38,39]. ASM is a glycoprotein that catalyzes sphingomyelin degradation to phosphorylcholine and ceramide, which is known to facilitate viral entry into the host cell [40–42]. SARS-CoV-2 activates the ASM/ceramide system, resulting in the formation of ceramide-enriched membrane domains that cluster ACE2 and thereby facilitate viral entry and infection [39,40,43]. Thus, inhibiting ASM represents a SARS-CoV-2 spike mutation-independent mechanism to interfere with viral entry and infection. In the context of other viral infections, our data indicate that FIASMAs are effective not only against, for example, rhinoviruses [44] or ebolaviruses [45], but also against SARS-CoV-2. In line with prior studies, our findings suggest that fluoxetine and sertraline use in early-stage SARS-CoV-2 infections with recent variants may reduce the risk of a severe course of COVID-19 or death [46].

However, we have demonstrated the antiviral efficacy of fluoxetine and sertraline against the respective SARS-CoV-2 variants in cell culture. Nonetheless, deriving the exact dosage for humans solely from cell culture experiments is not feasible, as there are additional crucial factors such as the immune system involved in fighting the infection in humans. Determining the effective dosage for human intervention is the subject of ongoing clinical studies.

Randomized clinical trials and retrospective studies have already demonstrated that treatment with antidepressants result in a milder course of COVID-19 and a reduced mortality rate [20,23,47–50]. Furthermore, there was a lower infection rate among patients using antidepressants, indicating that fluoxetine and sertraline may have a protective effect against SARS-CoV-2 infections [51].

In conclusion, the antidepressants fluoxetine and sertraline are promising candidates for the supportive treatment of SARS-CoV-2 infections, in particular during the early phase of infection. Especially in developing countries with a restricted medical health care system, these cheap and generally available drugs may contribute to improve the treatment of COVID-19 patients. Since their antiviral efficacy is unaffected by SARS-CoV-2 spike mutations, they may be used in new or even breakthrough infections with newly emerging SARS-CoV-2 variants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v16040545/s1, Table S1: Primers used for mutagenesis and sequencing; Table S2: Mutations in respective plasmids and corresponding SARS-CoV-2 variant; Table S3: M- and N-gene-specific primers used for RT-qPCR.

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

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3.6 COVID-19 in Elderly, Immunocompromised or Diabetic Patients- From Immune Monitoring to Clinical Management in the Hospital

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Adalbert Krawczyk, Hana Rohn, Sarah Jansen und Korbinian Wünsch haben das Konzept dieser Studie erstellt. Die Experimente wurden von Sarah Jansen, Korbinian Wünsch und Laura Thümmler durchgeführt. Sarah Jansen, Mira Alt und Korbinian Wünsch haben die Daten analysiert und statistisch ausgewertet. Das Manuskript wurde von Sarah Jansen und Korbinian Wünsch erstellt und von Hana Rohn, Adalbert Krawczyk und Rabea Julia Madel überarbeitet.

Laura Thümmler	Prof. Dr. med. Monika Lindemann

viruses

MDPI

Article

COVID-19 in Elderly, Immunocompromised or Diabetic Patients—From Immune Monitoring to Clinical Management in the Hospital

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Abstract: The novel, highly transmissible severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has triggered a pandemic of acute respiratory illness worldwide and remains a huge threat to the healthcare system's capacity to respond to COVID-19. Elderly and immunocompromised patients are at increased risk for a severe course of COVID-19. These high-risk groups have been identified as developing diminished humoral and cellular immune responses. Notably, SARS-CoV-2 RNA remains detectable in nasopharyngeal swabs of these patients for a prolonged period of time. These factors complicate the clinical management of these vulnerable patient groups. To date, there are no well-defined guidelines for an appropriate duration of isolation for elderly and immunocompromised patients, especially in hospitals or nursing homes. The aim of the present study was to characterize at-risk patient cohorts capable of producing a replication-competent virus over an extended period after symptomatic COVID-19, and to investigate the humoral and cellular immune responses and infectivity to provide a better basis for future clinical management. In our cohort, the rate of positive viral cultures and the sensitivity of SARS-CoV-2 antigen tests correlated with higher viral loads. Elderly patients and patients with diabetes mellitus had adequate cellular and humoral immune responses to SARS-CoV-2 infection, while immunocompromised patients had reduced humoral and cellular immune responses. Our patient cohort was hospitalized for longer compared with previously published cohorts. Longer hospitalization was associated with a high number of nosocomial infections, representing a potential hazard for additional complications to patients. Most importantly, regardless of positive SARS-CoV-2 RNA detection, no virus was culturable beyond a cycle threshold (ct) value of 33 in the majority of samples. Our data clearly indicate that elderly and diabetic patients develop a robust immune response to SARS-CoV-2 and may be safely de-isolated at a ct value of more than 35.

Keywords: SARS-CoV-2; COVID-19; comorbidities; immunocompromised; diabetes mellitus; elderly patients

1. Introduction

Coronavirus disease (COVID-19) is an ongoing global threat with more than 200 million confirmed cases and over 5 million deaths worldwide [1]. In December 2020, the number of COVID-19 patients rose dramatically to occupy 82% of the intensive care (ICU) beds in Germany [2]. The alpha variant was predominant in Germany until May 2021 [3]. One year later, global health systems continue to face a shortage of medical resources [4]. Despite the available and efficient vaccines, COVID-19 remains a major challenge for healthcare systems, requiring a massive number of medical resources. As a consequence of the spread of the even more contagious Omicron variant, the number of SARS-CoV-2 infections is rising again, and the occupancy of ICU beds is reaching 88%. The majority of patients requiring intensive care are over 60 years of age (more than 60%) [5].

Given the current evolution of the pandemic, it is more urgent than ever to provide appropriate treatment to all hospitalized COVID-19 patients. In Germany, isolation after recovery is recommended for at least 14 days [6,7]. The clinical management of COVID-19 patients requiring outpatient care or mobile care services after the acute phase of the disease, such as elderly patients or patients with comorbidities, remains challenging. End-of-isolation measures for these patients requires a negative SARS-CoV-2 polymerase chain reaction (PCR) test result or, at least, a cycle threshold (ct) value over 30. However, although most of these patients recover from infection, many remain SARS-CoV-2 PCR-positive for an extended period. Thus, they continue to receive inpatient treatment to minimize the risk of SARS-CoV-2 transmission when released to outpatient care facilities. Age over 60 years has been identified as a major risk factor for severe COVID-19 [8]. Further known comorbidities associated with severe COVID-19 include immunocompromisation [9] and diabetes mellitus (DM) [10–12]. Of note, patients with severe COVID-19 may exhibit infectious viruses and often have high ct values for a longer period than patients with a moderate COVID-19 infection [13], thus further limiting the capacity of the health care system. This results in a high number of patients for whom there are no clearly defined guidelines for the duration of isolation.

Viral excretion kinetics are less well-studied in very elderly patients with pre-existing conditions. To assess the potential risk of infection in recovered but still SARS-CoV-2 RNA-positive "at-risk" patients, we examined the cellular and humoral immune responses, SARS-CoV-2 RNA levels, and viral load of 79 COVID-19 patients. All patients were hospitalized at the Department of Infectious Diseases, University Hospital Essen, Germany, between December 2020 and April 2021.

2. Materials and Methods

2.1. Study Population

This prospective study included 79 patients with a polymerase chain reaction (PCR)-confirmed SARS-CoV-2 infection. Patients were hospitalized at the Department of Infectious Diseases, University Hospital Essen, Germany, between December 2020 and April 2021. Inclusion criteria were a PCR-confirmed SARS-CoV-2 infection, age of 60 years or older, immunocompromisation or diabetes mellitus, and ongoing hospitalization. A total of 56 patients manifested with severe COVID-19 and 23 patients had moderate COVID-19 at the time of enrollment. SARS-CoV-2 RNA was measured from nasopharyngeal swabs taken at the time of hospitalization and approximately 10 to 15 days after the first positive PCR test. A follow-up SARS-CoV-2 PCR was performed approximatel 7 days later, if the second SARS-CoV-2 PCR was positive and the patients were still hospitalized. Patients were classified into the following groups according to the predominant risk factors for severe COVID-19: (i) immunocompromised patients (regardless of age; mean age 63.3 years with a standard deviation (SD) of ±17.7 days), (ii) patients with diabetes mellitus (regardless of age; mean age 69.6 with an SD of ±9.9 years), and (iii) elderly patients over 60 years of age who were neither immunocompromised nor had diabetes mellitus (referred to as elderly patients in the following; mean age 73.4 with an SD of ±10.1 years). Detailed patient characteristics are shown in Table 1. Notably, none of the patients were vaccinated

against SARS-CoV-2 prior infection. Regarding the length of hospital stay, one patient was excluded from the analysis. This patient was hospitalized for more than 170 days and only a fraction of that time was due to COVID-19. The study was approved by the local ethics committee (approval numbers 20-9512-BO and 20-9225-BO) and was carried out according to the ethical standards of the Declaration of Helsinki of 1964 and its subsequent amendments or comparable ethical standards.

Table 1. Characteristics of the total patient cohort with three subgroups: elderly (60+), immunocompromised (IM) and diabetes (DM) patients. Patients were classified according to the COVID-19 category given by the European Centre for Disease Prevention and Control (ECDC) classification among all cases: hospitalization (a), severe hospitalization (b), and death (c). Comparison between sex, age, length of hospital stay in days, time of sampling, ECDC criteria, hypertension, COPD, obesity, artificial ventilation, antibody/plasma therapy, Remdesivir and dexamethason therapy of the patients was performed with an independent *t*-test. Statistical significance was set at the level of $p < 0.05$.

	Total	60+	IM	DM	*p* Value 60+/IM	*p* Value 60+/DM	*p* Value IM/DM
Number, *n*	79	20	36	23			
Sex, men/women	49/30	9/11	24/12	16/7	0.11	0.1	0.82
Age, y ± SD	67.7 ± 14.5	73.4 ± 10.1	63.3 ± 17.7	69.6 ± 9.9	0.03	0.66	0.22
Length of hospital stay, d (range)	24 (4–91)	19 (4–45)	28.6 (6–91)	21.3 (7–55)	0.38	>1	>1
Time of sampling, d ± SD	16 ± 7.7	14.4 ± 8.2	15.8 ± 7.5	17.8 ± 7.4	0.70	0.10	0.72
ECDC classification, *n*	23 a, 51 b, 5 c	9 a, 11 b, 0 c	13 a, 18 b, 5 c	1 a, 22 b, 0 c	0.2	0.003	0.15
Hypertension, *n* (%)	49 (62)	11 (55)	19 (53)	19 (83)	0.87	0.05	0.02
COPD, *n* (%)	8 (10)	2 (10)	4 (11)	2 (9)	0.9	0.88	0.76
Obesity, *n* (%)	27 (34)	6 (30)	9 (25)	12 (52)	0.64	0.14	0.03
Artificial ventilation, *n* (%)	11 (14)	1 (5)	6 (17)	4 (17)	0.21	0.21	0.94
Antibody/plasma therapy (%)	12 (15)	2 (10)	8 (22)	2 (9)	0.25	0.88	0.18
Remdesivir, *n* (%)	25 (32)	9 (45)	5 (14)	11 (48)	0.01	0.85	0.004
Dexamethason, *n* (%)	26 (33)	5 (25)	11 (31)	10 (43)	0.66	0.2	0.31
Antibiotic therapy, *n* (%)	50 (63)	10 (50)	24 (67)	16 (70)	0.23	0.2	0.82
Nosocomial infektion, *n* (%)	20 (25)	2 (10)	13 (36)	5 (23)	0.02	0.31	0.21

2.2. Cells and Virus

Vero E6 cells (American Type Culture Collection (ATCC), CRL-1586, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM Life Technologies Gibco, Darmstadt, Germany) supplemented with 10% (*v*/*v*) fetal calf serum (FCS), penicillin (100 IU/mL), and streptomycin (100 µg/mL) at 37 °C in an atmosphere of 5% CO_2. Nasopharyngeal swab specimens were titrated on Vero E6 cells within 48 h to detect infectious virus. The medium was additionally supplemented with Ciprofloxacin (10 mg/mL), and Amphotericin B (2.5 mg/mL) to avoid contamination. After 72 h, cell cultures were analyzed for cytopathic effects (CPE) by transmitted light microscopy (Carl Zeiss AG, Oberkochen, Germany). Supernatants from infected cell cultures were harvested after 7 days of infection, cleared from any cell debris by centrifugation, and stored at −80 °C. A SARS-CoV-2 clinical isolate from 04/2020 with the D614G mutation was used for the virus neutralization assays.

2.3. SARS-CoV-2 Specific Antibody Detection

SARS-CoV-2 immunoglobulin (Ig)G against the spike glycoprotein was detected using an anti-SARS-CoV-2 IgG chemiluminescence enzyme immunoassay (CLIA) (LIAISON® SARS-CoV-2 TrimericS IgG assay, Diasorin, Saluggia, Italy) according to the manufacturer's instructions. A result of ≥13.0 arbitrary Units per milliliter (AU/mL) was considered positive and a score of <13 AU/mL was considered negative. The upper limit of quantification was 800 AU/mL. The conversion factor for the results in AU/mL to Binding Antibody Units (BAU)/mL, which correlates with the WHO International Standard for COVID-19, was 2.6 [14,15].

2.4. Virus Neutralization Assay

The neutralizing capacity of serum samples was determined by a standard end-point dilution assay, as previously described [16]. In brief, 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. The mixtures were added to confluent Vero E6 cells cultured on 96-well microtiter plates supplemented with 10% FCS, penicillin (100 IU/mL) and streptomycin (100 µg/mL). To detect cytopathic effects (CPEs) via light microscopy, the cell cultures were stained with crystal violet (Roth, Karlsruhe, Germany) and dissolved in 20% methanol (Merck, Darmstadt, Germany) after 3 days of incubation. The neutralization titer was defined as the reciprocal of the highest serum dilution at which none of the triplicates showed signs of CPEs.

2.5. ELISpot Assay for SARS-CoV-2 S and M Proteins

SARS-CoV-2 specific T-cell responses were determined by interferon gamma (IFN-γ) enzyme-linked immunospot (ELISpot) assay using synthetic peptide pools of the SARS-CoV-2 spike (S1/S2) and membrane (M) protein (600 pmol/mL of each peptide; PepTivator®, Miltenyi Biotec, Bergisch Gladbach, Germany). The peptide pools mainly consisted of 15-mer sequences with 11 overlapping amino acids. In total, 250,000 mononuclear cells isolated from patient blood were incubated with the respective peptides and added onto anti-interferon-gamma-coated strip assay plates (Merck Millipore Ltd., Tullagreen, Ireland). After 19 h of incubation, substrate solution (Oxford Immunotec, Oxford, UK) was added and spots were analyzed by an ELISpot reader (AID Fluorospot, Autoimmun Diagnostika GmbH, Strassberg, Germany). Mean values of duplicate samples were considered and a total of at least three spots after subtraction of unstimulated (background) spots (spot increment) was defined as positive [17].

2.6. SARS-CoV-2 Specific Antigen Test

The quantification of SARS-CoV-2 nucleocapsid (N) antigen protein was performed with the same nasopharyngeal swabs as used for PCR testing and viral culture. SARS-CoV-2 N antigen was detected using the CLIA-based LIAISON® SARS-CoV-2 antigen assay (DiaSorin, Saluggia, Italy) according to the manufacturer's instructions. The assay has an analytical sensitivity of 22.0 $TCID_{50}/_{mL}$ [18,19].

2.7. Flow Cytometry

To assess T-cell, B-cell, and Natural Killer (NK) cell counts, a flow cytometry characterization was performed. The samples were hemolyzed and stabilized accordingly (Coulter Immunoprep, Beckman Coulter, Brea, CA, USA). Monoclonal antibodies were each conjugated to one of the following monochromes: PC5 (R Phycoerythrincyanin 5.1), Phycoerithrin (PE), Phycoerythrin—Texas Red-X (ECD) or Fluorescein isothiocyanate (FITC). The cells were stained for CD3 (mouse IgG1, PC5), CD4 (mouse IgG1, PE), CD19 (mouse IgG1, ECD), CD16 (mouse IgG1, PE) and CD56 (mouse IgG1, PE) surface proteins. Fluorescent microbeads (Flow-Count Fluorospheres, Beckman Coulter) were added to determine cell counts on a flow cytometer (Cytomitcs FC 500, Beckman Coulter).

2.8. SARS-CoV-2 PCR

Two different SARS-CoV-2 RT-PCR kits were used. The RealStar® SARS-CoV-2 RT-PCR kit (Altona Diagnostics, Hamburg, Germany) with a detection limit of 625 copies/mL which targets S (spike) and E (envelope) genes of SARS-CoV-2. The Alinity m SARS-CoV-2 Assay (Abbott Laboratories, Chicago, IL, USA), which targets RNA-dependent RNA polymerase (RdRp) and nucleocapsid (N) genes, with a detection limit of 50 copies/mL, was also used. Samples in which SARS-CoV-2 could not be detected up to a cycle threshold (ct) of 43 were considered negative. Both tests were performed according to manufacturer's instructions [19,20].

2.9. Statistics and Data Analysis

Statistical analyses were performed with IBM SPSS-Statistic 27 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) software. Datasets were analyzed with the independent *t*-test, Mann–Whitney test, Brown–Forsythe test, Welch ANOVA, Kruskal–Wallis test, Kaplan–Meier estimator and Spearman's rank correlation coefficient. Univariate linear regression models were used to estimate relationships between the independent variables. p values of ≤ 0.05 were marked as statistically significant with "*", p values of ≤ 0.01 were marked with "**" and p values of ≤ 0.001 were marked with "***". Correlation analyses were run for $CD19^+$ B-cell numbers, SARS-CoV-2 neutralizing antibody titers, SARS-CoV-2 IgG ELISA antibody titers and IFNγ ELISpot increment for the spike protein (S1/S2), independent of whether patients had diabetes mellitus or an immunocompromisation.

3. Results

Among others, age, immunodeficiency, and pre-existing diseases such as diabetes mellitus are known risk factors for a severe course of COVID-19. Accordingly, the clinical management of these vulnerable patient groups is challenging in terms of deisolation and transfer to outpatient medical care to warrant optimal primary healthcare. To understand the immunologic and virologic status (viral shedding) of these patients, we determined the humoral and cellular immune response of 79 hospitalized elderly ≥60 years old (n = 20), immunocompromised (n = 36) and diabetic (n = 23) unvaccinated COVID-19 patients and monitored them for SARS-CoV-2 RNA and virus shedding for up to 90 days upon hospitalization. SARS-CoV-2 RNA and viral loads were determined immediately after hospitalization, around days 10–15, and during follow-up (Figure 1). Blood samples for immunomonitoring and assessment of cellular and humoral immune responses were acquired at about 10–15 days after the first positive SARS-CoV-2-PCR result. If still hospitalized and with persistently positive SARS-CoV-2 RNA following the second PCR test, SARS-CoV-2 RNA was quantified again one week later. Figure 1 illustrates an overview of the study design.

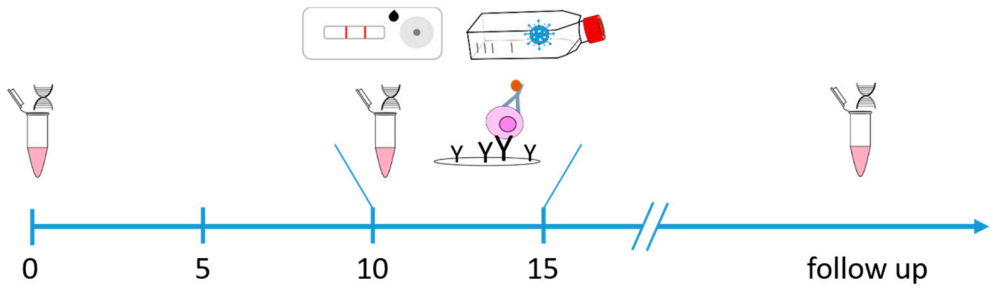

Figure 1. Study design. 79 patients were enrolled in the study; all of them had positive SARS-CoV-2 RT-PCR and were unvaccinated against SARS-CoV-2. Blood was drawn and nasopharyngeal swabs were taken approximately 10–15 days later to perform an antigen test, virus cultivation, an ELISpot assay and SARS-CoV-2 RT-PCR. Given the fact that the second SARS-CoV-2 PCR was positive, a follow-up PCR was performed around 7 days later.

3.1. Clinical Data

The characteristics of the patient cohorts investigated in the present study are summarized in Table 1. According to the risk factors for prolonged viral shedding and severe COVID-19, patients were divided into three subgroups: elderly patients (60 years or older; 60+), diabetes mellitus patients (DM) and immunocompromised patients (IM).

In total, 20 patients were over 60 years old and did not have immunodeficiency or diabetes mellitus. Thirty-six patients were immunocompromised (12 patients with malignant diseases, 8 solid organ transplant recipients (SOT), 4 patients with liver cirrhosis, 3 patients with permanent kidney replacement therapy, 3 patients with end-stage heart

failure, 2 pregnant patients, 1 SOT and permanent kidney replacement therapy, 1 patient with Crohn's disease, 1 bone marrow transplant recipient, 1 patient with chronic kidney disease). In this group, eight patients had a history of diabetes mellitus. Twenty-three patients suffered from diabetes mellitus without being immunocompromised.

There was a significant difference between the three patient groups regarding age ($p = 0.03$), severity of COVID-19 ($p = 0.003$), presence of the concomitant diseases hypertension ($p = 0.02$) and obesity ($p = 0.03$), therapy with Remdesivir ($p = 0.004$), and the number of nosocomial infections ($p = 0.02$; Table 1). Patients in the immunocompromised group were significantly younger than those in the 60+ group and were significantly less likely to suffer from hypertension than the diabetes mellitus group.

