Improved SARS-CoV-2 neutralization of Delta and Omicron variants of concern after fourth vaccination in hemodialysis patients

Cho-Chin Cheng¹,†, Louise Platen²,†, Catharina Christa¹, Myriam Tellenbach², Verena Kappler², Romina Bester¹, Bo-Hung Liao¹, Christopher Holzmann-Littig²,³, Maia Werz², Emely Schönhals², Eva Platen⁴, Peter Eggerer⁵, Laëtitia Tréguer⁶, Claudius Küchle², Christoph Schmaderer², Uwe Heemann², Lutz Renders²,³, Ulrike Protzer¹,⁷,⁸,#,* and Matthias Christoph Braunisch²,#,*

¹ Technical University of Munich, School of Medicine, Institute of Virology, Klinikum rechts der Isar, Munich Germany
² Technical University of Munich, School of Medicine, Department of Nephrology, Klinikum rechts der Isar, Munich, Germany
³ Technical University of Munich, School of Medicine, TUM Medical Education Center, Munich, Germany
⁴ Kidney Center Eifel Dialyse, Mechernich, Germany
⁵ KfH Kidney Center Harlaching, Munich-Harlaching, Germany
⁶ KfH Kidney Center, Traunstein, Germany
⁷ German Center for Infection Research (DZIF), Partner Site, Munich, Germany
⁸ Helmholtz Munich, Institute of Virology, Munich, Germany

† these authors share junior authorship
# these authors share senior authorship

Running title: COVID-19 vaccination in HD patients

* Correspondence to:
Prof. Dr. Ulrike Protzer, Technical University of Munich, School of Medicine, Institute of Virology, Helmholtz Center Munich, Klinikum rechts der Isar, Munich Germany and German Center for Infection Research (DZIF), Partner Site, Munich, Germany; Phone 0049 (0) 89 4140 6863; Fax: 0049 (0) 89 4140 6823; email: Protzer@tum.de
PD Dr. Matthias Christoph Braunisch, Technical University of Munich, Germany; School of Medicine, Department of Nephrology, Klinikum rechts der Isar; 0049 (0) 89 4140 2231; Fax: 0049 (0) 89 4140 7734; email: Matthias.Braunisch@mri.tum.de

NOTE: This preprint reports new research that has not been certified by peer review and should not be used to guide clinical practice.
Abstract

Background
Hemodialysis patients are exposed to a markedly increased risk when infected with SARS-CoV-2. To date it is unclear if hemodialysis patients benefit from a fourth vaccination.

Methods
A total of 142 hemodialysis patients (median age 72.6 years, 33.8% female) received four COVID-19 vaccinations between December 2020 and March 2022. RDB binding antibody titers were determined in a competitive surrogate neutralization assay. Vero-E6 cells were infected with SARS-CoV-2 variants of concern (VoC) Delta (B.1.617.2) or Omicron (B.1.1.529, sub lineage BA.1) in a biosafety level 3 laboratory to determine serum infection neutralization capacity before and after vaccination.

Results
After the fourth vaccination serum infection neutralization capacity significantly increased from a 50% inhibitory concentration (IC50, serum dilution factor 1:x) of 247.0 (46.3-1560.8) to 2560.0 (1174.0-2560.0) for the Delta VoC, and from 37.5 (20.0-198.8) to 668.5 (182.2-2560.0) for the Omicron VoC (each p<0.001). A significant increase of the neutralization capacity was even observed for patients who had high antibody titers after three vaccinations (p<0.001). Univariate regression analysis indicated immunosuppressive medication (p=0.001) and hepatitis B vaccination non-response (p=0.046), and multivariate analysis immunosuppressive medication as the only factor associated with a reduced effect against Delta (p<0.001). Ten patients with SARS-CoV-2 breakthrough infection before the fourth vaccination had by trend lower prior neutralization capacity for Omicron (p=0.051).

Conclusions
Our findings suggest that hemodialysis patients benefit from a fourth vaccination in particular in the light of the highly infectious SARS-CoV-2 Omicron variant. A routinely applied four-time vaccination seems to broaden immunity against variants and would be recommended in hemodialysis patients.