Importantly, immunocompromised patients had a significantly higher mortality and risk of severe COVID-19 when compared to elderly or diabetic patients. There were significantly more cases of severe COVID-19 among diabetes mellitus patients when compared to the elderly patients. COVID-19 treatment was conducted in accordance with international and national guidelines. In COVID-19 with low-dose oxygen requirements, antiviral therapy with Remdesivir was administered and, in case of further respiratory deterioration or in the later COVID-19 course, with Dexamethasone. With respect to therapy, there were significant differences between the patient groups. Remdesivir was used significantly less frequently in immunocompromised patients despite the often severe COVID-19 course due to impaired renal or hepatic function. Instead, therapy with Dexamethasone was conducted when necessary. Notably, immunocompromised patients showed significant higher rates of nosocomial infections than elderly and diabetes mellitus patients. In total, 13 of 20 patients with a nosocomial infection were in the immunocompromised group. Beyond that, no significant clinical differences could be detected between the three groups.

3.2. Immunomonitoring

3.2.1. Cellular Immune Status

The cellular immune status was examined in 45 patients during hospitalization (Figure 2). Of these, 12 patients were elderly patients (60+), 19 patients were immunocompromised, and 14 patients suffered from diabetes mellitus without being additionally immunocompromised. Immunocompromised patients had significantly fewer $CD3^+$ T-cells and $CD3^+CD4^+$ T-cells compared to elderly patients. Immunocompromised patients also had significantly fewer $CD3^+CD4^+$ T-cells than diabetes mellitus patients and fewer $CD3^-$, $CD16/56^+$ natural killer (NK) cells compared to elderly patients. No significant differences in terms of $CD19^+$ B-cells were detected between the three subgroups (Figure 2).

3.2.2. Cellular Immunity

To determine the extent of SARS-CoV-2-specific T-cell responses to SARS-CoV-2 spike (S1/S2) and membrane proteins (M), IFNγ-ELISpot analyses were performed from 51 blood samples. Therefore, peripheral blood mononuclear cells (PBMCs) isolated from 12 elderly patients, 25 immunocompromised patients, and 14 diabetes mellitus patients were stimulated with peptide pools of the SARS-CoV-2 spike (S1/S2) or membrane (M) protein. Subsequently, the cellular immune response was quantified by assessing the number of IFNγ-positive spots. T-cell responses of elderly, immunocompromised and diabetes mellitus patients were compared with the T-cell responses of a convalescent group. The convalescent group consisted of 20 younger convalescents with a mean age of 41 years (range 19 + 58 years) and a mean time of sampling after PCR-confirmed SARS-CoV-2 infection of 64 days (range 24–84 days). None of these convalescents were hospitalized due to SARS-CoV-2. In total, 40 patients showed a T-cell response to the S1/S2 peptide pool and 44 patients showed a T-cell response to the M peptide pool in the SARS-CoV-2-IFNγ-ELISpot assay. There was no detectable S1/S2 and M protein specific cellular immune response in five patients with immunosuppression.

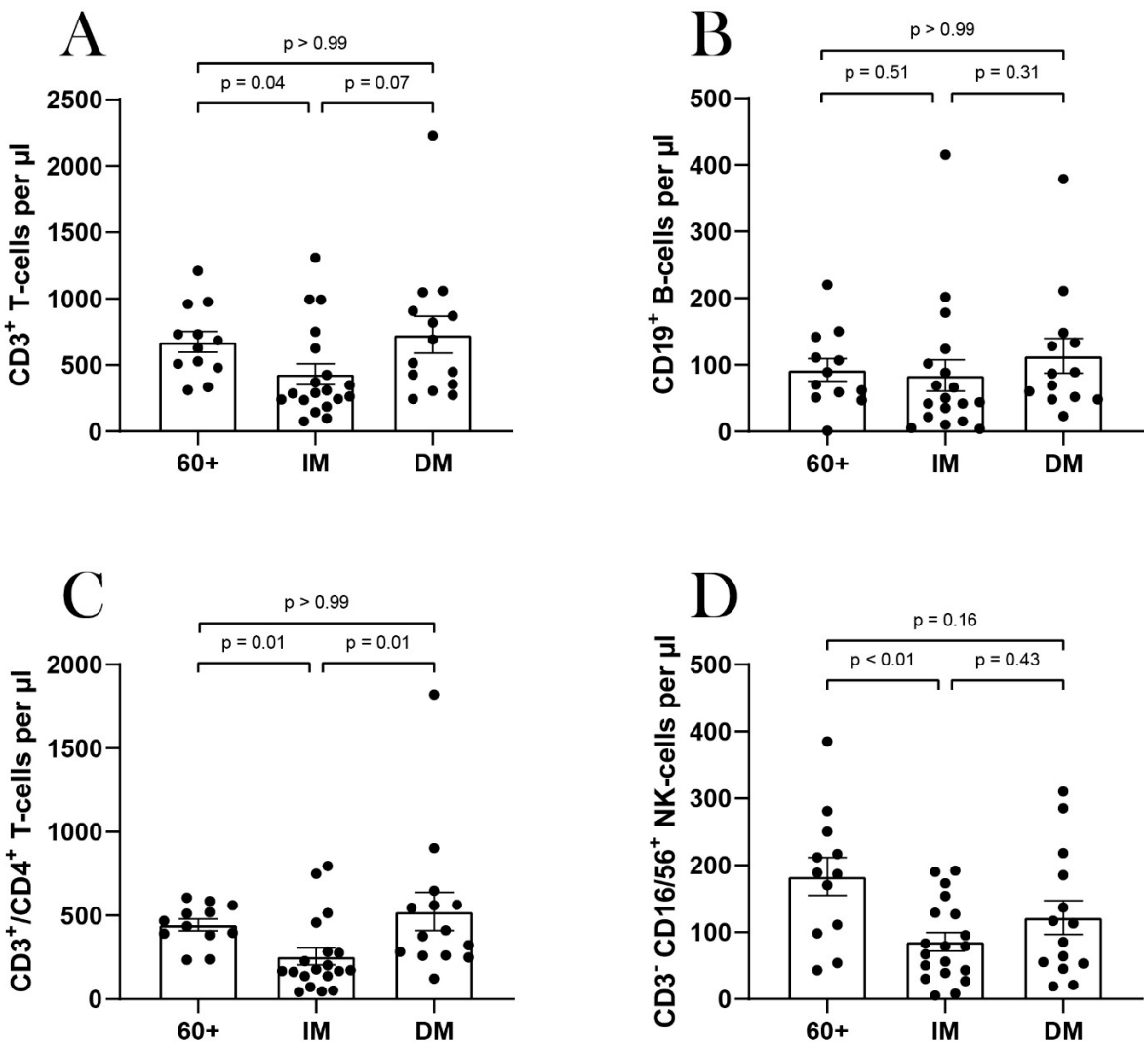

Figure 2. Cellular immune status of elderly (60+), immunocompromised (IM) and diabetes mellitus (DM) patients: Total numbers of $CD3^+$ cells (**A**); $CD19^+$ B-cells (**B**), $CD3^-$, $CD16/56^+$ cells (**C**) and $CD3^+$, $CD4^+$ T-cells (**D**) were determined for 45 patients. Each dot (•) represents one patient. A comparison between the groups was performed with the Mann–Whitney test. Statistical significance was set at the level of $p < 0.05$.

SARS-CoV-2-specific T-cell responses were significantly lower in immunocompromised patients compared to elderly 60+ patients regarding both the S1/S2 and the M peptide pool. No significant difference could be found in the cellular immune response for the convalescent and 60+, IM or DM patients (Figure 3A,B).

3.2.3. Humoral Immunity

The humoral immune response was assessed by determining SARS-CoV-2 IgG ELISA titers and neutralizing antibody titers. SARS-CoV-2 IgG ELISA titers were assessed in 70 out of 79 patients. A total of 9 out of 70 patients received anti-SARS-CoV-2 antibodies or convalescent plasma therapy and were excluded from this analysis. One patient received plasmapheresis due to concomitant disease before sampling and was also excluded. The SARS-CoV-2 IgG ELISA titers were determined in sera from 13 elderly (60+), 26 immunocompromised (IM), and 21 diabetes mellitus (DM) patients.

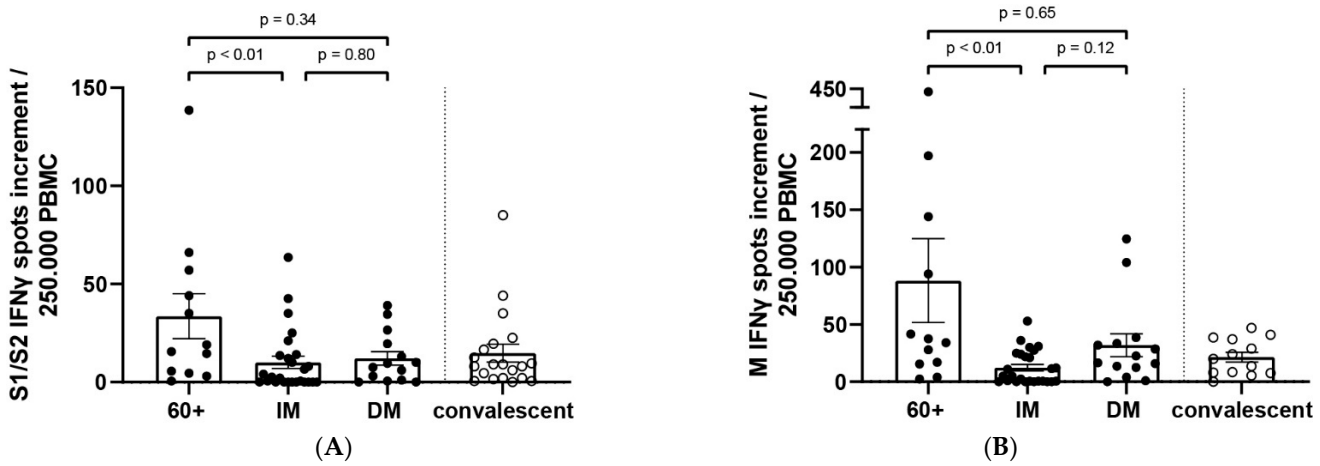

Figure 3. T-cell responses of elderly (60+), immunocompromised (IM) and diabetes mellitus (DM) patients compared to a convalescent cohort: S1/S2-protein-specific T-cell responses (**A**) and M protein specific T-cell responses (**B**) for 51 patients and 20 convalescent was determined via ELISpot assay. Each dot (●) represents one patient and each hollow dot (○) represents one convalescent. Comparison between the groups was performed with the Mann–Whitney test. Statistical significance was set at the level of $p < 0.05$.

Overall, in 16 patients, no SARS-CoV-2 IgG ELISA antibody response was detected. Most of these patients were immunocompromised ($n = 11$), but 3 elderly and 2 diabetic patients also did not show an antibody response. The patients that did not show SARS-CoV-2-neutralizing antibodies also had no measurable cellular immune response.

Thus, only about 73% of the total patient cohort ($n = 44$) had detectable SARS-CoV-2 IgG levels and immunocompromised patients showed significantly lower levels than diabetes mellitus patients. Figure 4 provides an overview of the SARS-CoV-2 IgG ELISA distribution in each group.

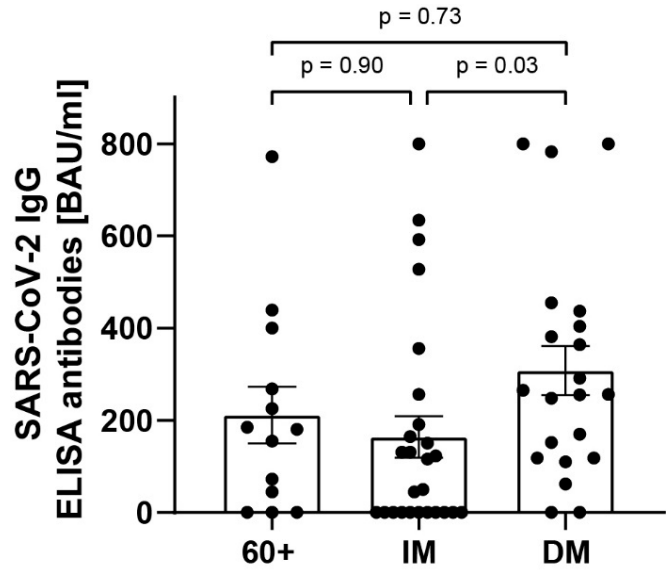

Figure 4. SARS-CoV-2 IgG ELISA levels of elderly (60+), immunocompromised (IM) and diabetes mellitus (DM) patients. SARS-CoV-2 IgG ELISA levels of 60 patients were determined via antibody assay. Each dot (●) represents one patient. Comparison between the groups was performed with the Kruskal–Wallis test. Statistical significance was set at the level of $p < 0.05$.

Neutralizing SARS-CoV-2 antibodies were evaluated in 71 out of 79 patients. A total of 15 patients were elderly, 33 were immunocompromised and 23 were diabetic patients. A total of 9 out of 71 patients received anti-SARS-CoV-2 antibodies or convalescent plasma therapy and were, therefore, excluded from this analysis. The reference group consisted of the same convalescent patients as the group for cellular immunity, excluding one patient that did not give a sample for the evaluation of neutralizing SARS-CoV-2 antibodies. A total of 13 elderly, 23 immunocompromised, 20 diabetic patients, and 16 convalescents had detectable SARS-CoV-2-neutralizing antibodies (Figure 5).

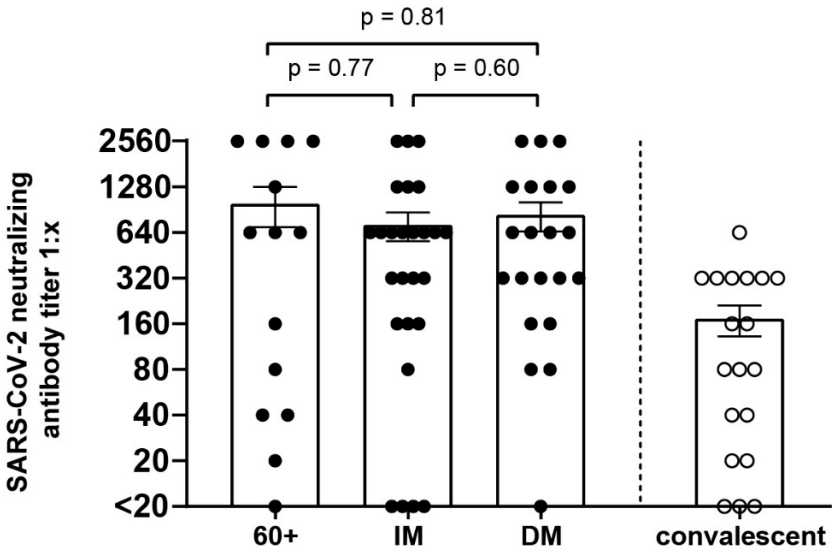

Figure 5. SARS-CoV-2-eutralizing antibody titers of elderly (60+), immunocompromised (IM) and diabetes mellitus (DM) patients compared with a group of convalescents: Virus neutralization assays were performed for 62 patients and 19 convalescent donors. Each dot (●) represents one patient. (○) represents one convalescent. Comparison between the groups was performed with the Mann–Whitney test. Statistical significance was set at the level of $p < 0.05$.

3.3. Correlation Analyses

To better understand the associations between different aspects of the humoral and cellular immune response, correlation analyses were performed. Our data indicated a significant correlation between SARS-CoV-2-neutralizing antibody titers and CD19+ B-cell numbers ($p = 0.01$, r2 = 0.16, $n = 39$). There was no correlation between SARS-CoV-2 IgG ELISA antibody titers and CD19+ B-cell numbers ($p = 0.26$, r2 = 0.06, $n = 22$). There was also no significant correlation between SARS-CoV-2-neutralizing antibody titers and SARS-CoV-2 IgG ELISA antibody titers ($p = 0.18$, r2 = 0.04, $n = 42$) or IFNγ ELISpot increment (S1/S2) ($p = 0.11$, r2 = 0.06, $n = 47$; Figure 6A–D).

3.4. Nasopharyngeal Sampling

Nasopharyngeal swabs were taken about 10–15 days after the first positive SARS-CoV-2 PCR test. This swab was used to perform a SARS-CoV-2 antigen test, SARS-CoV-2 RT-PCR and virus cultivation to determine if replication-competent virus was still present in the elderly, immunocompromised and diabetes mellitus patients after symptomatic COVID-19 over an extended period.

A total of 64 samples were tested for SARS-CoV-2 antigen, and SARS-CoV-2 was detectable in 9 out of 29 immunocompromised patients. This could not be detected in the 17 swabs of elderly patients or in the 18 swabs of diabetic patients. All nine samples with detectable SARS-CoV-2 antigen had a ct-value of 25 or lower.

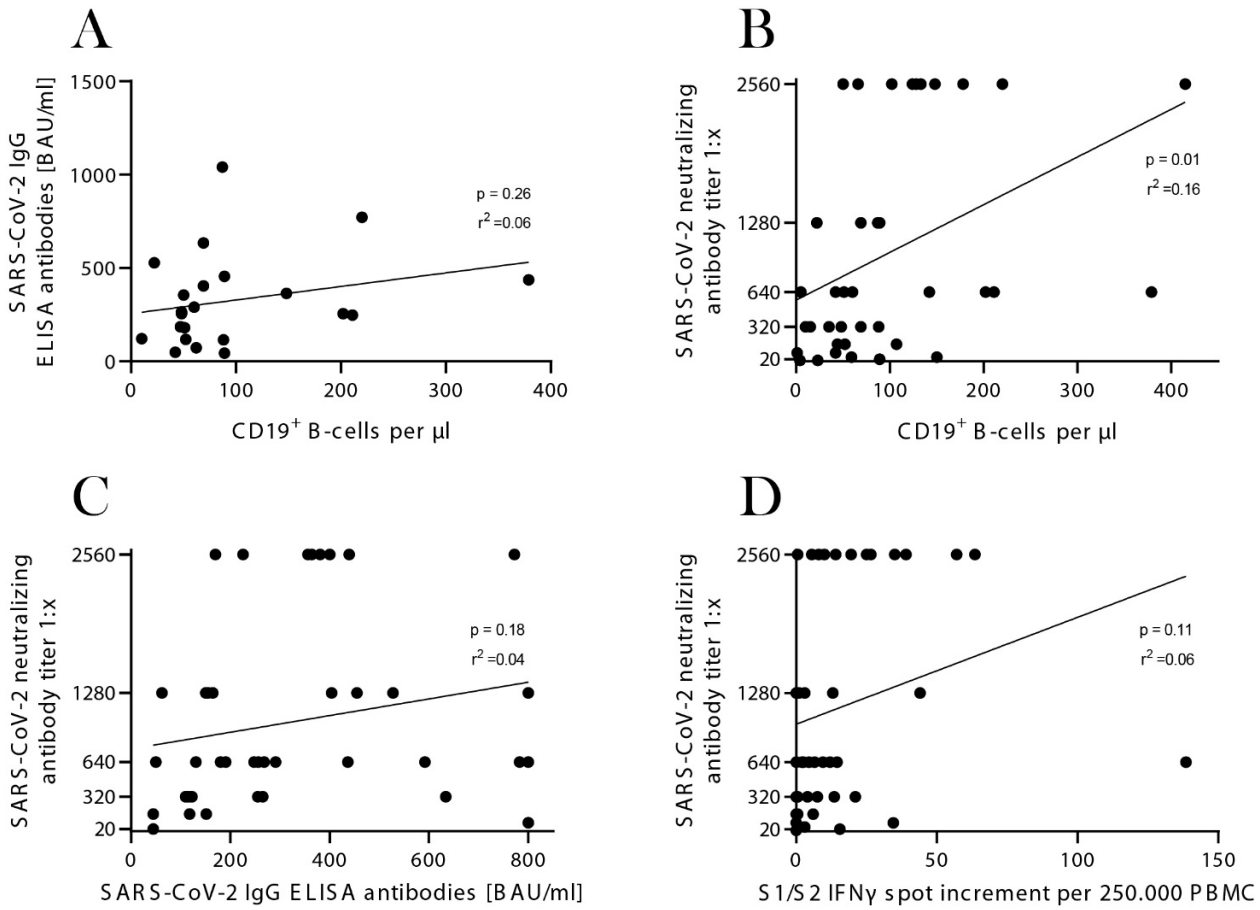

Figure 6. Correlation analyses for SARS-CoV-2 IgG ELISA antibody titers and CD19+ B-cell numbers (**A**), SARS-CoV-2 neutralizing antibody titers and CD19+ B-cell numbers (**B**), SARS-CoV-2 neutralizing antibody titers and SARS-CoV-2 IgG ELISA antibody titers (**C**) and SARS-CoV-2 neutralizing antibody titers and IFNγ ELISpot increment for the spike (S1/S2) protein (**D**). Each dot (•) represents one patient.

Nasopharyngeal swabs from 79 patients were tested via SARS-CoV-2 PCR. In total, 16/20 elderly patients (80%) showed SARS-CoV-2 RNA with a mean cycle threshold of 33. A total of 29/36 samples (80.65%) from immunocompromised patients were PCR-positive with a mean ct of 30 and 11/23 samples (47.83%) from diabetes mellitus patients were PCR-positive with a mean ct of 32. Most of these patients were deisolated and discharged from the hospital after consultation with the local health department and/or the Institute for Hospital Hygiene. However, 24 out of 56 patients showing positive PCR results were still hospitalized one week later. In a follow-up SARS-CoV-2 PCR testing, 3/5 elderly, 9/14 immunocompromised and 2/5 diabetes mellitus patients out of those 24 patients continued to be PCR-positive.

A total number of 39 nasopharyngeal swabs were tested in cell culture for infectious virus. Nine of these nasopharyngeal swabs were obtained from elderly patients, 20 from immunocompromised and 10 from diabetes mellitus patients. Plaque formation was observed in four samples, all with a ct-value below 33. A positive SARS-CoV-2 viral culture was obtained in four patients (Figure 7). Three out of those four patients were immunocompromised and one was an elderly patient. Virus could be cultivated in 1 out of 20 samples (5%) with a ct-value of 30 to 43 and in 3 out of 8 samples (37.5%) with a ct-value below 30.

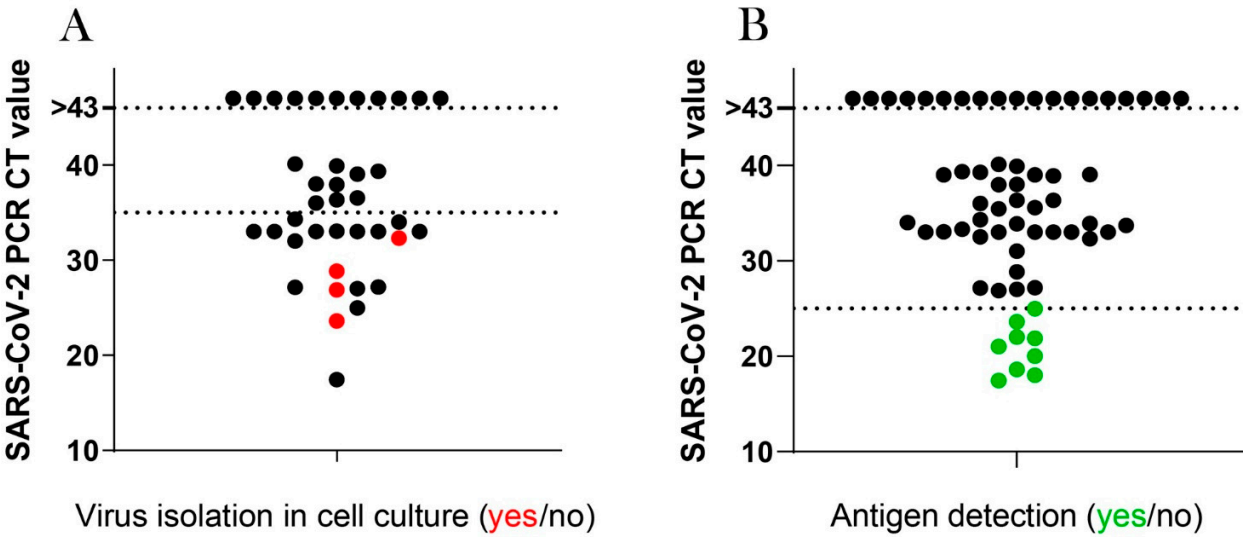

Figure 7. Correlation analyses for SARS-CoV-2 PCR ct-values and virus cultivation (A) or antigen detection (B). Nasopharyngeal swabs from 79 COVID-19 patients were tested for SARS-CoV-2 RNA via PCR. Additionally, 39 samples of patients with a positive SARS-CoV-2 PCR were inoculated on Vero E6 cells to detect infectious virus (**A**) and 64 samples were tested for SARS-CoV-2 N antigen (**B**). Red dots (•) in (**A**) indicate samples with cultivatable SARS-CoV-2 and green dots (•) in (**B**) indicate samples with positive SARS-CoV-2 antigen test.

A total of 24 patients received a follow-up nasopharyngeal swab, which was conducted around 7 days after the first virus cultivation. In four of those swabs, SARS-CoV-2 was isolated via PCR, and in two of those swabs, SARS-CoV-2 was cultivatable in vitro.

3.5. Clinical Outcome

The duration of hospitalization for elderly, immunocompromised and diabetes mellitus patients was compared. Mean length of hospitalization was 24 days (range 4–91 days) for the total patient cohort. On average, elderly patients were hospitalized for 19 days (range 4–45 d), immunocompromised patients for 28.6 days (range 9–91 d) and diabetes mellitus patients for 21.3 days (range 7–55 d) (Figure 8). Of note, some immunocompromised patients were hospitalized for more than 50 days; however, the groups did not differ significantly.

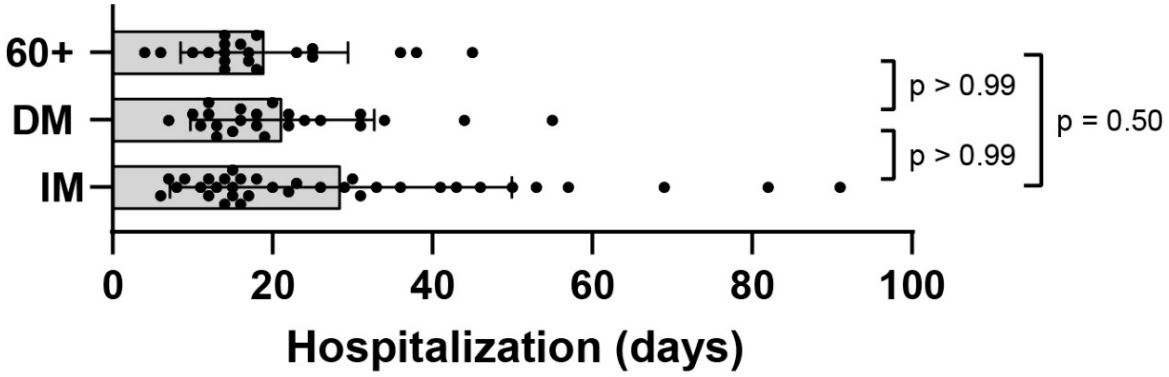

Figure 8. Duration of hospitalization of elderly (60+), immunocompromised (IM) and diabetes mellitus (DM) patients. Days in hospital for a cohort of 78 patients. Each dot (•) represents one patient. Brown–Forsythe test and Welch ANOVA were used as statistical tests. Statistical significance was set at $p \leq 0.05$.

3.6. Overall Survival

Next, we examined the differences in the survival probability of our study cohort over a follow-up period of 90-days. Importantly, immunocompromised patients showed a lower probability of survival (75%) compared to diabetes mellitus patients (80%) or elderly patients (100%). However, the difference was not significant (Figure 9).

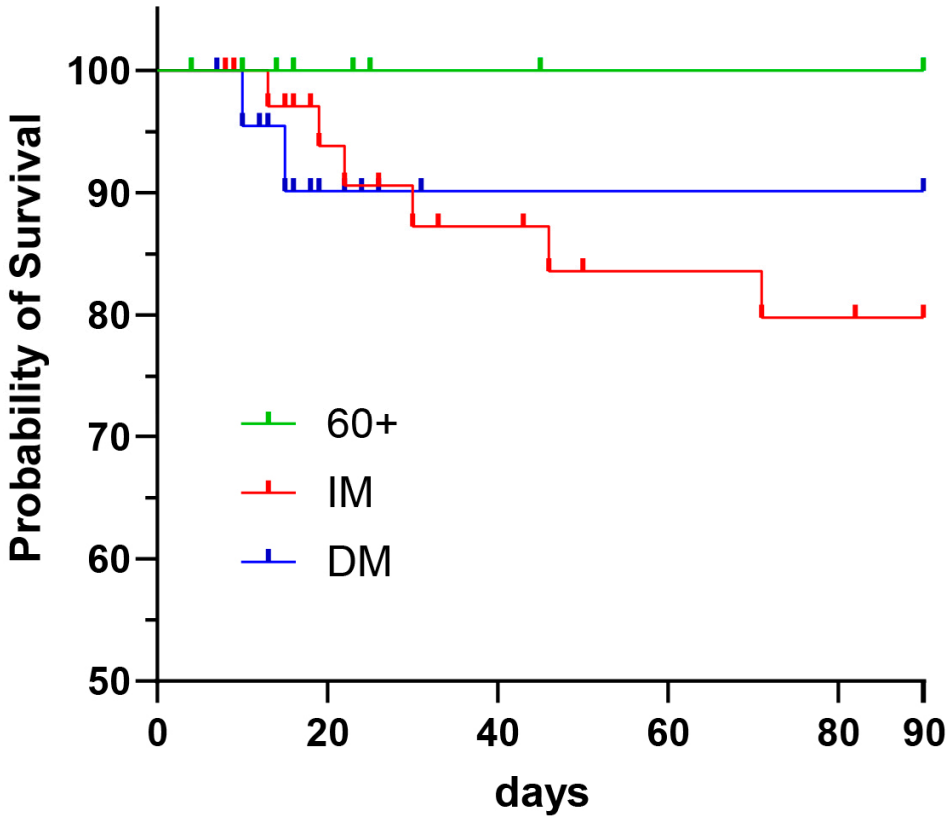

Figure 9. Kaplan–Meier estimate showing the probability of survival for elderly (60+), immunocompromised (IM) and diabetes mellitus (DM) patients. Patients' survival was monitored for 90 days after the first positive SARS-CoV-2 PCR.

4. Discussion

In the present study, we evaluated the humoral and cellular immunity, overall survival and infectivity in a cohort of 79 unvaccinated, hospitalized COVID-19 patients. The study cohort comprised patients with high risk factors predisposed to severe COVID-19, such as advanced age, immunocompromisation and diabetes mellitus. Our data suggest that immunocompromised patients have a weaker cellular immune response, while the humoral immune response seems to be decent. Immunosuppressed patients seem to shed virus over a prolonged period.

The combined results of our study not only characterize the immune response but should further have a meaningful impact on the in-hospital management of high-risk COVID-19 patients, which is often as delicate as it is important.

Regarding the humoral immune response to SARS-CoV-2 infection, it is still unclear if pre-existing comorbidities (especially great age and immunocompromisation) are associated with lower antibody levels [21]. All patients examined in our study had a moderate to severe COVID-19 course with hospitalization and, in some cases, oxygen support, whereas none of the convalescents were hospitalized.

We compared SARS-CoV-2-neutralizing antibody titers of the study cohort with those of convalescent patients recovered from a mild or asymptomatic SARS-CoV-2 infection without relevant comorbidities. Of note, convalescent group samples were obtained after

SARS-CoV-2 infection. The neutralizing antibody titers of the convalescents did not significantly differ from those of the elderly, immunocompromised or diabetes mellitus patients. However, the heterogeneity in terms of infection severity and sample period could have masked a difference in neutralizing antibody titers. Although SARS-CoV-2-neutralizing antibodies remain detectable in the first few months after mild COVID-19 [22,23], their levels may decrease with time after SARS-CoV-2 infection.

In addition, it has been reported that the severity of COVID-19 affects antibody levels, e.g., milder COVID-19 symptoms lead to lower antibody levels [24,25]. According to these data, we identified significantly reduced SARS-CoV-2 IgG ELISA antibody levels in immunocompromised patients compared to diabetes mellitus patients, but not compared to elderly patients. The elderly patients in our cohort showed significantly more moderate COVID-19 cases than the diabetes mellitus patients, possibly leading to lower SARS-CoV-2 antibody titers. This evidences a lower humoral immune response in immunocompromised patients. Of note, one-third of the immunocompromised patients in our cohort did not develop any humoral immune response during the acute COVID-19 phase. This is consistent with recent studies showing that immunocompromised patients are significantly more likely to remain seronegative after COVID-19 [26–28].

Likewise, significantly lower T-cell-specific immunity was observed in immunocompromised patients compared to elderly patients. This is in accordance with recently published data showing a significantly reduced T-cell response in solid organ transplant recipients (SOT) in the acute phase of COVID-19 [29]. Overall, the cellular immune response after COVID-19 is not well characterized in immunocompromised patients. Of note, immunocompromised patients that did not develop measurable SARS-CoV-2-neutralizing antibodies also had no measurable T-cell responses. Significantly lower T-cell-specific immune response in immunocompromised patients after SARS-CoV-2 vaccination could support the hypothesis of a reduced response [15,30–32]. However, recent studies also demonstrated no difference between the T-cell-specific immune response of immunocompromised and immunocompetent patients [33–35]. Therefore, further research is needed to define possible differences in T-cell-specific immunity in immunocompromised and immunocompetent patients.

Patients with diabetes mellitus not only had more severe COVID-19 compared with geriatric patients, but, similarly to immunocompromised patients, had a lower T-cell-specific immune response, although this did not reach the significance level. This is consistent with recent evidence suggesting that impaired T-cell responses attributable to multifactorial factors may predispose diabetic patients to a more severe COVID-19 course [36].

Our correlation analyses revealed that higher $CD19^+$ B-cell numbers were associated with higher SARS-CoV-2-neutralizing antibody titers. This is consistent with a prior study showing that patients barely develop antibody responses if they have a deficiency of B-cells [37]. No correlation was observed between SARS-CoV-2 IgG ELISA antibody titers and neutralizing antibody titers, which is contrary to the findings of others [37,38]. Furthermore, there was no correlation between SARS-CoV-2-neutralizing antibody titers and IFNγ ELISpot increment. These findings are in line with prior studies [26]. However, the correlation analyses are limited due to the low number of samples.

In line with our findings showing no deficiency in humoral or cellular immune response in elderly and diabetic SARS-CoV-2-positive patients, infectious virus was isolated in only one case. Of note, in samples from the immunocompromised patients, infectious virus was isolated in only three cases. Thus, our results indicate that elderly, immunocompromised and diabetic patients are not susceptible to prolonged virus shedding. This is in line with recent data demonstrating that the median duration of infectious-virus excretion was 8 days after the onset of symptoms, and excretion rates reached lower than 5% after 15.2 days [39]. Other study groups were unable to culture virus at all 8 days after the onset of symptoms [40]. Other authors suggest that persistent viral RNA shedding might be associated with different factors, such as receiving glucocorticoid treatment or suffering

from severe COVID-19 [13,39]. One third of our patients—most of them immunocompromised or diabetic—received dexamethasone therapy. However, we cannot exclude that the prolonged detection of SARS-CoV-2 RNA was not caused solely by the underlying comorbidities, but also by the glucocorticoid therapy those patients received.

In previous studies, vital virus could not be cultured when ct-values were above 24 [41]. In contrast to those findings, we were able to isolate virus in samples with a ct-value of 27 (from an elderly patient) and in samples with a ct-value of 24, 33 and 29 (from immunocompromised patients). The elderly, immunocompromised and diabetic patients in our study were identified as PCR-positive for a longer period than reported in recent studies. The mean time from symptom onset to the first negative RT-PCR result reported in the literature was 9.5 (6.0–11.0) days [42]. In our study population, mean time to SARS-CoV-2 PCR control was 16 days. These findings are in line with recently published data showing prolonged virus-shedding in patients with chronic kidney disease or tumor patients [43,44]. At that time most patients remained persistently PCR-positive for SARS-CoV-2, and thus had to be isolated according to the Robert Koch Institute (RKI) recommendations at that time. The consequence of the strict isolation measures was a prolonged hospitalization for the patients in our cohort (24 days) compared to the average duration for inpatients with COVID-19 in Germany (14.3 days) [45]. Of importance, despite persistent SARS-CoV-2 PCR detection, only low levels of replication-competent virus could be cultured in our cohort.

Those elongated hospitalization times do not only burden the medical system with high numbers of avoidable inpatients but could additionally endanger patients. Our clinical data included information about the frequency of antibiotic therapy and the necessity of this therapy for a nosocomial infection. The analysis displayed that 36% of immunocompromised patients suffered from a nosocomial infection (13 of 36 patients). This number stands in contrast to the average percentage of nosocomial infections in Germany for the year 2020 (6.8%). Diabetic (23%) and elderly patients (10%) also showed a higher number of nosocomial infections, although the rate not as high as for the immunosuppressed patients. Before the COVID-19 pandemic, only 5.6% of all hospital patients had a nosocomial infection [46]. The authors suggested an increase in infections during the pandemic due to a higher number of patients being treated per nurse and the lack of protective gear. However, an elongated hospitalization time due to unconclusive isolation requirements can also represent a hazard, especially as patients with imprecise isolation requirements also have a higher risk of nosocomial infections, namely, immunocompromised patients [46]. Nosocomial infections are associated with higher mortality. In addition, this leads to rising economical costs.

However, in our study, the mortality in our subgroups was below the average at that time in Germany [45]. The experience of all our medical staff ward members with high-risk patients' treatment might be a possible explanation for this.

It is key to discharge patients right after appropriate treatment has been given to decrease the risk of developing nosocomial infections and optimize the usage of medical health care system capacity. To date, most national and international recommendations advocate extending isolation and precautions for severely ill COVID-19 or severely immunocompromised persons to up to 20 days after the onset of symptoms and concurrent improvement in other symptoms. No clear procedural evidence-based guidance on handling at-risk individuals with persistent RNA detection is available at present. Recent work has suggested that seroconversion could be used as an additional parameter for deciding when to end isolation regimens [42]. Other recent studies even suggest non-infectivity in PCR-positive patients with a neutralizing antibody titer above 1:20. Thus, independent predictors for the detection of infectious SARS-CoV-2 from the respiratory tract were high viral loads and an absence of neutralizing antibodies in serum, but not immunodeficiency [39]. Most of our patients, had a neutralizing antibody titer well above 1:50, despite a positive SARS-CoV-2 PCR result. Since the presence of infectious virus could not be detected in most cases, further quarantine measures probably could have been omitted.

Our study has some limitations. Firstly, it is a monocentric study, only considering patients from one university hospital. Secondly, the patients in the immunocompromised cohort are quite heterogeneous with respect to their underlying disease. Our data indicate that this heterogenous group seems to have comparable immune responses. Neither age, concomitant diabetes nor subgroups within immunocompromised patients indicated strong deviations in the immune response or viral shedding. Our data also show no association between the kind of treatment for COVID-19 and the occurrence of nosocomial infection or virus shedding. Our results are limited due to the number of patients in these subgroup, and further studies are required. Thirdly, our prospective study includes immunocompromised patients, elderly patients and patients with diabetes mellitus, but not a younger control group. Comparing the immune status of our study cohort with a younger control group may reveal possible age-dependent differences in SARS-CoV-2-specific humoral and cellular immunity.

In conclusion, our data indicate significantly lower SARS-CoV-2 IgG ELISA antibodies in immunocompromised patients compared to diabetic patients, and a lower T-cell-specific immune response compared to geriatric patients. Infectious virus was only isolated from four samples with a cycle threshold of 33 or lower. Furthermore, no difference could be found in the magnitude of SARS-CoV-2-neutralizing antibody levels in the immunocompromised patients who developed antibody titers compared to elderly and diabetes mellitus patients. Therefore, the earlier discharge of high-risk patients (including immunocompromised patients) after the appearance of antibodies could be a way to stratify isolation measures. However, additional studies are needed to confirm these findings in larger cohorts.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee of the University Hospital Essen (approval no. 20-9512-BO and 20-9225-BO).

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors upon request.

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3.7 The GNB3 c.825C > T (rs5443) polymorphism and protection against fatal outcome of corona virus disease 2019 (COVID-19)

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2022

Anteile:

- Durchführung der Experimente: 20 %

Birte Möhlendick hat das Konzept der Studie entwickelt, die Daten analysiert, statistisch ausgewertet und das Manuskript erstellt. Die Experimente wurden von Laura Thümmler und Christoph Zacher durchgeführt. Monika Lindemann, Winfried Siffert und Karl-Heinz Jöckel haben das Manuskript überarbeitet.

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The *GNB3* c.825C>T (rs5443) polymorphism and protection against fatal outcome of corona virus disease 2019 (COVID-19)

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Background and aims: Albeit several factors which influence the outcome of corona virus disease (COVID-19) are already known, genetic markers which may predict the outcome of the disease in hospitalized patients are still very sparse. Thus, in this study, we aimed to analyze whether the single-nucleotide polymorphism (SNP) rs5443 in the gene *GNB3*, which was associated with higher T cell responses in previous studies, might be a suitable biomarker to predict T cell responses and the outcome of COVID-19 in a comprehensive German cohort.

Methods: We analyzed the influence of demographics, pre-existing disorders, laboratory parameters at the time of hospitalization, and *GNB3* rs5443 genotype in a comprehensive cohort (N = 1570) on the outcome of COVID-19. In a sub cohort, we analyzed SARS-CoV-2-specific T cell responses and associated *GNB3* rs5443 genotypes. We investigated the influence of all factors on COVID-19 fatality in multivariable analysis.

Results: We found a younger patient age, normotension or absence of diabetes mellitus or cardiovascular diseases, normal blood cell counts, and low inflammatory markers at hospital admission were protective factors against fatal course of disease. In addition, the rs5443 TT genotype was significantly associated with protection against COVID-19 fatality (OR: 0.60, 95% CI: 0.40–0.92, p = 0.02). We also observed significantly increased SARS-CoV-2-specific T cell responses in rs5443 TT genotype carriers (p = 0.01). Although we observed a significant association of the factors described previously in univariate analysis, only a younger age of the patients, normal blood cell counts, and the *GNB3* rs5443 TT genotype remained independent predictors against COVID-19 fatality in multivariable analysis.

Conclusion: Immutable predictors for COVID-19 fatality are relatively rare. In this study we could show that the TT genotype of the SNP rs5443 in the gene *GNB3* is associated with protection against COVID-19 fatality. It was as well correlated to higher SARS-CoV-2-specific T cell responses, which could result in a milder course of disease in those patients. Based on those observations we hereby provide a further prognostic biomarker, which might be used in routine diagnostics as a predictive factor for COVID-19 mortality already upon hospitalization.