Keywords: hemodialysis, SARS-CoV-2, COVID-19 vaccination, in-vitro viral neutralization
Introduction

In hemodialysis patients, a SARS-CoV-2 infection is associated with a markedly increased morbidity and mortality in comparison to the general population with a mortality rate of more than 20% in hospitalized patients \(^1\-^3\). In the last two years we have learned that double vaccination might not be enough to achieve adequate long-term immune protection in all hemodialysis patients and triple vaccination offers significantly better protection against COVID-19 in this patient group \(^1\,^4\). Even if an infection cannot always be prevented after booster vaccination, the course of the COVID-19 disease in general is milder depending on the number of vaccinations in hemodialysis patients \(^5\). However, even in hemodialysis patients with an inadequate immune response after multiple vaccinations, morbidity remains significantly increased \(^6\).

A third vaccination is associated with an increased virus neutralization capacity in the general population \(^7\,^8\). Therefore, to date, the third vaccination became part of the standard vaccination regimen, and meanwhile, a fourth vaccination is recommended in risk groups like hemodialysis patients as a so-called second booster \(^9\). The usefulness of a third and now of a fourth vaccination is based on data from the general population and was obtained during the SARS-CoV-2 Delta wave. However, infections with the Omicron variant of concern (VoC) became predominant in 2022 \(^1^0\). Omicron resulted in dramatically increased infection rates in the general population and in hemodialysis patients.

The question remains, whether the currently recommended vaccination regimen (four vaccinations, i.e. a second booster) in hemodialysis patients also offer effective protection towards VoC Omicron. It has not been clarified yet with certainty as to whether hemodialysis patients can further extend their immune response after a fourth vaccination. Interestingly, even if the immune response of a COVID-19 vaccination is less pronounced in hemodialysis patients compared to the normal population, there are indications that immune responses might be sustained in those hemodialysis patients who develop antibody responses \(^1^1\).

The aim of this study was to investigate whether hemodialysis patients benefit from a fourth vaccination and if the immune response after the fourth vaccination has a comparable efficacy towards the VoCs Delta and Omicron. Furthermore, we examined the serum neutralization capacity of those hemodialysis patients who experienced a breakthrough infection after their third vaccination.

Here, we present the results of the live-virus infection neutralization of SARS-CoV-2 Delta and Omicron VoCs and antibody-mediated immunity before and after the fourth COVID-19 vaccination in a cohort of 142 hemodialysis patients.
Material and Methods

Study design

The COVIIMP study (German: “COVID-19-Impfansprechen immunsupprimierter Patient*innen”) is a prospective observational study examining the COVID-19 immunization success and the clinical course of COVID-19 in immunocompromised patients who received active or passive immunization against SARS-CoV-2 as recommended by the German health authorities. Patients were enrolled between April 1st, 2021 and March 20th, 2022. All patients are immunocompromised due to immunosuppressive medication after kidney transplantation, a rheumatologic disease demanding immunomodulatory therapy or End Stage Kidney Disease (ESKD) requiring maintenance hemodialysis.

The study is conducted at the university hospital Klinikum rechts der Isar of the Technical University of Munich in Munich and collaborating outpatient care centers. All participants provided written informed consent. The study, conforming to the ethical guidelines of the Helsinki Declaration, was approved by the Medical Ethics Committee of the Klinikum rechts der Isar of the Technical University of Munich (approval number 163/21 S-SR, March 19th, 2021) and registered at the Paul Ehrlich Institute (NIS592).

Study population

Of 513 enrolled patients, a total of 142 patients requiring maintenance hemodialysis was selected. These patients received four COVID-19 vaccinations between December 29th, 2020 and March 20th, 2022 and underwent blood analysis before and after the fourth vaccination (Figure 1A). This subpopulation was recruited in four dialysis centers (Klinikum rechts der Isar, KfH Kidney Center Traunstein, Kidney Center Eifeldialyse, KfH Kidney Center München-Harlaching).

Demographic data, medical history including current dialysis vintage, underlying kidney disease, history of transplantation and comorbidities as assessed by the Charlson Comorbidity Index (CCI) were collected. Immunosuppressive medication during the vaccination period was documented.