KEYWORDS

GNB3, rs5443, genetic association, T cell response, G protein, COVID-19, SARS-CoV-2, disease severity

Introduction

Heterotrimeric guanine-binding proteins (G proteins) transmit signals from the cell surface, trigger intracellular signal cascades, and involve in a wide variety of physiological processes (Klenke et al., 2011). The gene *GNB3* encodes the G protein subunit β3 and is located on chromosome 12p13.31. The ß-subunits are not only the important regulators of the *a*-subunits of G proteins but also intracellular effectors. The synonymous single-nucleotide polymorphism (SNP) rs5443 (c.825C>T; p.S275=) in the gene *GNB3* is associated with several disorders and affects the pharmacodynamics of many different drugs (Klenke et al., 2011). The T allele of this SNP gives rise to the splice variant Gβ3-s, which lacks 123 nucleotides or 41 amino acids. Aberrant splicing results in a dominant gain of function and G protein activation (Siffert et al., 1998). We could show in previous studies that the rs5443 T allele is associated with increased chemotaxis, migration, and proliferation of B lymphoblasts, neutrophils, and T lymphocytes (Virchow et al., 1998; Virchow et al., 1999; Lindemann et al., 2001; Tummala, 2013). Lindemann et al. (2001) could show that $CD4^+$ T cell counts are increased in individuals carrying the rs5443 T allele. Therefore, it appears that individuals carrying the T allele show an increased function of their cellular immune system.

Adaptive immune responses, especially those of the T cells, are of major importance in SARS-CoV-2 infection. Virus-specific $CD4^+$ and $CD8^+$ T cells produce effector cytokines and exert cytotoxic activity in most patients with SARS-CoV-2 infection, whereas neutralizing antibodies directly interfere with viral entry of host cells (Jung and Shin, 2021). Nevertheless, patients with corona virus disease 2019 (COVID-19) not only show lower proportions of SARS-CoV-2-specific $CD4^+$ or $CD8^+$ T cells but also B cells and NK cells, with increasing disease severity (Huang et al., 2020; Peng et al., 2020; Zeng et al., 2020; Olea et al., 2021). Zeng et al. (2020) observed $CD4^+$ T cell lymphopenia in all severe and fatal cases with SARS-CoV-2 infection in their study. Furthermore, the authors could show that prolonged activation and exhaustion of $CD8^+$ T cells were associated with COVID-19 severity. In single-cell transcriptomic analyses, encompassing over 80,000 virus-reactive $CD8^+$ T single cells, Kusnadi et al. (2021) could show that SARS-CoV-2-reactive $CD8^+$ cells exhibited exhausted phenotypes with a decreased capacity to produce cytokines in severely ill COVID-19 patients.

In light of these observations, we hypothesized that the SNP rs5443 in the gene *GNB3* might influence the T cell response in COVID-19 patients as well and, thereby, the outcome of the disease. To answer this question we analyzed the SNP rs5443 in the gene *GNB3* in a comprehensive retrospective German cohort with SARS-CoV-2 infection and its influence upon T cell response and course of COVID-19.

Methods

Study participants, recruitment, and outcome of the patients

The study was conducted following the approval of the Ethics Committee of the Medical Faculty of the University of Duisburg-Essen (20-9230-BO) and in cooperation with the West German Biobank (WBE; 20-WBE-088). Written informed consent was obtained from the study patients.

Enrollment started on 11 March 2020, and ended on 18 May 2021. Altogether, 1,570 SARS-CoV-2-positive patients with at least one positive real-time reverse transcription polymerase chain reaction (RT-PCR) test result were consecutively recruited for the study. Follow-up was completed on 30 June 2021, and at that time all patients either were discharged from the hospital as "cured" or had a fatal outcome of the disease. The clinical outcome was defined as follows according to the criteria of the ECDC (European Center of Disease Prevention and Control, 2021)—"mild': outpatients (N = 205); "hospitalized": inpatients (N = 760); "severe": hospitalized patients admitted to an intensive care unit and/or became dependent on mechanical ventilation (N = 292); "fatal" all cases of COVID-19-related deaths during the hospital stay or within a follow-up of 30 days (N = 313). In contrast to the ECDC classification, where patients counted up to three times, every patient counted only once, according to the worst clinical outcome

observed during the hospital stay in our study. The patients included in this study were of Caucasian origin.

For further statistical analyses, demographic data, medical history, and hematological parameters (erythrocyte, platelet, neutrophil, and lymphocyte counts) at the time of hospital admission were documented for each patient. The medical history included pre-existing disorders of the cardiovascular system (e.g., myocardial infarction, coronary heart disease but not arterial hypertension), arterial hypertension, and diabetes mellitus.

Neutrophil–lymphocyte ratio, platelet–lymphocyte ratio, and systemic immune-inflammation index were calculated as inflammatory markers. The neutrophil-lymphocyte ratio (NLR) is calculated by dividing the number of neutrophils per nanoliter (nl) by the number of lymphocytes per nl from a peripheral blood sample. Similarly, the platelet-lymphocyte ratio (PLR) is calculated, where the number of platelets per nl is divided by the number of lymphocytes per nl in a peripheral blood sample. For the systemic immune-inflammation index (SII), the platelet counts per nl were multiplied by the number of neutrophils per nl and then divided by lymphocyte counts per nl in a peripheral blood sample.

Interferon-γ ELISpot assay

SARS-CoV-2-specific T cell responses were analyzed in 182 randomly selected SARS-CoV-2-positive patients using interferon-γ (IFN-γ) ELISpot assays as previously described (Schwarzkopf et al., 2021). Briefly, ELISpot stripes containing polyvinylidene difluoride (PVDF) membranes (MilliporeSigma™ MultiScreen™ HTS, Fisher Scientific, Schwerte, Germany) were activated with 50 µl of 35% ethanol for 10 s and washed with distilled water. Plates were then coated for 3 hours with 60 µl of monoclonal antibodies against IFN-γ (10 µg/ml of clone 1-D1K, Mabtech, Nacka, Sweden). Thereafter, ELISpot plates were washed and then blocked with 150 µl AIM-V® (Thermo Scientific, Dreireich, Germany). After 30 min at 37°C, AIM-V® was discarded, and duplicates of 250,000 peripheral blood mononuclear cells (PBMC) were grown in the presence or absence of either PepTivator® SARS-CoV-2 protein S1/S2 (600 pmol/ml, Miltenyi Biotec, Bergisch Gladbach, Germany) in 150 µl of AIM-V®. The peptide mix of the S1/S2 protein consists mainly of 15-mer sequences with 11 amino acids overlap, covering the immunodominant sequence domains of the surface glycoprotein of SARS-CoV-2. After 19 h of incubation at 37°C, the ELISpot plates were washed, and captured IFN-γ was detected by incubation for 1 hour with 50 µl of the alkaline phosphatase-conjugated monoclonal antibody against IFN-γ (clone 7-B6-1, Mabtech, Stockholm, Sweden), diluted 1:200 with PBS plus 0.5% bovine serum albumin (BSA). After further washing, 50 µl of nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) was added, and purple spots appeared within 7 min. Spot numbers were analyzed by an ELISpot reader (AID Fluorospot, Autoimmun Diagnostika GmbH, Strassberg, Germany). Mean values of duplicate cell cultures were considered. We determined SARS-CoV-2-specific spots by spot increment, defined as stimulated minus non-stimulated values. Stimulated spot numbers > 3-fold higher than negative (unstimulated) controls combined with an increment value of >3 to the antigen were considered positive. Of note, the negative controls reached a mean value of less than one spot.

Genotyping of *GNB3* rs5443 (c.825C>T)

Genomic DNA was extracted from 200 µl EDTA-blood using the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was performed with 2 µl genomic DNA and 30 µl *Taq* DNA-Polymerase 2x Master Mix Red (Ampliqon, Odense, Denmark), with the following conditions: initial denaturation 94°C for 3 min; 35 cycles with denaturation 94°C for 30 s, annealing at 66°C for 30 s, and elongation 72°C for 30 s each; final elongation 72°C for 10 min (forward primer: 5′ GCT GCC CAG GTC TGA TCC C 3' and reverse primer 3′ TGG GGA GGG TCC TTC CAG C 5′). PCR products were digested with *Bse*DI (Thermo Scientific, Dreireich, Germany), and restriction fragments were analyzed by agarose gel electrophoresis. The various genotype results from restriction fragment length polymorphism (RFLP)-PCR were validated by Sanger sequencing.

Statistical analyses

Correlation of demographics (sex and medical history) and outcome of COVID-19 were calculated using Pearson's chi square (χ^2) statistics using the Baptista–Pike method for the odds ratio (OR) and 95% confidence interval (CI). One-way analysis of variance (ANOVA) was performed using the Kruskal–Wallis test with Dunn's multiple comparison to assess the influence of age, hematological parameters, or inflammatory markers on COVID-19 severity. To calculate thresholds for the laboratory values, which correlate with fatal course of disease receiver operating characteristic (ROC) analysis, Youden`s J statistic was performed.

The number of patients with fatal outcome of disease, for whom IFN-γ ELISpot analyses could be performed, was relatively small. Thus, we defined additional groups to perform statistical analyses to estimate the influence of the T cell response on COVID-19 severity in our cohort. Therefore, patients from the categories "mild" and "hospitalized" were grouped together to the group "moderate," whereas the patients with "severe" and "fatal" COVID-19 were consolidated to the group "serious." The differences in T cell responses as analyzed by IFN-γ ELISpot between patients with "moderate" and "serious" COVID-19 was estimated by Mann–Whitney test.

TABLE 1 Demographics, clinical characteristics, and outcome of the disease in SARS-CoV-2-positive patients. Classification according to the COVID-19 surveillance report of the ECDC: category "mild" is a case that has not been reported as hospitalized or dead. A "severe" case has been admitted to intensive care and/or required mechanical respiratory support. All values are given in medians and interquartile ranges (IQR), except from sex and medical history, which are reported in absolute counts and percentages.

Characteristics	All patients (N = 1570)	Mild (N = 205)	Hospitalized (N = 760)	Severe (N = 292)	Fatal (N = 313)	p-value
Age–years	62.0 (49.0–76.0)	47.0 (34.5–64.0)	62.0 (48.3–76.0)	59.0 (50.0–70.0)	71.0 (59.5–82.0)	$p < 0.0001$
Male sex	910 (58.0)	107 (52.2)	416 (54.7)	185 (63.4)	202 (64.5)	$p = 0.002$
Medical history						
Cardiovascular system[a]	547 (34.8)	11 (5.4)	257 (33.8)	111 (38.0)	168 (53.7)	$p < 0.0001$
Arterial hypertension	748 (47.6)	29 (14.1)	373 (49.1)	149 (51.0)	197 (62.9)	$p < 0.0001$
Diabetes mellitus	404 (25.7)	14 (6.8)	214 (28.2)	76 (26.0)	100 (31.9)	$p = 0.001$
Hematological parameters						
Erythrocytes/nl	4.4 (3.8–4.8)	4.6 (4.2–4.9)	4.4 (4.0–4.9)	4.4 (3.8–4.8)	4.0 (3.4–4.6)	$p < 0.0001$
Platelets/nl	202.0 (156.0–260.0)	204.0 (164.0–270.5)	205.0 (157.0–255.0)	209.0 (169.0–292.0)	189.0 (135.0–242.0)	$p < 0.0001$
Neutrophils/nl	4.9 (3.1–7.5)	3.7 (2.7–5.1)	3.9 (2.6–5.8)	6.3 (4.2–9.3)	7.7 (5.2–11.7)	$p < 0.0001$
Lymphocytes/nl	0.9 (0.7–1.3)	1.1 (0.9–1.5)	1.0 (0.7–1.4)	0.8 (0.6–1.1)	0.7 (0.5–1.1)	$p < 0.0001$
Inflammatory markers						
NLR	5.0 (2.9–9.9)	3.1 (2.1–4.7)	3.8 (2.4–6.2)	7.9 (4.5–13.0)	11.1 (6.0–18.5)	$p < 0.0001$
PLR	217.8 (151.1–326.7)	176.3 (139.1–268.9)	197.7 (140.7–285.5)	269.8 (181.4–418.4)	252.6 (162.9–414.2)	$p < 0.0001$
SII	1031.0 (523.9–2206.0)	717.2 (385.7–1055.0)	769.6 (399.8–1417.0)	1680 (921.9–3466.0)	1917.0 (1010.0–4019.0)	$p < 0.0001$

[a]Cardiovascular system: for example, myocardial infarction, coronary heart disease but not arterial hypertension. Abbreviations: nl = nanoliter; NLR, neutrophil–lymphocyte ratio; PLR, platelet–lymphocyte ratio; SII, systemic immune-inflammation index.

Hardy–Weinberg equilibrium (HWE) was calculated using Pearson's chi square (χ^2) goodness of fit test, and samples were considered as deviant from HWE at a significance level of $p < 0.05$.

For genetic association, we calculated OR and 95% CI by Pearson's chi square (χ^2) statistics using the Baptista–Pike method for OR and 95% CI, respectively. p-values are reported two-sided, and values of <0.05 were considered significant. One-way analysis of variance (ANOVA) was performed using Kruskal–Wallis test with Dunn's multiple comparison test to determine the influence of *GNB3* rs5443 genotype on T cell response as measured by IFN-γ ELISpot assay.

Multivariable analysis was performed to estimate independency of the variables age, sex, medical history, laboratory parameters, and *GNB3* rs5443 genotypes by stepwise Cox regression (likelihood ratio test, backward).

Results

From 11 March 2020 to 30 June 2021, we enrolled and studied 1,570 SARS-CoV-2-positive patients to determine the association of the SNP rs5443 in the gene *GNB3*, with severity of COVID-19. In a sub group of patients (N = 182), who were representative for all severity groups, we additionally analyzed the T cell response to SARS-CoV-2-specific antigens. The demographics and clinical characteristics of the patients are summarized in Table 1. We observed that about 20% of all patients (inpatients and outpatients) and 23% of the hospitalized patients had a fatal outcome of COVID-19. With increasing severity of the disease, we found significantly more elderly and male patients and those who had arterial hypertension, cardiovascular disorders, or diabetes mellitus as pre-existing medical disorders (Table 1). The number of platelets, erythroctytes, and lymphocytes decreased significantly, whereas the neutrophil counts increased with disease severity ($p < 0.0001$, ANOVA). Regarding the inflammatory markers, NLR, PLR, and SII, we observed significantly higher values with increasing severity of COVID-19 as well.

SARS-CoV-2-specific T cell response and *GNB3* rs5443 genotype

In 182 patients, we performed IFN-γ ELISpot assays to determine T cell response to SARS-CoV-2-specific antigens. We were able to analyze patients from all severity groups: "mild" (N = 79); "hospitalized" (N = 82); "severe" (N = 17), and "fatal" (N = 4). The number of patients with fatal outcome of disease, for whom IFN-γ ELISpot analyses could be performed, was relatively small. Thus, we defined additional groups to perform statistical analyses to estimate the influence of the T cell response on COVID-19 severity in

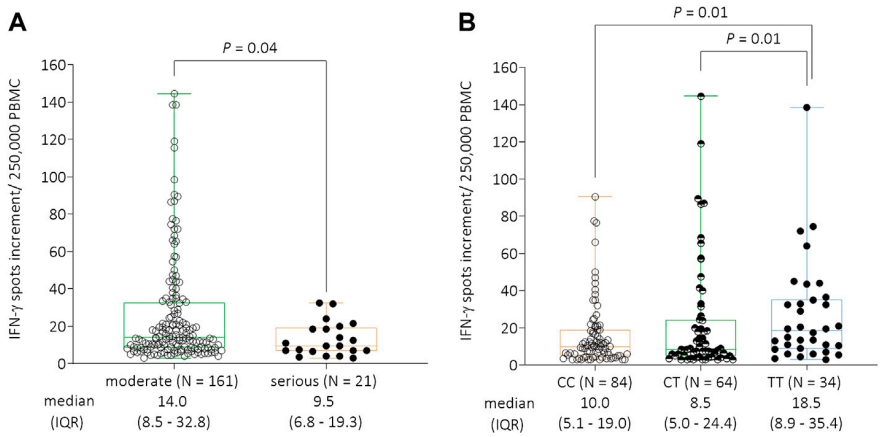

FIGURE 1
(A) IFN-γ ELISpot responses to S1/S2 protein of SARS-CoV-2 per 250,000 peripheral blood mononuclear cells stratified by COVID-19 severity. Due to the low number of cases in the individual groups, patients from the categories "mild" and "hospitalized" were grouped together to the group "moderate," whereas the patients with "severe" and "fatal" COVID-19 were consolidated to the group "serious." There was a significant decline of IFN-γ spots increment comparing the "serious" (N = 21, median = 9.5, and IQR = 6.8–19.3) to the "moderate" (N = 161, median = 13.5, and IQR = 8.0–35.0) groups (p = 0.04). **(B)** IFN-γ ELISpot responses to S1/S2 protein of SARS-CoV-2 per 250,000 peripheral blood mononuclear cells stratified by the *GNB3* rs5443 genotype. Individuals with the TT genotype had significantly higher spots increment (median = 18.5, and IQR = 8.9–35.4) compared to CT (median = 8.5, IQR = 5.0–24.4, and p = 0.01) or CC genotype carriers (median = 10.0, IQR = 5.1–19.0, and p = 0.01). Abbreviations: IFN-γ = interferon gamma; PBMC = peripheral blood mononuclear cells; IQR = interquartile range.

TABLE 2 *GNB3* rs5443 (c.825C>T) genotype distribution among all patients with SARS-CoV-2 infection and subdivided according to the severity of COVID-19.

	All patients (N = 1,570)	Mild (N = 205)	Hospitalized (N = 760)	Severe (N = 292)	Fatal (N = 313)
GNB3 rs5443 CC	700 (44.6)	89 (43.4)	330 (43.4)	121 (41.4)	160 (51.1)
GNB3 rs5443 CT	666 (42.4)	90 (43.9)	324 (42.6)	130 (44.5)	122 (39.0)
GNB3 rs5443 TT	204 (13.0)	26 (12.7)	106 (13.9)	41 (14.0)	31 (9.9)
Minor allele frequency (T)	0.34	0.35	0.35	0.36	0.29

our cohort. Therefore, patients from the categories "mild" and "hospitalized" were grouped together to the group "moderate," whereas the patients with "severe" and "fatal" COVID-19 were consolidated to the group "serious." We observed a significant decline of spots increment in the IFN-γ ELISpot assay comparing the "serious" group (N = 21, median = 9.5, and IQR = 6.8–19.3) to the "moderate" group (N = 161, median = 14.0, IQR = 8.5–32.8, p = 0.04, Figure 1A).

In a next step, we analyzed the influence of *GNB3* rs5443 genotypes on IFN-γ production against SARS-CoV-2-specific antigens. Here, we found a significant increase of IFN-γ spots increment in TT genotype carriers (median = 18.5 and IQR = 8.9–35.4) compared to those with CC genotype (median = 10.0, and IQR = 5.1–19.0) or CT genotype (median = 8.5, and IQR = 5.0–24.4) (both p = 0.01, respectively, Figure 1B).

GNB3 rs5443 as a protective factor against COVID-19 fatality

Overall, the observed genotypes for *GNB3* rs5443 were compatible with HWE in patients with "mild" (p = 0.66), "hospitalized" (p = 0.07), "severe" (p = 0.52), and "fatal" (p = 0.28) SARS-CoV-2 infection. Genotype distributions for all patients and the different groups according to severity of SARS-CoV-2 infection are shown in Table 2. Notably, we observed very similar rs5443 T allele frequencies (35%–36%) in all groups, except from those patients with a "fatal" outcome of COVID-19 (29%). Thus, we estimated, whether T allele or TT genotype carriers might be protected more effectively against fatal outcome of the disease. We found a significant association for protection against COVID-19 fatality in rs5443 TT genotype carriers comparing all patients ("mild," "hospitalized," and "severe") with SARS-CoV-2 infection

TABLE 3 Protective factors against COVID-19 fatality. Abbreviations: nl = nanoliter; NLR = neutrophil-lymphocyte ratio; PLR = platelet-lymphocyte ratio; SII = systemic immune-inflammation index; OR = odds ratio; CI = confidence interval, NS = not significant in stepwise multivariable analysis.

	Univariate analysis		Multivariable analysis	
Factor	**OR (95% CI)**	***p*-value**	**Or ([95% CI)**	***p*-value**
Age (<62 years)	0.35 (0.27–0.45)	<0.0001	0.47 [0.34–0.64)	<0.0001
Sex (female)	0.71 (0.55–0.92)	0.01	NS	NS
Absence of				
Diseases of cardiovascular system	0.41 (0.32–0.53)	<0.0001	NS	NS
Arterial hypertension	0.53 (0.41–0.68)	<0.0001	NS	NS
Diabetes mellitus	0.75 (0.57–0.98)	0.04	NS	NS
Hematological parameters				
Erythrocytes (≥4.0/nl)	0.27 (0.21–0.34)	<0.0001	0.70 (0.52–0.94)	0.02
Platelets (≥133.5/nl)	0.40 (0.29–0.54)	<0.0001	0.42 (0.30–0.60)	<0.0001
Neutrophils (≤6.6/nl)	0.28 (0.21–0.36)	<0.0001	0.32 (0.23–0.45)	<0.0001
Lymphocytes (≥0.9/nl)	0.41 (0.32–0.53)	<0.0001	0.55 (0.41–0.74)	<0.0001
Inflammatory markers				
NLR (<7.3)	0.24 (0.18–0.31)	<0.0001	NS	NS
PLR (<224.6)	0.60 (0.46–0.78)	<0.0001	NS	NS
SII (<1206.4)	0.32 (0.24–0.42)	<0.0001	NS	NS
GNB3 rs5443 TT genotype	0.60 (0.40–0.92)	0.02	0.65 (0.44–0.96)	0.03

and with those who died from COVID-19 (OR: 0.60, 95% CI: 0.40–0.92; $p = 0.02$, Table 3).

Thereupon, we performed multivariable analysis to analyze the independence of the *GNB3* rs5443 TT genotype in comparison to the other predictive parameters: age, pre-existing disorders, hematological parameters, and inflammatory markers. We performed ROC analysis and Youden`s statistic for the numeric variables to estimate a threshold above which the risk for COVID-19 fatality significantly decreased. We found that a younger patient age (<62 years; $p < 0.0001$), erythrocyte (≥4.0/nl; $p = 0.02$), platelet (≥133.5/nl; $p < 0.0001$), neutrophil (<6.6/nl; $p < 0.0001$), and lymphocyte (≥0.9/nl; $p < 0.0001$) counts above these respective thresholds at the time of admission to hospital, and the *GNB3* rs5443 TT genotype ($p = 0.03$) remained independent predictors for protection against COVID-19 fatality (Table 3).

Discussion

Remarkably, we observed that the TT genotype of the SNP rs5443 in the gene *GNB3* was associated with a higher T cell response as estimated by IFN-γ ELISpot assay in our patients. We could not find an association of *GNB3* genotype to lymphocyte or T cell counts. Thus, it seems that the increased T cell response in TT genotype carriers might be related to an increased activation of T cells. Early development of $CD8^+$ T cell responses is correlated to a more effective viral clearance and a mild course of COVID-19. Patients with severe disease display early onset of inflammation as well as delayed and relatively excessive adaptive immune response (Moss, 2022). The SNP rs5443 in the gene *GNB3* was not only correlated to higher T cell responses but also to a significantly reduced risk for COVID-19 fatality in our study in univariate and multivariable analyses.

The underlying mechanism of the influence of *GNB3* genotype on T cell response remains elusive. Juno (2014) could show that *GNB3* TT genotype carriers had a significantly lower *LAG-3* gene expression. The *LAG-3* (lymphocyte activation gene 3) gene is localized on chromosome 12 nearby to *GNB3*, nevertheless there are no SNPS in the gene *LAG-3* in high linkage disequilibrium with rs5443, which could be causative for the different gene expression in *GNB3* TT genotype carriers. LAG-3 was found to be expressed on dysfunctional or exhausted T cells in chronic viral infections and correlated with severity of the infection (Blackburn et al., 2009; Richter et al., 2010). Further studies are needed to analyze whether a reduced *LAG-3* expression is responsible for the T cell activation in *GNB3* TT genotype carriers.

We found that the comorbidities arterial hypertension, other disorders of the cardiovascular system and diabetes mellitus were associated with COVID-19 fatality in univariate analysis. This has already been extensively described in a multitude of studies and meta-analyses (Zhou et al., 2020). A variety of other factors, for example, age, sex, or laboratory parameters, have also been identified to influence the course of COVID-19 (Hobohm et al., 2022). The

infection-fatality ratio of COVID-19 significantly increases through ages 30, 60, and 90 years (COVID-19 Forecasting Team, 2022). Thus, we observed that a younger age (<62 years) was an independent protective factor against COVID-19 fatality in our study as well. Nevertheless, we could not confirm the independent influence of other consistent factors, like sex or pre-existing disorders, in a multivariable analysis.

Normal cell counts of lymphocytes and platelets upon hospital admission are associated with a significantly reduced risk for fatal outcome of COVID-19. Impaired adaptive immune responses as reflected by low counts of white blood cells together with augmented inflammation serve as a good predictor for the course of the disease (Qin et al., 2022). In our study, we noticed impaired white blood cell counts in individuals with severe COVID-19 as well. Several studies could show that the inflammatory markers NLR, PLR, and SII determined upon hospital admission are good predictive markers for in-hospital mortality (Fois et al., 2020; Wang et al., 2021; Sarkar et al., 2022). We also found a significant association for COVID-19 fatality and high NLR, PLR, and SII in the univariate analysis in our study. Nonetheless, those markers did not reach statistical significance in the multivariable analysis. Therefore, it seems even more important to find persistent markers that can predict the course of COVID-19 disease.

Together with a younger patient age, a normal white blood cell count at hospital admission, the *GNB3* rs5443 TT genotype remained an independent protective factor against COVID-19 fatality in our study. Immutable predictors are still relatively rare, thus analyses of genetic host factors might be useful in predicting severity, which could be implemented in routine diagnostics.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials; further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the Medical Faculty of the University of Duisburg-Essen. The patients/participants provided their written informed consent to participate in this study.

Author Contributions

BM: conceptualization, resources, methodology, formal analysis, investigation, supervision, data curation, funding acquisition, project administration, visualization, writing—original draft, and writing—review and editing. KS: resources, methodology, data curation, formal analysis, validation, and writing—review, and editing. CZ, CE, HR, MK, LT, and VR: data curation, resources, and investigation. ML: validation and writing—review and editing. WS and K-HL: conceptualization, validation, supervision, and writing—review, and editing.

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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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3.8 GNB3 c.825c>T polymorphism influences T-cell but not antibody response following vaccination with the mRNA-1273 vaccine

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Anteile:

- Durchführung der Experimente: 10 %

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GNB3 c.825c>T polymorphism influences T-cell but not antibody response following vaccination with the mRNA-1273 vaccine

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Background: Immune responses following vaccination against COVID-19 with different vaccines and the waning of immunity vary within the population. Genetic host factors are likely to contribute to this variability. However, to the best of our knowledge, no study on G protein polymorphisms and vaccination responses against COVID-19 has been published so far.

Methods: Antibodies against the SARS-CoV-2 spike protein and T-cell responses against a peptide pool of SARS-CoV-2 S1 proteins were measured 1 and 6 months after the second vaccination with mRNA-1273 in the main study group of 204 participants. Additionally, antibodies against the SARS-CoV-2 spike protein were measured in a group of 597 participants 1 month after the second vaccination with mRNA-1273. Genotypes of *GNB3* c.825C>T were determined in all participants.

Results: The median antibody titer against the SARS-CoV-2 spike protein and median values of spots increment in the SARS-CoV-2 IFN-γ ELISpot assay against the S1-peptide pool were significantly decreased from months 1 to 6 ($p < 0.0001$). Genotypes of *GNB3* c.825C>T had no influence on the humoral immune response. At month 1, CC genotype carriers had significantly increased T-cell responses compared to CT ($p = 0.005$) or TT ($p = 0.02$) genotypes. CC genotype carriers had an almost 6-fold increased probability compared to TT genotype carriers and an almost 3-fold increased probability compared to T-allele carriers to mount a SARS-CoV-2-specific T-cell response above the median value.

Conclusion: CC genotype carriers of the *GNB3* c.825C>T polymorphism have an increased T-cell immune response to SARS-CoV-2, which may indicate better T-cell-mediated protection against COVID-19 after vaccination with mRNA-1273.

KEYWORDS

GNB3 c.825C>T, COVID-19, SARS-CoV-2, mRNA-1273, antigen-specific T-cell response, ELISpot, SARS-CoV-2 spike antibody titer

1 Introduction

Antibodies and T-cells play an important role in both the outcome of COVID-19 and vaccination against it. Interaction between the angiotensin-converting enzyme 2 receptor expressed on the host cells and the receptor-binding domain in the spike (S) 1 subunit of the SARS-CoV-2 spike protein allows the virus to enter the host cell (Harrison et al., 2020). Vaccines against COVID-19 encode this SARS-CoV-2 spike protein and induce an immune response (Martinez-Flores et al., 2021). Immune responses following vaccination against COVID-19 with different vaccines and the waning of immunity vary within the population (Collier et al., 2021). Common factors such as age, sex, pre-existing conditions, or immunosuppressive therapy have been investigated and shown to contribute to this variability (Geisen et al., 2021; Lindemann et al., 2021; Simon et al., 2021; Steensels et al., 2021; Widge et al., 2021). In addition, genetic host factors are also likely to contribute to this variability (Crocchiolo et al., 2022; Gutierrez-Bautista et al., 2022). However, to the best of our knowledge, no studies on vaccination against COVID-19 and G protein polymorphisms have been published so far.

Here, we investigated whether genotypes of the c.825C>T polymorphism in the gene *GNB3* (rs5443) may influence the immune response after vaccination against COVID-19. This polymorphism exerts diverse influences on G protein-mediated signaling by generating a splice variant of the G protein subunit beta-3 (Siffert et al., 1998). Previous studies have shown that the *GNB3* c.825C>T polymorphism affects the immune response after stimulation with various recall antigens and after vaccination against the hepatitis B virus (HBV) (Lindemann et al., 2001; Lindemann et al., 2002).

2 Methods

2.1 Study group

For the study, 2,526 healthcare workers from the University Hospital Essen (Essen, Germany) were recruited. From this study cohort, we gathered a homogeneous group of 204 participants aged between 18–40 years for further investigations. All participants in this study group were non-obese, non-smokers, and were healthy or had minor health issues, but no immunosuppressive conditions or cardiovascular diseases. Immune responses after the vaccination in the study group did correlate neither with age nor with BMI. Furthermore, there were no differences in immune responses between healthy participants and participants with minor health issues. The allele frequencies of *GNB3* c.825C>T are differently distributed in African and East Asian populations. In this study, merely two participants belonged to these populations and they constituted less than 1% of our study group. The selection was based on questionnaires and the flow chart of enrollment is shown in Supplementary Figure S1. For additional investigations of antibody titers, we established an age-matched replication group of 597 participants. All participants in both study groups were vaccinated twice with the COVID-19 vaccine mRNA-1273 (Moderna Inc.). None of the participants had a history of SARS-CoV-2 infection and all tested negative for antibodies against the SARS-CoV-2 nucleocapsid protein. The investigations were reviewed and approved by the Ethics Committee of the Medical Faculty of the University of Duisburg-Essen (21–10005-BO). All participants provided their written informed consent to participate in this study.

2.2 Study design

Blood samples were taken from all participants 1 and 6 months after the second vaccination with mRNA-1273. We measured antibody titers against the SARS-CoV-2 S protein and the SARS-CoV-2 nucleocapsid protein and determined genotypes of the *GNB3* c.825C>T polymorphism. In addition, in the main study group of 204 participants, the T-cell response against the S1 peptide pool was measured using the SARS-CoV-2 IFN-γ ELISpot assay 1 and 6 months after the second vaccination.

2.3 *GNB3* c.825C>T genotyping

Genomic DNA was extracted from 200 µl EDTA blood using the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was performed with 2 µl genomic DNA and 30 µl Taq DNA-Polymerase 2x Master Mix Red (Ampliqon, Odense, Denmark) under the following conditions: initial denaturation 94°C for 3 min, 38 cycles with denaturation at 94°C for 30 s, annealing at 60°C for 30 s, elongation at 72°C for 30 s each, and final elongation at 72°C for 10 min (forward primer: 5′ GCCCTCAGTTCTTCC CCAAT 3'; reverse primer 3′ CCCACACGCTCAGACTTCAT 5′). PCR products were digested with BseDI (Thermo Scientific, Dreireich, Germany), and restriction fragments were analyzed by agarose gel electrophoresis. For the various genotypes, results from restriction fragment length polymorphism (RFLP)-PCR were validated by Sanger sequencing.

2.4 Detection of antibodies against SARS-CoV-2 spike protein

Determination of anti-spike SARS-CoV-2 antibody concentrations was performed using the SARS-CoV-2 S1 receptor-binding domain (RBD) IgG/sCOVG test (Siemens Healthcare GmbH, Erlangen, Germany) according to the manufacturer's instructions. Anti-Spike SARS-CoV-2 antibody concentration results were reported in binding antibody units per ml (BAU/ml). The limit of detection for positivity was 21.8 BAU/ml.

2.5 Detection of antibodies against the SARS-CoV-2 nucleocapsid protein

All samples were also analyzed for SARS-CoV-2 IgG antibodies against the nucleocapsid protein to exclude participants with prior SARS-CoV-2 infection. The Architect i2000SR CoV-2 IgG assay (Abbott Diagnostics, IL, United States) was used according to the manufacturer's instructions. Results with an index ≥1.4 were considered evidence of the previous infection.

2.6 ELISpot assay

To assess SARS-CoV-2-specific cellular immunity, we performed ELISpot assays using an overlapping peptide pool of SARS-CoV-2 S1 proteins (Miltenyi Biotec, Bergisch Gladbach, Germany) without the addition of any cytokines. We tested 250,000 peripheral blood mononuclear cells (PBMCs) per sample and measured IFN-γ production after 20 h of incubation. Mean values of duplicate cell cultures were considered. The median and mean spot numbers of autologous (unstimulated) controls were 0 and 0.05, respectively. SARS-CoV-2-specific spots were determined as stimulated minus unstimulated values (spots increment). The cut-off definition for positive results was based on negative control values (non-stimulated cultures) and the consideration that three times higher values for stimulated versus non-stimulated cells in cellular assays are often interpreted as a positive T-cell response. Using these criteria, the cut-off was 1.5 spot increments. Further details on the ELISpot assay and the cut-off definition have been published previously (Schwarzkopf et al., 2021).

2.7 Statistical analysis

Statistical analysis was performed with GraphPad Prism 7 (Graph Pad Software, San Diego, California, United States) and IBM SPSS Statistics 27 (IBM Software, Ehningen, Germany). Comparisons between three groups were made using the Kruskal–Wallis test and between two groups using the Mann–Whitney test. For genetic associations, we calculated the odds ratio (OR) and 95% confidence interval (CI) by Fisher's exact test using the Baptista–Pike method for the OR. *p*-values are given two-sided and values <0.05 were considered significant.

3 Results

3.1 Descriptive statistics of study groups

In the main study group of 204 participants, 105 participants were tested 1 month and 163 participants 6 months after the second vaccination with mRNA-1273. At both time points, sixty-four subjects participated. At month 1, the median age of the study group was 24 years (range 18–39), the BMI 22.5 kg/m^2 (range 17.0–29.9), and 69.5% (n=73) of participants were female. At month 6, the median age was 26 years (range 18–40), the BMI was 22.5 kg/m^2 (range 17.0–29.1), and 75.5% (n=123) were female. In the additional study group of 597 participants, the median age was 28 years (range 18–40), the BMI was 23.0 kg/m^2 (range 16.7–53.8), and 74.4% (n=444) of the participants were female.

3.2 Antibody titer against SARS-CoV-2 S1-RBD and T-cell response to SARS-CoV-2 S1 ELISpot assay one and six months after the second vaccination with mRNA-1273

The median antibody titer against SARS-CoV-2 S1-RBD was 3,887 BAU/ml (range 1,058–52,213) at month 1 (Figure 1A), which significantly ($p < 0.0001$) decreased to 644 BAU/ml (range 91–6,491) at month 6 (Figure 1B).

At month 1, 93.3% and at month 6, 41.7% of participants had a positive T-cell response in the SARS-CoV-2 IFN-γ ELISpot assay against the S1-peptide pool. Median values of spots increment decreased from 7.5 (range 0.5–60.5) to 0.5 (range -0.5–22.5) ($p < 0.0001$, Figures 1C,D).

Samples of 64 participants were available at both time points, 1 and 6 months after the second vaccination. The median antibody titer against SARS-CoV-2 S1-RBD was 3,682 BAU/ml (range 1,058–52,213) at month 1, which significantly ($p < 0.0001$) decreased to 731 BAU/ml (range 128–6,491) at month 6 (Figure 2A). Median values of spots increment in the SARS-CoV-2 IFN-γ ELISpot assay against the S1-peptide pool decreased from 6.0 (range 0.5–49) to 1.8 (range 0.0–22.5) ($p < 0.0001$, Figure 2B).

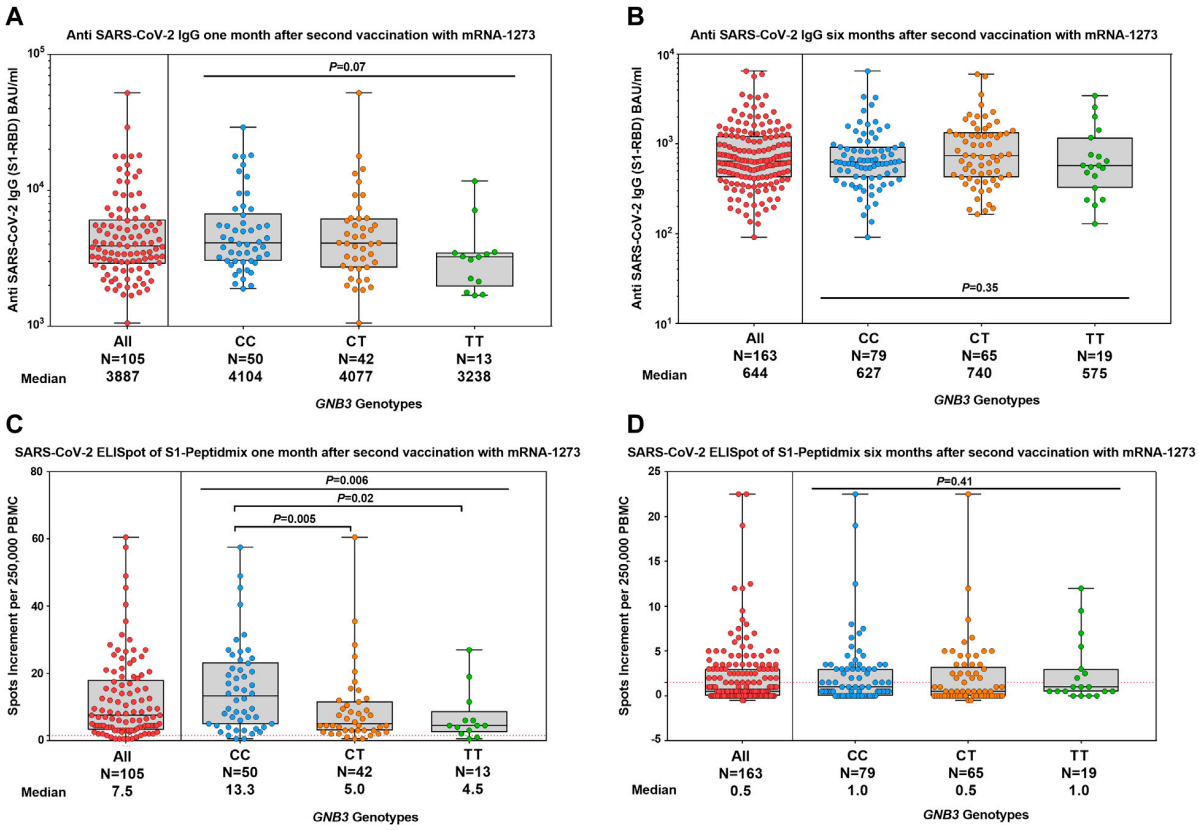

FIGURE 1
Humoral and cellular immune responses were stratified by *GNB3* genotypes 1 and 6 months after the second vaccination with mRNA-1273. Distribution of antibody concentrations against SARS-CoV-2 S1-RBD 1 month **(A)** and 6 months **(B)** after the second vaccination. ELISpot responses to the S1-protein of SARS-CoV-2 at 1 month **(C)** and 6 months **(D)** after the second vaccination. Antibody titers are reported in BAU/ml and T-cell response as spots increment. Red dashed lines indicate the cut-off for positivity (1.5 spots increment per 250,000 peripheral blood mononuclear cells).

3.3 Influence of *GNB3* c.825C>T on the immune response one and six months after the second vaccination with mRNA-1273

We investigated the impact of *GNB3* c.825C>T genotypes on the humoral immune response. We found a slightly albeit non-significantly lower anti-spike antibody titer in TT genotype carriers at month 1, which was no longer detectable in month 6 (Figures 1A,B). We validated these results in a larger cohort of 597 individuals (Figure 3).

At month 1, the median values of spots increment in the ELISpot assay were 13.3 (range 0.5–57.5) for CC, 5.0 (range 0.5–60.5) for CT, and 4.5 (range 0.5–27.0) for TT genotype carriers (p = 0.006, Figure 1C). CC genotype carriers had significantly increased T-cell responses compared to CT or TT genotypes (p = 0.005 and p = 0.02, respectively, Figure 1C). The effect was even more pronounced when comparing the CC genotype with T-allele carriers (13.3 vs. 4.5 spots increment, p = 0.001). At month 6, T-cell responses were strongly reduced and, therefore, genotype-dependent differences were no longer detectable (Figure 1D).

We analyzed the frequency distribution of *GNB3* genotypes above and below the median of 7.5 spots increment 1 month after the second vaccination to estimate if there is a genotype-related probability for a T-cell response above this cutoff. We found that CC genotype carriers had an almost 6-fold increased probability compared to TT genotype carriers (OR: 5.9, 95% CI: 1.6–21.5, p = 0.01) and an almost 3-fold increased probability compared to T-allele carriers (OR: 2.9, 95% CI: 1.3–6.2, p = 0.01) to mount a SARS-CoV-2-specific T-cell response above the median value.