Hepatitis B vaccination

Hepatitis B vaccination status was based on medical reports and, if available, serological laboratory data on anti-HBs antibodies. Patients were considered as non-responder if an anti-HB titer below 10 IU/l despite three hepatitis B vaccinations was documented or if their treating physicians classified them as a hepatitis B non-responder, according to local standards.

SARS-CoV-2 infection
We identified participants as SARS-CoV-2 convalescent, if they had a prior positive SARS-CoV-2 PCR or at least one positive serological SARS-CoV-2 nucleocapsid-specific IgG measurement. The clinical course and treatment were documented by structured interrogations.

Sample collection
Blood was collected for analysis in median 2 (2.0 - 3.25) days before (analysis 1) and 26 (26.0-26.0) days after (analysis 2) the fourth vaccination.

SARS-CoV-2 IgG assay
Antibodies in patients’ sera were detected using commercial surrogate paramagnetic particle chemiluminescence immunoassays (CLIA, Yhlo Biotechnology, Shenzhen, China) performed on the iFlash 1800 platform. Nucleocapsid-specific IgG antibodies (anti-N IgG) were determined using the 2019-nCoV IgG kit. The surrogate neutralization assay (NAb) was performed with the iFlash 2019-nCoV NAb kit based on the competition of serum antibodies with recombinant angiotensin-converting enzyme 2 for binding the SARS-CoV-2 Wuhan strain receptor binding domain (RBD) and has been adapted for quantification to manufacturer’s instructions. The cut-off level for seropositivity was set at 10 neutralizing units per milliliter (AU/ml) according to manufacturer’s instructions. Surrogate neutralization activity expressed as AU/ml can be adapted to WHO standard (AU/ml x 2.4 = BAU/ml [binding units / ml]). The maximum measurable value for NAb was 800 AU/ml, lower level of detection was 4 AU/ml. If values exceeded the upper limit of quantification a value of 801 AU/ml was used for statistical analysis. NAb high-response was defined as levels ≥700 AU/ml before the fourth vaccination. N-specific IgGs ≥10 AU/ml were qualitatively determined as reactive.

SARS-CoV-2 infection-neutralization assay
Serum infection-neutralization capacity was analyzed as previously described. Briefly, SARS-CoV-2 isolates, which were kindly provided by Prof. Oliver Keppeler’s group from the Institute of Virology and the Max von Pettenkofer Institute in Munich, were isolated from nasopharyngeal swabs of COVID-19 infected individuals. To obtain high titer of virus stock, Vero-E6 cells were infected with VoC Delta (B.1.617.2, GISAID EPI ISL: 2772700) or Omicron (B.1.1.529, sub lineage BA.1, GISAID EPI ISL: 7808190) and incubated in Dulbecco’s modified Eagle’s medium. After 2-3 days inoculation, cell culture medium was collected, centrifuged and the virus-containing supernatant was stored at -80°C. Prior to the neutralization experiments, viral titers were verified by plaque assay and strain identity was confirmed by next-generation sequencing. All measurements were performed using serum
samples that were stored at -80°C and defrosted and stored at 4°C on the day before the analysis. Samples from all patients were analyzed in parallel. For quantification of the neutralization capacity, two-fold serial dilutions of the sera from 1:20 to 1:2560 were incubated with a predefined multiplicity of infection (MOI) of 0.03 (450 PFU/15,000 cells/well) of either of the VoCs for 1 hour at 37°C. The MOI was determined from an in-cell ELISA pre-test by which we observed viral signal saturation 24 hours after infection. After the 1-hour inoculation, the inoculum was transferred onto pre-seeded Vero E6 cells for another one-hour incubation at 37°C. The infection was terminated after one day and followed by an in-cell ELISA for the detection of SARS-CoV-2 N-protein. Cells were fixed with 4% paraformaldehyde and permeabilized by 0.5% saponin buffer. After blocking with 10% goat serum, cells were stained using anti-SARS-CoV-2-N primary (40143-T62, Sino Biological) and goat anti-rabbit IgG2a-HRP secondary antibody (EMD Millipore / #12-348), and eventually transformed into luminescence signal by adding substrate tetramethylbenzidine (TMB). To determine serum IC50 values, a nonlinear regression curve was applied and the dilution factor at which 50% inhibition was observed and was calculated using PRISM software (GraphPad). Patients were classified as low or non-responder if the IC50 value of the infection neutralization was ≤1:20.