4 Discussion

In this study, we observed a nearly 6-fold decrease in antibody titers from 1 to 6 months after the second

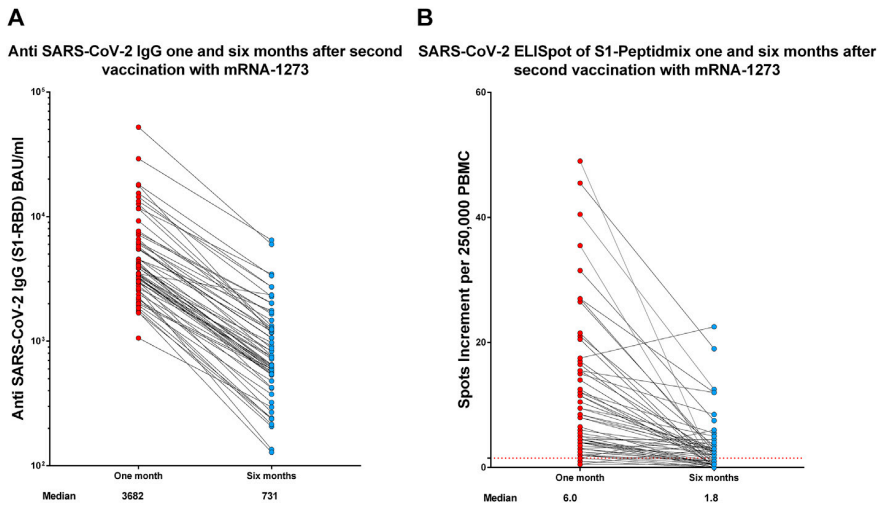

FIGURE 2
Humoral **(A)** and cellular **(B)** immune response 1 and 6 months after the second vaccination with mRNA-1273 in the group of 64 participants who were available at both time points. Antibody titers are reported in BAU/ml and T-cell response as spots increment. Red dashed lines indicate the cut-off for positivity (1.5 spots increment per 250,000 peripheral blood mononuclear cells).

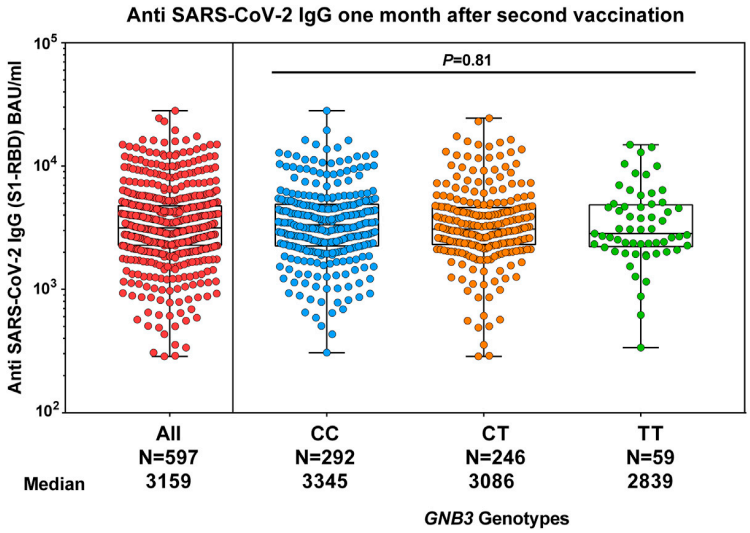

FIGURE 3
Comparison of humoral response and *GNB3* genotypes 1 month after the second vaccination with mRNA-1273 in the replication group. The median of anti-spike antibody levels is given in BAU/ml.

vaccination with mRNA-1273; nevertheless, all participants remained seropositive 6 months after the second vaccination. Despite many studies on the immune response after vaccination against COVID-19, there are only a few studies on the course of antibody titers over a longer period of time after vaccination with mRNA-1273. Those studies also reported a significant drop in antibody titers after vaccination with mRNA-1273 (Collier et al., 2021; Doria-Rose et al., 2021; Tre-Hardy et al., 2021; Gallagher et al., 2022).

In addition, Tré-Hardy *et al.* investigated whether different demographic characteristics such as age, BMI, or pre-existing conditions may influence the kinetics of antibody titers and

found no statistically significant relationship. Despite a very homogeneous study group of young, non-obese, and non-smoking participants without systemic immunosuppressive therapies or serious pre-existing conditions, we observed an almost 6-fold decrease in antibody titers, which confirms the findings of Tre-Hardy et al. (2021).

Some individuals generate lower antibody titers due to older age, pre-existing conditions, or immunosuppressive therapy (Geisen et al., 2021; Lindemann et al., 2021; Simon et al., 2021; Steensels et al., 2021; Widge et al., 2021). In our study, we present, for the first time, data on the potential influence of a G protein polymorphism on the immune response after vaccination with mRNA-1273. For this project, we chose the *GNB3* c.825C>T polymorphism because it was shown to correlate with T-cell responses to vaccination against HBV and to different recall antigens (Lindemann et al., 2001; Lindemann et al., 2002). In our current study, we observed that C-allele carriers had higher antibody titers, but this trend escaped statistical significance. In addition, no statistically significant differences were found between the genotypes of *GNB3* c.825C>T and the antibody titers after the booster vaccination against HBV (Lindemann et al., 2002). However, it has been shown that CT genotype carriers tend to have higher antibody titers after booster vaccination against HBV. It seems that *GNB3* c.825C > T may have a slight impact on the humoral immune response.

Data on T-cell kinetics after the vaccination with mRNA-1273 are scarce. Many studies tested T-cell immunity only once after vaccination or after a very short follow-up time. However, Gallagher *et al.* investigated the kinetics of T-cell responses after vaccination with mRNA-1273 at long-term follow-up and demonstrated approximately 30% decreased T-cell responses at a median of 223 days after the first vaccination with mRNA-1273 (Gallagher et al., 2022).

Our analysis of the cellular immunity also reveals a decrease in T-cell responses 6 months after the second vaccination with mRNA-1273. We observed a 15-fold decrease in T-cell responses in the SARS-CoV-2 ELISpot assay against the S1 peptide pool from 1 to 6 months after the second vaccination. In addition, at 6 months, only half of the participants had a T-cell response above the cut-off. It is also worth noting that our study is the first to measure T-cell responses in such a large cohort and, in addition, all tests were performed on freshly collected PBMC.

Our data show that CC genotype carriers have a stronger T-cell-mediated response and may be better protected against COVID-19 or have a milder COVID-19 infection after vaccination with mRNA-1273. This may also be an advantage for CC genotype carriers when antibodies cannot neutralize the virus and T-cell immunity is critical, e.g., after infection with immune escape variants of SARS-CoV-2 or when the humoral immune response is impaired.

However, at this time point, further studies are needed. First, our data should be replicated in an independent cohort. Further studies after booster vaccination causing a stronger immune response are also needed to see the influence of *GNB3* c.825C>T on the T-cell response after a longer follow-up. Last, the molecular mechanisms by which the *GNB3* c.825C>T polymorphism influences the T-cell response after SARS-CoV-2 vaccination and the potential clinical implications of these findings are to be yet unraveled.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

Ethics statement

The cohort study was reviewed and approved by the Ethics Committee of the Medical Faculty of the University of Duisburg-Essen (21–10005-BO). All participants provided their written informed consent to participate.

Author contributions

IC, BM, ML, and WS conceptualized and designed this study. BM obtained ethics approval. IC and BM obtained informed consent, demographic data, and samples from the participants, organized logistics, and performed laboratory analyses. Additionally, LT, NF, CE, and UD performed laboratory investigations. IC performed statistical analyses. IC, BM, ML, and WS drafted and wrote the manuscript, which was reviewed by all authors. All authors had full access to the data and approved submission of this manuscript. IC and BM had the final responsibility to submit this manuscript.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.932043/full#supplementary-material

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3.9 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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Anteile:

- Durchführung der Experimente:20 %

Adalbert Krawczyk, Andreas Schönfeld, Hana Rohn und Maren Bormann haben das Konzept für die vorliegende Studie erstellt. Laura Thümmler, Maren Bormann, Mira Alt, Mona Otte, Leonie Brochhagen, Lukas van de Sand und Peer Braß haben die Experimente durchgeführt. Die Daten wurden von Maren Bormann, Alexander Thomas, Sebastian Dolff, Ulf Dittmer, Oliver Witzke, Folker Meyer und Monika Lindemann analysiert und statistisch ausgewertet. Adalbert Krawczyk und Maren Bormann haben das Manuskript erstellt und überarbeitet.

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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

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.

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

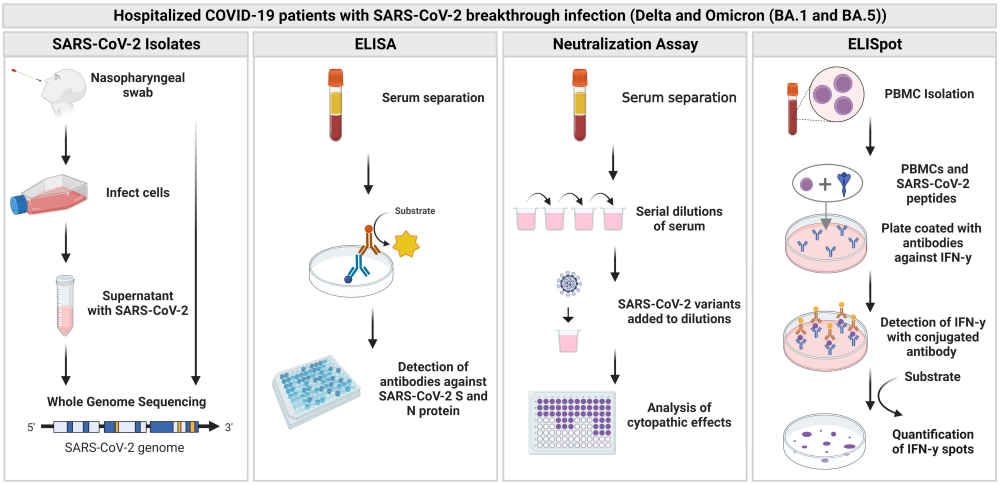

FIGURE 1
Overview of the study. Blood samples and nasopharyngeal swabs were collected from hospitalized patients with SARS-CoV-2 breakthrough infection (Delta and Omicron). Blood samples were further analyzed with an enzyme-linked immunosorbent assay (ELISA), neutralization assay as well as enzyme-linked-immuno-spot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

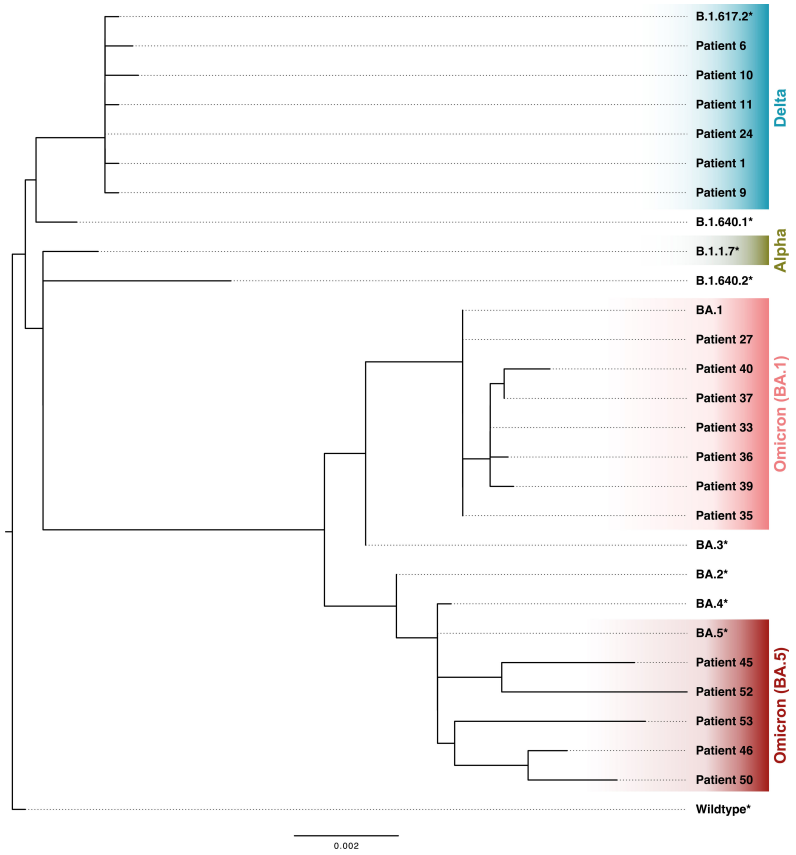

FIGURE 2
Phylogenetic tree of assembled SARS-CoV-2 spike (S) region sequences of clinical isolates of patients with SARS-CoV-2 breakthrough infection. * S region extracted from GISAID reference genomes (GISAID).

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

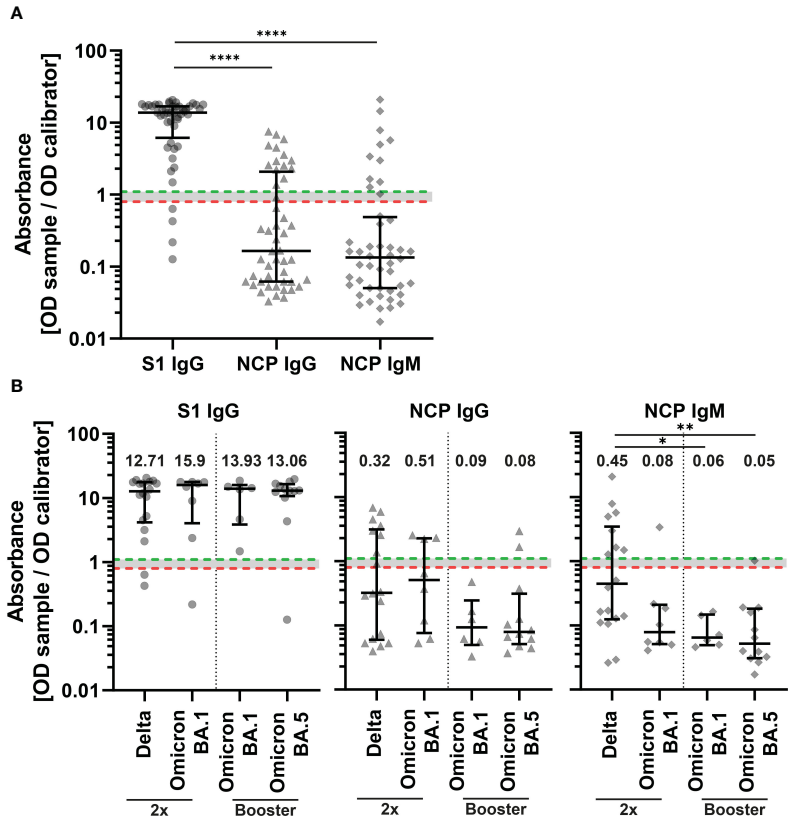

FIGURE 3
Binding serum antibody levels in COVID-19 patients with SARS-CoV-2 breakthrough infection. IgG antibodies against the subunit 1 of spike protein (S1) (Wuhan-Hu-1 isolate) and IgG and IgM antibodies against the nucleocapsid protein (NCP) of all patients (n=48) **(A)** and double vaccinated patients (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

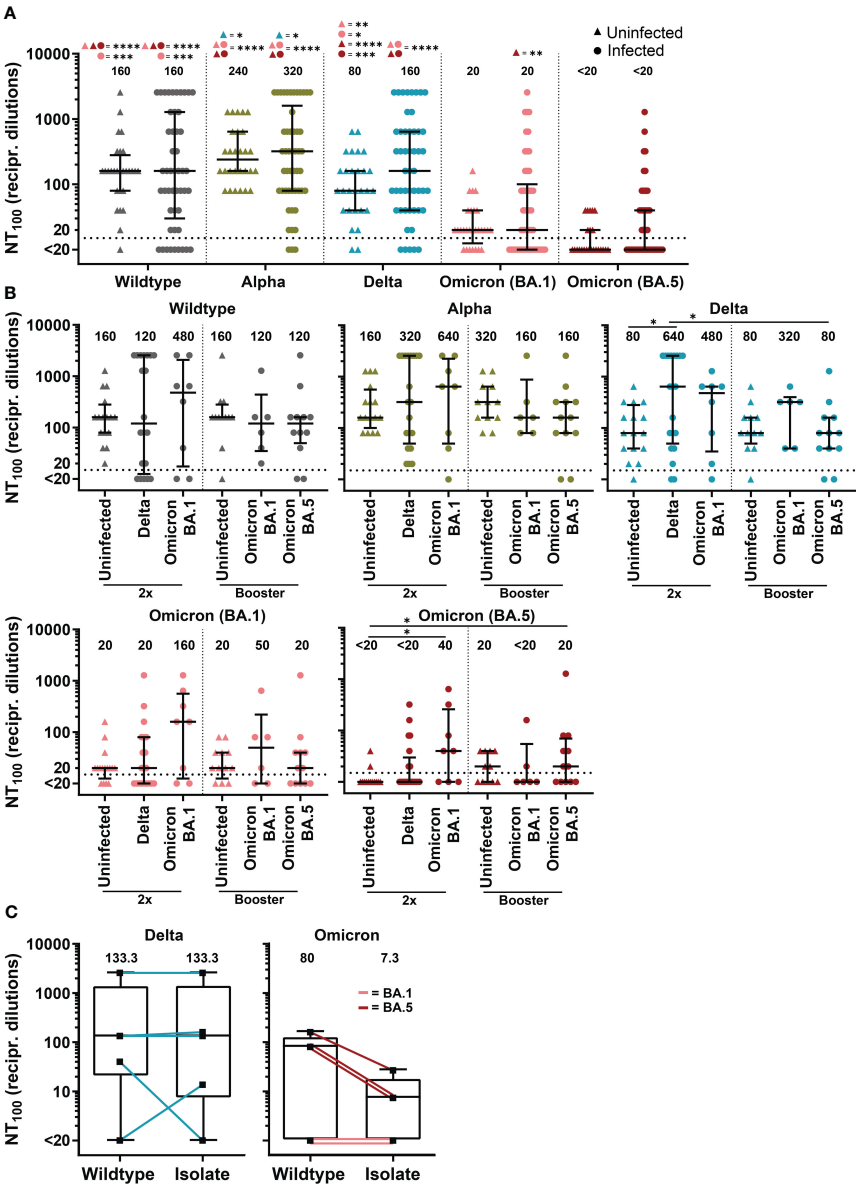

FIGURE 4
Neutralizing antibody titers against SARS-CoV-2 variants of COVID-19 patients with SARS-CoV-2 breakthrough infection and uninfected, vaccinated controls. **(A)** Complete neutralization titer (NT_{100}) against clinical isolate with D614G mutation (wildtype), Alpha (B.1.1.7), Delta (AY.43) and 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

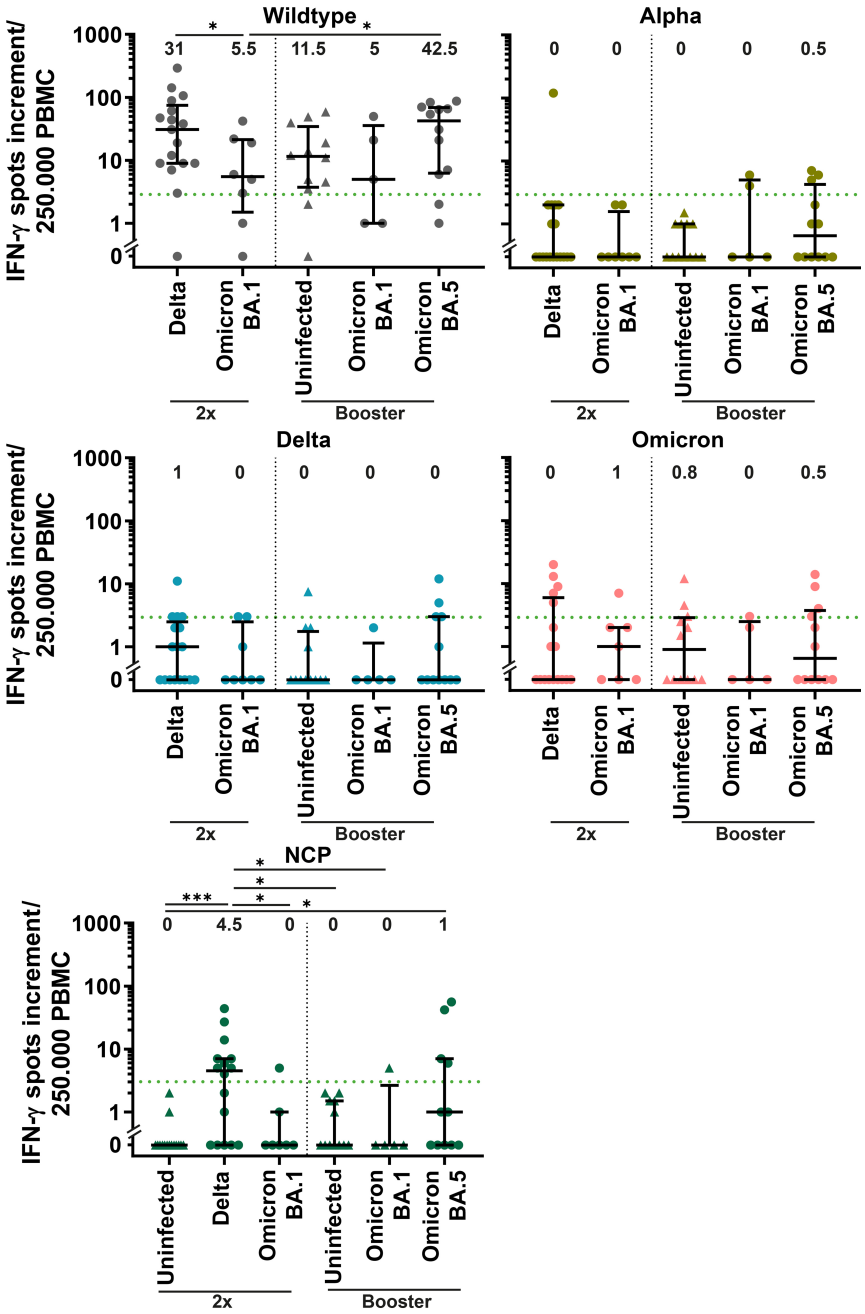

FIGURE 5
Cellular response against SARS-CoV-2 variants in COVID-19 patients with SARS-CoV-2 breakthrough infection. Cellular immunity was assessed by an IFN-γ enzyme-linked-immuno-spot (ELISpot) assay using peripheral blood mononuclear cells (PBMCs) and is displayed for double vaccinated patients (2x) 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.

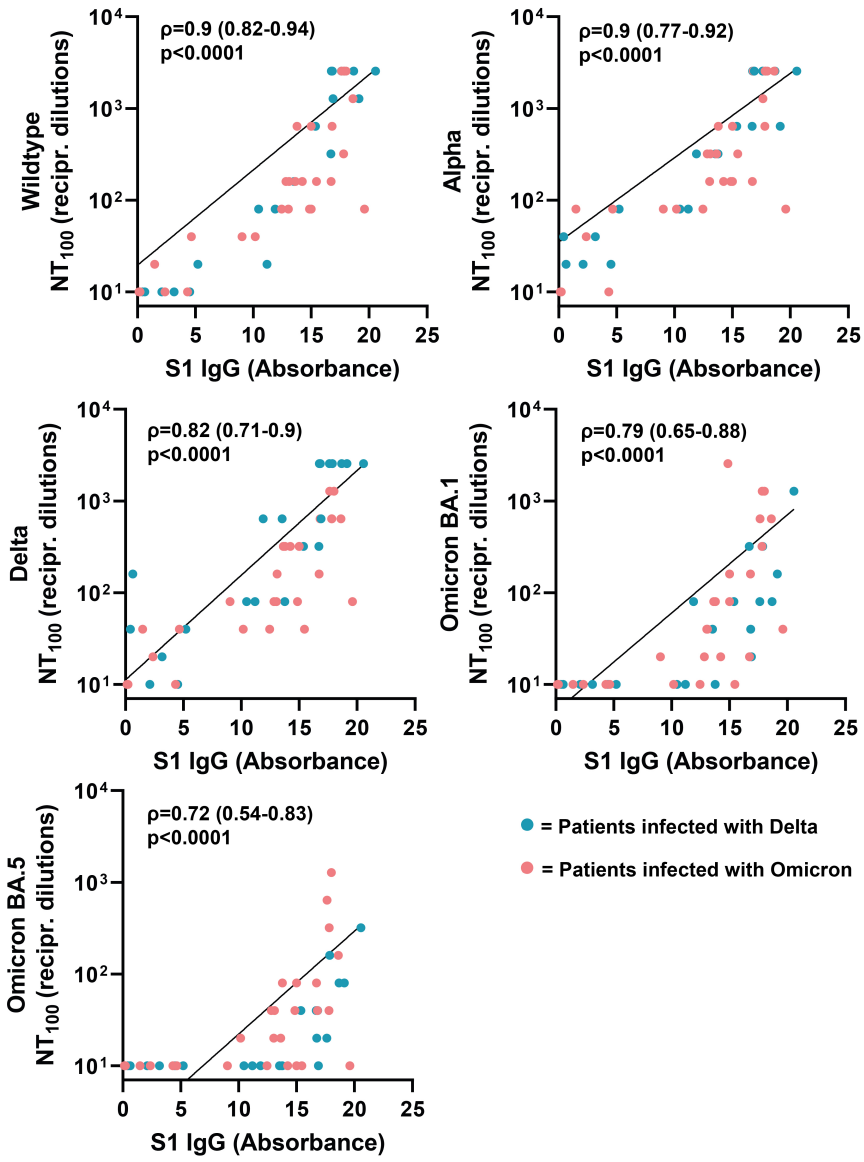

FIGURE 6
Correlation between serum antibody levels and neutralizing antibody titers against SARS-CoV-2 variants. Correlation coefficients (ρ) and *p*-values were calculated using Spearman's rank analysis.

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

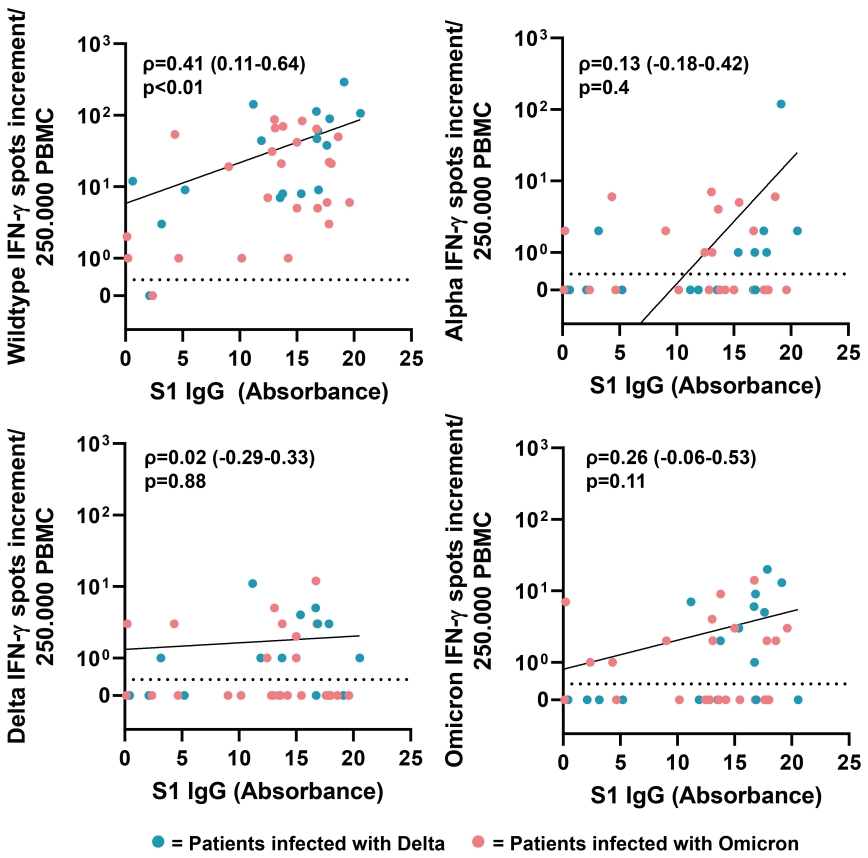

FIGURE 7
Correlation between serum antibody levels and cellular IFN-γ response after stimulation with SARS-CoV-2 variants. Correlation coefficients (ρ) and *p*-values were calculated using Spearman's rank analysis.

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. Diskussion

Die durch SARS-CoV-2 ausgelöste Pandemie stellte die Gesundheitssysteme vor große Herausforderungen und forderte eine rasche Entwicklung von Therapie- und Präventionsmaßnahmen. Die Untersuchung der Immunantwort nach Impfung und Infektion sollte wichtige Erkenntnisse über den Status der Immunität und der daraus resultierenden protektiven Wirkung gegenüber Reinfektionen und schweren Verläufen bringen. Insbesondere immunsupprimierte Personen mussten aufgrund ihres hohen Risikos für schwere Verläufe geschützt werden. Im weiteren Verlauf der Pandemie mutierte das Virus mehrmals. Es wurden zunehmend Durchbruchsinfektionen beobachtet, da die bis dahin verfügbaren Impfstoffe gegenüber den neu auftretenden Varianten zunehmend an Wirkung verloren haben. Ebenso waren Therapeutika wie beispielsweise monoklonale Antikörper nicht mehr wirksam. Die Erforschung von breit wirksamen, weltweit zugänglichen und Varianten-unabhängigen alternativen Therapien war von großer Bedeutung, um eine Behandlung von SARS-CoV-2 zu ermöglichen. In der vorliegenden Arbeit haben wir die Immunantwort bei verschiedenen Kohorten nach Infektion und Impfung analysiert. Darüber hinaus untersuchten wir bei COVID-19 die Auswirkungen einer Immuntherapie mit monoklonalen Antikörpern auf die Langzeitimmunität. Des Weiteren haben wir die Wirksamkeit der Antidepressiva Fluoxetin und Sertralin, in Bezug auf ihre antivirale Wirkung gegenüber verschiedenen SARS-CoV-2-Varianten, analysiert.

4.1 Immunreaktion bei geriatrischen, ungeimpften COVID-19-Patienten

Zu Beginn der SARS-CoV-2 Pandemie lagen noch keine standardisierten Richtlinien zum Umgang mit hospitalisierten, an COVID-19 erkrankten Personen vor. Insbesondere war unklar, ob das Virus bei Immunsupprimierten aufgrund einer verminderten Immunantwort länger infektiös persistiert. Im Rahmen der vorliegenden Arbeit wurde die humorale und zelluläre Immunantwort gegen SARS-CoV-2, die Dauer der Nachweisbarkeit von SARS-CoV-2-RNA sowie die Hospitalisierungsdauer bei drei immunsupprimierten Kohorten untersucht (Wünsch *et al.*, 2022). Insgesamt wurden 79 ungeimpfte COVID-19-Patienten mit Risikofaktoren für einen schweren Krankheitsverlauf in die Studie eingeschlossen und in drei Kohorten unterteilt. Die erste Kohorte bestand aus 20 Personen im Alter von über 60 Jahren, die zweite Kohorte aus 23 Personen über 60 Jahre mit Diabetes mellitus und die dritte Kohorte

aus 36 Personen, welche aufgrund anderer Vorerkrankungen oder Medikation als immunkompromittiert galten. Es konnte gezeigt werden, dass die zelluläre Immunantwort bei älteren Patienten signifikant stärker war als bei geriatrischen Patienten mit Diabetes mellitus oder Immunsuppression. Die schwächere zelluläre Immunantwort bei Diabetikern und Immundefizienten könnte durch eine geringere Anzahl von T-Zellen aufgrund von schwereren Verläufen zustande kommen. Frühere Studien konnten zeigen, dass es zu einem stärkeren Abfall der Anzahl an T-Zellen bei Patienten mit schwerem COVID-19-Verlauf kommt, verglichen mit milderen Verläufen (Bao *et al.*, 2020; Vabret *et al.*, 2020; Zavaglio *et al.*, 2021). Wünsch *et al.* zeigten zudem, dass die Konzentration an Antikörpern gegen SARS-CoV-2 bei Diabetikern und älteren Patienten höher ist als bei immunsupprimierten Patienten. Dies ist konsistent mit Studien die zeigten, dass schwerere Verläufe von COVID-19 mit höheren Antikörpertitern assoziiert sind, jedoch nur in geringem Maß bei Immunkompromittierten mit geringer Anzahl an $CD19^+$ B-Zellen (Burack *et al.*, 2021; Konik *et al.*, 2021; Mrak *et al.*, 2021). In der vorliegenden Arbeit konnte SARS-CoV-2 mittels quantitativer Polymerase-Kettenreaktion (engl.: *quantitative polymerase chain reaction*, qPCR) im Durchschnitt bis 16 Tage nach Auftreten der Symptome nachgewiesen werden. Diese Daten entsprechen Daten anderer Studien bei denen ebenfalls ein verlängertes Auftreten von SARS-CoV-2 bei Patienten mit Immunsuppression beobachtet werden konnte (Rogado *et al.*, 2021; Tang *et al.*, 2022). Aufgrund der fortgeführten Isolation wegen positiver qPCR-Ergebnisse kam es zu einer längeren Hospitalisierung am Universitätsklinikum Essen, insbesondere bei immunsupprimierten Patienten, verglichen mit anderen Krankenhäusern in Deutschland (Karagiannidis *et al.*, 2020).

4.2 Einfluss von Einzelnukleotid-Polymorphismen auf die Immunantwort gegenüber SARS-CoV-2

Erkenntnisse über genetische Faktoren, die den Verlauf von COVID-19 sowie das Ansprechen auf die Impfung gegen SARS-CoV-2 beeinflussen, sind von besonderer Bedeutung. Allgemein gelten bislang Übergewicht, höheres Alter und Vorerkrankungen als Risikofaktoren für einen schweren Verlauf von COVID-19 (Bonanad *et al.*, 2020; Fang *et al.*, 2020; Gao *et al.*, 2021). Studien konnten allerdings auch protektive Faktoren nachweisen, wie beispielsweise einen erhöhten Vitamin-D-Spiegel im Serum oder bestimmte Polymorphismen, die einen Einfluss auf das

Immunsystem haben (Shelef *et al.*, 2022; Xu *et al.*, 2023). Der Einzelnukleotid-Polymorphismus c.825C>T im Gen *GNB3* führt zu einer verstärkten Migration von B-Zellen und einer erhöhten Proliferation von T-Lymphozyten (Lindemann *et al.*, 2001; Tummala *et al.*, 2013). Wir haben in einer 1570 Patienten umfassenden Studie untersucht, welchen Einfluss der Polymorphismus auf die zelluläre Immunantwort und den Verlauf einer SARS-CoV-2-Infektion hat (Möhlendick *et al.*, 2022). Patienten mit dem Genotyp TT wiesen eine signifikant stärkere zelluläre Immunantwort auf, verglichen mit Patienten mit den Genotypen CC oder CT. Auch hatten Patienten mit Genotyp TT eine höhere Anzahl an $CD4^+$ T-Lymphozyten. Diese Ergebnisse sind konsistent mit Daten aus vorherigen Studien zum Hepatitis-B-Virus (Lindemann *et al.*, 2002; Lindemann *et al.*, 2001). In der Gruppe der COVID-19-Patienten mit Genotyp TT kam es trotz höherem Durchschnittsalter und vermehrt auftretenden Erkrankungen des kardiovaskulären Systems zu signifikant weniger Todesfällen, was auf einen möglichen protektiven Einfluss des Polymorphismus hinweist. Da der genaue Wirkmechanismus des Polymorphismus auf die T-Zellantwort bislang jedoch unklar ist, sind weitere Untersuchungen notwendig, um einen möglichen Zusammenhang zwischen der stärkeren zellulären Immunantwort und der geringeren Mortalitätsrate aufzuklären.

Die Entwicklung und Zulassung der Impfstoffe gegen SARS-CoV-2 war ein wichtiger Meilenstein in der Bekämpfung der Pandemie. Verschiedene Faktoren beeinflussen das Ansprechen auf eine Impfung. Auch für die Impfung gegen SARS-CoV-2 konnte eine Variabilität in der Generierung einer Impfantwort nachgewiesen werden (Collier *et al.*, 2021). Wir sind der Frage nachgegangen, welchen Einfluss der Einzelnukleotid-Polymorphismus *GNB3* c.825C>T auf die Impfantwort gesunder Probanden einen Monat und sechs Monate nach zweifacher Impfung mit dem mRNA-Impfstoff mRNA-1273 von Moderna hat (Čiučiulkaitė *et al.*, 2022). Es zeigte sich bei allen Probanden ein Abfall der humoralen und zellulären Immunantwort gegenüber dem SARS-CoV-2 Spike-Protein sechs Monate nach der zweiten Impfung. Diese Ergebnisse sind konsistent mit vorherigen Studien bezüglich der Immunantwort nach Impfung (Doria-Rose *et al.*, 2021; Gallagher *et al.*, 2022; Tré-Hardy *et al.*, 2021). Probanden mit dem homozygoten Genotyp TT wiesen zu beiden ausgewählten Zeitpunkten nach Impfung sowohl eine signifikant geringere Antikörperkonzentration als auch eine signifikant schwächer ausfallende zelluläre Immunreaktion auf, als Personen mit dem Genotyp CC oder CT. Dieses Ergebnis ist konträr zu der bereits oben genannten Studie, in der Probanden gegen das Hepatitis-B-Virus geimpft wurden. Hier zeigte sich bei

Probanden mit dem Genotyp TT eine 2,5-fach höhere T-Zellantwort (Lindemann *et al.*, 2001). Ein Grund für die unterschiedlichen Ergebnisse der Studien könnte der Impfstoff sein. In der Studie von Lindemann *et al.* wurde der rekombinante Impfstoff Gen H-B-Vax von Chiron Behring verwendet, wohingegen in der vorliegenden Arbeit der mRNA-Impfstoff mRNA-1273 von Moderna gegen SARS-CoV-2 verwendet wurde. Da durch den mRNA-Impfstoff eine Vervielfältigung des S-Proteins im Organismus über einen längeren Zeitraum möglich ist, könnte dies zu einem anderen Ansprechen auf die Impfung geführt haben als bei rekombinanten Impfstoffen. Zusätzlich wurde in der vorherigen Studie eine höhere Anzahl an $CD4^+$ T-Lymphozyten nachgewiesen (Lindemann et al., 2001). Eine Studie bei HIV-Patienten beobachtete jedoch, dass Personen mit dem Genotyp TT signifikant weniger $CD4^+$ T-Zellen aufweisen (Brockmeyer *et al.*, 2005). Eine Limitierung der vorliegenden Arbeit ist die fehlende Bestimmung der Anzahl an $CD4^+$ T-Lymphozyten, wodurch ein Vergleich mit vorangegangenen Studien zu anderen Viren erschwert ist. Bei einer Impfung wird eher das adaptive Immunsystem stimuliert, wohingegen es bei einer Infektion zum Zusammenspiel des angeborenen und adaptiven Immunsystems kommt. Es wurde bereits beobachtet, dass der Einzelnukleotid-Polymorphismus *GNB3* c.825C>T ebenfalls zu einer erhöhten Chemotaxis bei neutrophilen Granulozyten führt (Virchow *et al.*, 1998). Möglicherweise führt die zusätzliche Stimulation durch Chemokine zu einer höheren T-Zellantwort bei Patienten mit SARS-CoV-2-Infektion, die den Genotyp TT tragen, wohingegen fehlende Stimuli nach einer Impfung zu einer schwächeren zellulären Antwort führen könnten.

4.3 Behandlung von COVID-19 mit monoklonalen Antikörpern gegen SARS-CoV-2

Gegen Ende des Jahres 2020 wurden monoklonale Antikörper (engl.: *monoclonal antibodies,* mAb) zur Therapie von COVID-19 in Deutschland zugelassen. Zuvor wurden Patienten mit schwerem Verlauf im Rahmen von Studien mit Rekonvaleszentenplasma (RKP) behandelt. Die Untersuchung der Wirksamkeit von monoklonalen Antikörpern bei der Behandlung von COVID-19 war von großer Bedeutung, um eine bestmögliche Therapie sicherstellen zu können. Wir haben retrospektiv die 30-Tage-Mortalisitätsrate von Personen analysiert, die mit mAb oder RKP behandelt wurden und mit der Mortalitätsrate von Patienten verglichen, welche zu Beginn der Pandemie eine symptomorientierte Therapie erhalten haben (Thümmler

et al., 2022d). Die Behandlung von COVID-19 Patienten mit mAb oder RKP hat die Überlebensrate signifikant verbessert. Vorherige Studien zur Hospitalisierung und Mortalität bei Behandlung mit monoklonalen Antikörpern zeigten ebenfalls, dass es insbesondere bei Personen mit hohem Risiko für einen schweren Verlauf von COVID-19 mit Gabe von mAb zu signifikant weniger Hospitalisierungen und Todesfällen kam (Bachmann *et al.*, 2022; Cheng *et al.*, 2023; Rainwater-Lovett *et al.*, 2021; Sarrell *et al.*, 2022). In der Gruppe der mit RKP behandelten Patienten überlebten neun von 11 Personen im Zeitraum von 30 Tagen. De Santis *et al.* konnten ebenfalls eine niedrigere 30-Tage-Mortalitätsrate bei Gabe von RKP nachweisen (De Santis *et al.*, 2022). Die Arbeit von Pei *et al.* zeigte zusätzlich zur Überlebensrate, dass die Gabe von RKP zu einem schnelleren Sinken der Viruslast führt und Plasmen mit einem hohen Titer an neutralisierenden Antikörpern besser für die Behandlung geeignet sind (Pei *et al.*, 2021). Die Gabe von RKP ist jedoch umstritten, da es zu Transfusionszwischenfällen kommen kann und die Behandlung zu einem frühen Zeitpunkt erfolgen muss (Beraud *et al.*, 2022; Nashaat *et al.*, 2022). Spätere Studien zur Behandlung neuer Varianten von SARS-CoV-2 mit mAb zeigten allerdings, dass die neutralisierende Wirkung der Antikörper gegenüber den neuen Varianten, insbesondere Omikron, abnimmt (Cao *et al.*, 2022; Iketani *et al.*, 2022). Eine neuere Studie von Tayyar *et al.* beobachtete, dass die Gabe von RKP bei Immunsupprimierten, die mit der Omikron-Variante infiziert waren, zu milderen Verläufen und einer 30-Tage-Mortalitätsrate von 4,5 % führte (Tayyar *et al.*, 2023). Diese Ergebnisse weisen darauf hin, dass die Gabe von RKP eine alternative Behandlung für bestimmte Patientenkohorten darstellen kann, um einen schweren oder tödlichen Verlauf von COVID-19 zu verhindern. Eine Limitierung unserer Studie stellt das höhere Durchschnittsalter der Patienten dar, welche eine symptomorientierte Therapie erhalten haben, da ein hohes Alter allgemein als Risikofaktor für eine höhere Mortalität bei COVID-19 bekannt ist. Aufgrund der damaligen Impfempfehlungen der STIKO wurden diese Personen zuerst geimpft, wodurch sie vor einem schweren und tödlichen Verlauf von COVID-19 geschützt waren und nicht mehr mit mAb oder RKP behandelt wurden (STIKO, 2021).