**Statistical Analysis**

Categorical variables are presented as frequencies and percentages. Continuous variables are expressed as mean ± standard deviation (SD) or median and interquartile range (IQR), as appropriate. Group differences were tested with the $\chi^2$ test or Fisher test. The independent samples $t$-test or Mann-Whitney-U test was used for continuous variables, as appropriate. Paired samples were examined with the Wilcoxon test and the McNemar test, as appropriate. Spearman correlation was used for correlation analysis.

Univariate and multivariate linear regression models were applied to identify possible predictors of the infection-neutralizing capacity of VoC Delta or Omicron (IC50) out of the following candidate variables: age, dialysis vintage, presence of immunosuppression, comorbidities, and hepatitis B vaccination non-response. Possible predictors were preselected prior to the statistical analysis. Logistic regression was used to examine the neutralization capacity towards an infection with SARS-CoV-2.

All tests were conducted two-sided and $p < 0.05$ was considered significant. Statistical analysis was performed using R version 4.0.2 (R Foundation for statistical Computing, Vienna, Austria).
**Results**

Overall, 142 patients on maintenance hemodialysis were included (Figure 1A). Patients had a median age of 72.6 (61.5-80.6) years. 48 (33.8%) patients were female. The median dialysis vintage was 48.9 (21.3 - 83.7) months. At the time of the first, second, third and fourth vaccination, 124 (87.3%), 125 (88.0%), 136 (95.8%) and 142 (100%) were on maintenance hemodialysis, respectively. Further details of patient characteristics can be found in Table 1 for all patients, and stratified by infection neutralization response against VoC Omicron.

**COVID-19 and vaccinations**

All patients received four vaccinations. Eight and six patients received their first and second vaccination with AZD1222 (Vaxzevria®) by AstraZeneca, respectively. All other vaccinations were done with mRNA-based vaccines (BNT162b2 by BioNTech-Pfizer or mRNA-1273 by Moderna). Fifteen patients received two or more vaccinations with mRNA-1273 (Spikevax®, Moderna), the remaining patients received BNT162b2 (Comirnaty®, BioNTech-Pfizer). Median duration between the first and the fourth vaccination was 338.0 (333.0-342.0) days and between the third and the fourth vaccination was 126.0 (105.0-126.0) days, respectively. Median duration between the third vaccination and the first blood sampling was 4.1 (3.4-4.1) months.

A SARS-CoV-2 breakthrough infection indicated by SARS-CoV-2 nucleocapsid-specific IgG antibody positivity occurred in 22 (15.5%) individuals before the second blood sampling after the fourth vaccination (Figure 1B). In these patients, the average time between the SARS-CoV-2 infection and the second blood collection was 215.7 ±223.3 days. Of these, seven patients had no known history of SARS-CoV-2 infection, but were classified as convalescent due to positive anti-nucleocapsid IgG detection. Four (18.2%) of the 22 infected patients were treated with SARS-CoV-2 specific monoclonal antibodies. Ten (7.0%) patients had a SARS-CoV-2 infection between the two blood drawings before and after fourth vaccination. No patient reported recurrent SARS-CoV-2 infections (Figure 1B).

**Immunosuppression**

Immunosuppressive medication was prescribed in 16 (11.3%) patients during the observation period. Reasons for immunosuppression were history of organ transplantation in eight, cancer treatment in four, underlying kidney disease in two and unknown causes in two other patients. Six patients received more than one immunosuppressive. Immunosuppressive agents were
glucocorticoids in 14, tacrolimus in four, mycophenolate mofetil in three, and others in two patients (lenalidomide, rituximab and reduced dose CHOP).