Monoklonale Antikörper erwiesen sich bei frühzeitiger Gabe als äußerst wirksame Medikamente, um schwere bzw. tödliche COVID-19-Verläufe zu verhindern. Es blieb jedoch unklar, ob Patienten nach Gabe von mAb eine ausreichende Immunantwort aufbauen konnten, um vor einer erneuten Infektion mit SARS-CoV-2 geschützt zu sein. Da diese Behandlung bei Personen durchgeführt wurde, welche ein erhöhtes Risiko

für einen schweren bis tödlichen Verlauf haben, sind Erkenntnisse über eine Immunantwort nach der Infektion von großer Wichtigkeit. In der vorliegenden Arbeit haben wir die SARS-CoV-2-spezifische zelluläre Immunantwort mittels IFN-γ *Enzyme Linked Immuno Spot* (ELISpot) Assay fünf Monate nach Behandlung mit mAb untersucht (Thümmler *et al.*, 2022c). Als Kontrollgruppen dienten infizierte, geimpfte, immunsupprimierte Patienten ohne mAb-Behandlung, nicht-infizierte, geimpfte Immunkompromittierte und gesunde, geimpfte Personen ohne Immunsuppression. Die niedrigste zelluläre Immunantwort gegenüber dem S-Protein zeigten nicht-infizierte, geimpfte, Immunsupprimierte, gefolgt von infizierten, ungeimpften, immunsupprimierten Personen mit mAb-Behandlung. Infizierte, geimpfte Immunkompromittierte ohne Behandlung mit mAb wiesen die drittniedrigste T-Zellantwort auf und die gesunde, geimpfte Kontrollkohorte hatte die höchste zelluläre Immunantwort gegenüber dem S-Protein. Die Ergebnisse der mit mAb behandelten Gruppe wiesen allerdings keine signifikanten Unterschiede zu den Kontrollgruppen auf. Nicht-infizierte, geimpfte, immunsupprimierte Personen zeigten hingegen eine signifikant geringere zelluläre Immunantwort als immunsupprimierte, geimpfte Personen mit SARS-CoV-2-Infektion und gesunde, geimpfte Kontrollpersonen. Grund hierfür könnte eine fehlende Verstärkung der Immunantwort durch eine Infektion mit SARS-CoV-2 sein. Eine Arbeit von Bertrand *et al.* legt dagegen nahe, dass COVID-19 bei Immunkompromittierten zu einer T-Zellantwort führt, welche mit der von gesunden Personen vergleichbar ist (Bertrand *et al.*, 2021). Ungeimpfte, immunkompromittierte Patienten nach mAb-Therapie wiesen zudem eine deutlich geringere T-Zellantwort gegenüber dem M- und N-Protein auf. Studien konnten belegen, dass die Gabe von mAb die Viruslast senkt und zu milderen Verläufen führt (Pei *et al.*, 2021; Sarrell *et al.*, 2022). Es ist gut dokumentiert, dass die T-Zellantwort vom Schweregrad der SARS-CoV-2-Infektion abhängt, sodass es aufgrund des milden Verlaufs bei immunsupprimierten Personen nach Behandlung mit mAb zu einer geringeren zellulären Immunantwort gekommen sein kann (Konik *et al.*, 2021; Peng *et al.*, 2020). Eine Limitierung unserer Arbeit ist die fehlende Kontrollgruppe an ungeimpften, immunsupprimierten COVID-19-Patienten ohne Behandlung mit mAb. Da zum Zeitpunkt der Studie die Behandlung mit mAb bei immunkompromittierten Personen empfohlen wurde, um einen schweren Verlauf zu verhindern, stand keine ungeimpfte, infizierte, immunsupprimierte Kontrollgruppe zur Verfügung.

4.4 Immunantwort bei Immunsupprimierten nach Booster-Impfung gegen SARS-CoV-2

Die Einführung der zweifachen Impfung gegen SARS-CoV-2 führte zu einer deutlichen Eindämmung der Pandemie. Insbesondere für Immunsupprimierte mit erhöhtem Risiko für einen schweren Verlauf von COVID-19 ist ein Schutz vor einer Infektion durch Impfung von besonderer Bedeutung. Studien beobachteten eine reduzierte Impfantwort bei Personen mit Immundefizienz, etwa nach Nierentransplantation oder bei hämatologischen Erkrankungen (Gagelmann *et al.*, 2022; Grupper *et al.*, 2021; Huang *et al.*, 2022). Das Auftreten neuer SARS-CoV-2-Varianten und die Abnahme der Immunantwort führten zunehmend zu Durchbruchsinfektionen (Lipsitch *et al.*, 2022; Wang *et al.*, 2021). Eine Studie aus Israel konnte zeigen, dass eine Booster-Impfung effektiv zur Eindämmung von Infektionen mit SARS-CoV-2 und zu einer Serokonversion beitragen kann (Bar-On *et al.*, 2021; Petrelli *et al.*, 2022). Erkenntnisse darüber zu gewinnen, ob eine Booster-Impfung zu einer adäquaten Impfantwort bei Immunsupprimierten führt, war zu diesem Zeitpunkt sehr wichtig, da immer neue Infektionswellen mit neuen Varianten von SARS-CoV-2 auftraten.

Wir haben die zelluläre und humorale Immunantwort vor und nach Booster-Impfung gegen SARS-CoV-2 bei Patienten nach Stammzelltransplantation untersucht (Thümmler *et al.*, 2022b). Es konnten keine signifikanten Unterschiede in der zellulären Immunantwort zwischen Stammzelltransplantierten und gesunden Kontrollpersonen festgestellt werden, jedoch zeigten Kontrollpersonen tendenziell höhere Werte. Die Ergebnisse sind konsistent mit der Studie von Murray *et al.*, bei der ebenfalls vergleichbare zelluläre Immunantworten gegenüber SARS-CoV-2 zwischen Patienten nach Knochenmarkstransplantation und gesunden Kontrollpersonen beobachtet wurden (Murray *et al.*, 2022). Bei Patienten nach Stammzelltransplantation war nach Booster-Impfung ein leichter, nicht signifikanter Anstieg der neutralisierenden Antikörpertiter, verglichen zum Vorwert, zu verzeichnen. Kontrollpersonen zeigten hingegen einen deutlichen und signifikanten Anstieg der humoralen Immunantwort. In anderen Studien konnte ebenfalls eine reduzierte oder ausbleibende humorale Immunantwort bei Stammzelltransplantierten nach Impfung nachgewiesen werden (Atanackovic *et al.*, 2021; Dhakal *et al.*, 2021). Zusammen mit den Ergebnissen unserer Arbeit deuten die Daten darauf hin, dass eine stärkere Impfantwort generiert wird, wenn die immunsuppressive Therapie nach Stammzelltransplantation bereits abgesetzt ist.

Da bei Empfängern von Organen kein Absetzen der Immunsuppression möglich ist, sind Erkenntnisse über die Wirksamkeit von SARS-CoV-2-Impfstoffen bei dieser vulnerablen Gruppe besonders wichtig. In der vorliegenden Arbeit haben wir die zelluläre und humorale Immunantwort nach Booster-Impfung gegen SARS-CoV-2 bei Nierentransplantierten untersucht (Thümmler *et al.*, 2022a). Bei Patienten nach Nierentransplantation wurde eine signifikant geringere IFN-γ-Antwort beobachtet, verglichen mit gesunden Kontrollpersonen. In einer vorherigen Studie konnte ebenfalls eine reduzierte zelluläre Immunantwort gegen SARS-CoV-2 bei Hämodialyse-Patienten gezeigt werden (Simon *et al.*, 2022). Bei IL-2 zeigten sich vergleichbare Antworten zwischen beiden Kohorten. Diese Ergebnisse sind konsistent mit vorherigen Studien zur Impfantwort bei Nierentransplantierten nach erster Booster-Impfung (Affeldt *et al.*, 2022; Schrezenmeier *et al.*, 2021). Schrezenmeier *et al.* konnten zeigen, dass es bei Nierentransplantierten eine Woche nach der dritten Impfung gegen SARS-CoV-2 zu keinem Anstieg der IFN-γ-sezernierenden T-Zellen kommt, jedoch zu einem Anstieg der IL-2-sezernierenden T-Zellen (Schrezenmeier *et al.*, 2021). Der Großteil der Nierentransplantierten wies zudem eine signifikant schwächere humorale Immunantwort nach Booster-Impfung auf, verglichen mit gesunden Kontrollpersonen. Die Ergebnisse stimmen mit anderen Studien zur Impfantwort nach Booster-Impfung bei Nierentransplantierten und Dialyse-Patienten überein (Del Mastro *et al.*, 2023; Schrezenmeier *et al.*, 2021; Stumpf *et al.*, 2021). Simon *et al.* konnten bereits feststellen, dass ein Teil der geimpften Personen keine Immunantwort generiert (Simon *et al.*, 2022). In unserer Arbeit entwickelten rund 64 % der Nierentransplantierten eine humorale Immunantwort, sodass wir das Ergebnis von Simon *et al.* bestätigen konnten. Ferner zeigte unsere Arbeit vergleichbare Titer neutralisierender Antikörper gegenüber der Wildtyp-Variante, der Alpha- und der Delta-Variante von SARS-CoV-2 bei Patienten nach Nierentransplantation und gesunden Kontrollpersonen. Gegenüber der Omikron-Variante von SARS-CoV-2 fiel die humorale Antwort deutlich schwächer aus, sowohl bei Nierentransplantierten als auch bei der Kontrollkohorte. Studien anderer Arbeitsgruppen konnten zeigen, dass die Omikron-Variante von SARS-CoV-2 aufgrund von Fluchtmutationen der neutralisierenden Wirkung vieler Antikörper entgeht (Cao *et al.*, 2022; Tuekprakhon *et al.*, 2022). Del Mastro *et al.* konnten in einer Studie ebenfalls zeigen, dass Antikörper von Nierentransplantierten und gesunden Kontrollpersonen die Wildtyp-Variante von SARS-CoV-2 neutralisieren können, diese Wirkung bei Omikron jedoch stark reduziert ist (Del Mastro *et al.*, 2023)..

4.5 Immunantwort gegenüber den SARS-CoV-2-Varianten Delta und Omikron nach Durchbruchsinfektion

Während der Pandemie entwickelten sich fortlaufend neue Varianten von SARS-CoV-2, darunter die Delta- und Omikron-Variante (Hill *et al.*, 2022; Tegally *et al.*, 2022). Trotz mehrfacher Impfung und/oder Infektion mit vorherigen Varianten traten im Zusammenhang mit Delta und Omikron gehäuft Durchbruchsinfektionen auf. Dies ist vor allem auf die Fluchtmutationen dieser Varianten zurückzuführen, wodurch die neutralisierende Wirkung von Antikörpern stark vermindert ist und es zu Durchbruchsinfektionen kommt (Cao *et al.*, 2022; Carabelli *et al.*, 2023; Tuekprakhon *et al.*, 2022). Erkenntnisse über die Immunantwort bei Durchbruchsinfektionen sind wichtig, um ein Verständnis dafür zu entwickeln, ob diese Immunantworten vor einer erneuten Infektion schützen können. Wir haben in der vorliegenden Arbeit die humorale und zelluläre Immunantwort bei hospitalisierten Patienten mit COVID-19 bei vorliegender Infektion mit den Varianten Delta, Omikron BA.1 oder Omikron BA.5 analysiert (Bormann *et al.*, 2023). Bei *Enzyme-linked Immunosorbent Assay* (ELISA)-Antikörpertitern gegen das Spike- und das Nukleokapsid-Protein wurden keine signifikanten Unterschiede beobachtet. Dieses Ergebnis ist konsistent mit den Daten einer vorherigen Studie, bei welcher die Antikörpertiter nach Booster-Impfung und Infektion untersucht wurden (Rose *et al.*, 2022). Allerdings konnten wir eine starke Reduktion der neutralisierenden Antikörpertiter bei Patienten mit Infektion mit Omikron BA.1 oder BA.5 detektieren. Diese Ergebnisse stimmen mit vorherigen Studien überein, in denen gezeigt wurde, dass die Omikron-Varianten aufgrund der vielen Fluchtmutationen schlechter von Antikörpern neutralisiert werden (Cao *et al.*, 2022; Tuekprakhon *et al.*, 2022; Wang *et al.*, 2023). Wir konnten zudem feststellen, dass Durchbruchsinfektionen mit Delta zu höheren Titern an neutralisierenden Antikörpern gegenüber der Wildtyp-, Alpha- und Delta-Variante führten, wohingegen Durchbruchsinfektionen mit Omikron BA.5 nicht zu höheren Titern führten. Eine vorherige Studie konnte ebenfalls einen höheren Titer gegen verschiedene, vorherige SARS-CoV-2-Varianten nach Durchbruchsinfektion mit der Delta-Variante beobachten (Seaman *et al.*, 2022). Patienten, die mit der Delta- oder Omikron BA.5-Variante infiziert waren, hatten eine höhere zelluläre Immunantwort, verglichen mit Patienten mit einer Omikron BA.1-Infektion. Dies könnte daran liegen, dass die mononukleären Zellen des peripheren Blutes (engl.: *peripheral blood mononuclear cells*, PBMC) mit

dem Spike-Protein stimuliert wurden, welches selektiv die Mutationen der Variante Delta bzw. Omikron BA.5 enthielt und nicht die von BA.1.

4.6 Antidepressiva als alternative Behandlung und Prävention von COVID-19

Die sich immer neu entwickelnden Varianten von SARS-CoV-2 führen weiterhin zu Durchbruchsinfektionen. Eine durchgemachte Infektion und die derzeit verfügbaren Impfstoffe können nur unzureichend Schutz vor einer Infektion mit den neuen Varianten vermitteln. Die Wirksamkeit von Medikamenten wie beispielsweise von monoklonalen Antikörpern ist ebenfalls reduziert (Kurhade *et al.*, 2023; Wang *et al.*, 2023). Es bedarf einer ständigen Weiterentwicklung der Medikamente und Impfungen, was ein zeit- und kostenintensiver Prozess ist. Wir haben in dieser Arbeit die bereits zugelassenen und gut charakterisierten Antidepressiva Fluoxetin und Sertralin auf ihre inhibierende Funktion gegenüber der Replikation von SARS-CoV-2-Varianten *in vitro* untersucht (Thümmler *et al.*, 2024). Wir konnten zeigen, dass Fluoxetin und Sertralin die Infektion von Zellen mit Pseudotyp Virus-ähnlichen Partikeln des Spike-Proteins verschiedener SARS-CoV-2-Varianten bis zu 80 % inhibieren können. Diese Ergebnisse sind konsistent mit Daten vorheriger Studien zur antiviralen Wirkung von Fluoxetin und Sertralin gegenüber SARS-CoV-2 (Chen *et al.*, 2022; Fred *et al.*, 2021). Die antivirale Wirkung der Antidepressiva wurde initial gegenüber acht verschiedenen SARS-CoV-2-Varianten gezeigt. In Versuchen mit Virus-Isolaten von Patienten konnten wir eine komplette Inhibition der Replikation von SARS-CoV-2 D614G, Alpha, Delta, Omikron BA.1 und Omikron BA.5 bei 30 µM Fluoxetin bzw. 10 µM Sertralin beobachten. Für diese Versuche wurden A549-AT-Zellen genutzt, eine Zelllinie humaner Lungenepithelzellen, welche unter anderem den Serotoninrezeptor 5-HT exprimieren, an welchen Antidepressiva wie Fluoxetin und Sertralin binden (Bayer *et al.*, 2007). Ein möglicher Grund für die stärkere Wirkung von Sertralin bei geringerer Konzentration könnte sein, dass Sertralin eine höhere Affinität zum Rezeptor 5-HT aufweist als Fluoxetin (DAZ, 2000). Unsere Daten deuten zudem darauf hin, dass die Antidepressiva unabhängig von Mutationen im Spike-Protein von SARS-CoV-2 wirken und somit gegenüber den bisher bekannten SARS-CoV-2-Varianten wirksam sind. Fluoxetin und Sertralin wurden als funktionelle Inhibitoren der sauren Sphingomyelinase (engl.: *functional inhibitors of the acid sphingomyelinase*, FIASMA) identifiziert, welche eine entscheidende Rolle beim Eintritt von SARS-CoV-2 in Wirtszellen spielt, indem sie den Abbau von Sphingomyelin zu Phosphoylcholin und

Ceramid katalysiert (Geiger *et al.*, 2022; Schloer *et al.*, 2020). Es ist bereits bekannt, dass SARS-CoV-2 an Ceramid-angereicherten Regionen in die Wirtszelle eindringt (Kornhuber *et al.*, 2022; Törnquist *et al.*, 2021). Eine Hemmung dieser Sphingomyelinase stellt einen Mechanismus dar, das Eindringen von SARS-CoV-2, unabhängig von der Variante, zu inhibieren. Konsistent mit unseren Ergebnissen konnten klinische Studien bei Gabe von Fluoxetin oder Sertralin einen milderen Verlauf von COVID-19 und eine geringere Mortalitätsrate beobachten (Hoertel, 2021; Lenze *et al.*, 2020; Reis *et al.*, 2022).

4.7 Schlussfolgerung

Die im Rahmen der vorliegenden Dissertation durchgeführten Arbeiten leisten einen wichtigen Beitrag zum Verständnis der Immunantwort gegenüber SARS-CoV-2 nach Impfung und Infektion bei verschiedenen Kohorten. Des Weiteren konnten wichtige Erkenntnisse über die Wirksamkeit verschiedener Therapieansätze wie die Gabe von monoklonalen Antikörpern und Rekonvaleszentenplasma gewonnen werden. Zusätzlich konnten wir zeigen, dass Fluoxetin und Sertralin die Replikation von SARS-CoV-2-Varianten *in vitro* inhibieren können.

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6. Anhang

6.1 Abkürzungsverzeichnis

%	Prozent
ACE2	*angiotensin-converting enzyme 2* / Angiotensin-konvertierende Enzym 2
APC	*antigen presenting cells* / antigenpräsentierende Zellen
ARDS	*acute respiratory distress syndrome* / akutes Atemnotsyndrom
BMG	Bundesministerium für Gesundheit
BMI	*Body Mass Index* / Körpermassenindex
CD	*cluster of differentiation* / Differenzierungscluster
COVID-19	*coronavirus disease 2019*
CYP3A4	Cytochrom P450 3A4
DC	*dendritic cells* / dendritische Zellen
DNA	*deoxyribonucleic acid* / Desoxyribonukleinsäure
E	*envelope* / Hülle
ELISpot	*Enzyme Linked Immuno Spot*
ER	*endoplasmatic reticulum* / endoplasmatisches Retikulum
EU	Europäische Union
FFP-2	*filtering face piece 2* / filternder Gesichts-Aufsatz der Schutzklasse 2
FIASMA	*functional inhibitors of acid sphingomyelinase activity* / funktionelle Inhibitoren der sauren Sphingomyelinase-Aktivtät
GNB3	*G protein subunit beta 3* / Protein G Untereinheit Beta-3
HR	*heptad repeat* / Heptadwiederholungen
IFN	Interferon
IL	Interleukin
JAK-I	Januskinase-Inhibitor
kDa	Kilodalton
mAb	*monoclonal antibodies* / monoklonale Antikörper
M	*membrane* / Membran
MHC	*major histocompatibilty complex* / Haupthistokompatibilitätskomplex
M^{Pro}	*main protease* / Hauptprotease
MERS-CoV	*middle east respiratory syndrome-coronavirus*
mRNA	*messenger-RNA*

N	*nucleocapsid* / Nucleocapsid
NK	*natural killer cells* / natürliche Killerzellen
nm	Nanometer
NSP	*nonstructural proteins* / Nichtstrukturproteine
ORF	*open reading frame* / offener Leserahmen
PAMP	*pathogen-associated molecular patterns* / Pathogen-assoziierte molekulare Muster
PBMC	*peripheral blood mononuclear cells* / mononukleäre Zellen des peripheren Blutes
PRR	*pathogen recognition receptors* / Mustererkennungsrezeptoren
RBD	*receptor-binding domain* / Rezeptor-bindende Domäne
RKP	Rekonvalszentenplasma
RLR	*retinoic acid inducible gene I (RIG-I)-like receptor* / Retinsäure-induzierbaren Gen-I-ähnliche Rezeptor
RNA	*ribonucleic acid* / Ribonukleinsäure
S	*spike* / Spike
s/n/c	*substitutions per nucleotide per cell infection* / Substitution pro Nukleotid pro Zellinfektion
SARS-CoV	*severe acute respiratory syndrome-coronavirus* / schweres akutes respiratorisches Syndrom Coronavirus
SARS-CoV-2	*severe acute respiratory syndrome-coronavirus type 2* / schweres akutes respiratorisches Syndrom Coronavirus Typ 2
STIKO	Ständige Impfkommission
TMPRSS2	*transmembrane protease serine subtype 2* / transmembrane Serinprotease 2
TLR	*toll-like-receptor* / Toll-ähnlicher Rezeptor
TNF-α	*tumor necrosis factor alpha* / Tumornekrosefaktor-alpha
VOC	*variants of concern* / besorgniserregende Varianten
WHO	*World Health Organization* / Weltgesundheitsorganisation

6.2 Abbildungsverzeichnis

6.3 Publikationsliste

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„Die Menschen, die wir lieben, gehen niemals wirklich von uns weg.
Sie leben in unseren Herzen weiter.“
(aus „Der kleine Prinz“)

6.5 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

6.6 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 „SARS-CoV-2: Immunantwort und antivirale Behandlung“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Laura Thümmler befürworte und die Betreuung auch im Falle eines Weggangs, wenn nicht wichtige Gründe dem entgegenstehen, weiterführen werde.

Essen, den _____ _____
Prof. Dr. med. Monika Lindemann

Hiermit erkläre ich, gem. § 7 Abs. (2) d) + f) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient, bei der Abfassung der Dissertation nur die angegeben Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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Laura Thümmler

Hiermit erkläre ich, gem. § 7 Abs. (2) e) + g) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät/Fachbereich abgelehnt worden ist.

Essen, den _____ _____
Laura Thümmler