**Impact of four vaccinations on neutralization capacity and NAbs**

After the fourth vaccination significantly increased serum neutralization capacities were found for both VoCs, Delta and Omicron. Infection neutralization capacity for Delta increased from a median IC50 (serum dilution factor, 1:x) of 247.0 (46.3-1560.8) to 2560.0 (1174.0-2560.0), and for Omicron from 37.5 (20.0-198.8) to 668.5 (182.2-2560.0) (each p<0.001) ([Figure 2 A, B](#)). NAb levels significantly increased from 721.0 (184.5-801.0) to 801.0 (801.0-801.0, p<0.001) ([Figure 2C](#)). Serum neutralization capacity after the fourth vaccination was significantly lower for Omicron compared to Delta (668.5 [182.2-2560.0] vs 2560.0 [1174.0-2560.0], p<0.001). Similar to the overall cohort, when analyzing only NAb high-responder, we found a significant increase for the neutralization capacity for both VoCs, Delta (1172.5 [382.8-2560.0] vs 2560.0 [2560.0-2560.0], p<0.001) and Omicron (170.5 [56.3-468.5] vs 2553.0 [640.2-2560.0], p<0.001).

Patients with a serum IC50 ≤20 were classified as low, those with no detectable neutralization as non-responder. Regarding both, Delta and Omicron infection neutralization capacity, significantly fewer patients (Delta: 30 vs 5; Omicron: 61 vs 12, each p<0.001) were low or non-responders after the fourth vaccination. The percentage of NAb responder was already very high before the fourth vaccination and did not further increase significantly (136 vs 139, p=0.13) ([Figure 3](#)).

After the fourth vaccination, infection neutralization of Delta and NAb titers were correlated highly significantly (p<0.0001) but moderately (rho=0.50) positive. Similarly, the correlation of the infection neutralization capacity of Omicron and NAb was highly significant (p<0.0001) and moderately (rho=0.44) positive.

Univariate regression analysis showed significantly reduced neutralization capacity for Delta after the fourth vaccination if immunosuppressive medication (p=0.001) or hepatitis B vaccination non-response (p=0.046) was present ([Table 2A, left column](#)). Multivariate analysis showed a reduced Delta neutralization capacity after the fourth vaccination if immunosuppressive medication (p<0.001) was taken and - by trend - if hepatitis B vaccination non-response was present (p=0.070) ([Table 2A, right column](#)). For Omicron infection neutralization, no such association was present in univariate or multivariate analyses ([Table 2B](#)). Univariate and multivariate analyses showed reduced NAbs after the fourth vaccination if immunosuppressive medication was prescribed ([Table 2C](#)).
When comparing serum neutralizing capacities after the fourth vaccination between subgroups we saw significant differences in Delta infection neutralization if immunosuppression was prescribed (716.5 [176.2-2560.0] vs 2560.0 [1678.0-2560.0], p=0.002) (Figure 4A), and by trend for Omicron (193.5 [80.0-1481.8] vs 820.5 [214.3-2560.0], p=0.067) (Figure 4B). Patients with a history of SARS-CoV-2 infection had by trend a higher IC50 value for Delta (2560.0 [2560.0-2560.0] vs 2560.0 [955.2-2560.0], p=0.069) and significantly higher values for Omicron neutralization (1952.0 [893.2-2560.0] vs 489.0 [157.8-2560.0], p=0.013) (Figure 4C, D). If patients were classified as hepatitis B vaccine non-responder, they had significantly lower IC50 values for Delta neutralization (2460 [531.0-2560.0] vs 2560.0 [1765.0-2560.0], p=0.018) (Figure 4E), but not for Omicron neutralization (553.0 [103.5-1762.5] vs 760 [254.0-2560.0], p=0.18) (Figure 4F).

**Impact of NAb and infection neutralization capacity on breakthrough infections**

Finally, the ten patients with a SARS-CoV-2 infection before the fourth vaccination had by trend lower serum neutralization capacity for Omicron at the first blood sampling being almost significant (10.0 [0.0-26.8] vs 42.5 [20.0-217.5], p=0.051) (Figure 5). No difference was detected for serum neutralization capacity of Delta (189.5 [42.5-1167.0] vs 257.5 [50.8-1583.8], p=0.54). The VoC causing the SARS-CoV-2 infection was not determined. Omicron serum neutralization capacity after infection but before the fourth vaccination was not able to predict the COVID-19 breakthrough infection (p=0.29) when using logistic univariate regression.

**Discussion**

This prospective observational study demonstrates that hemodialysis patients benefit from a fourth COVID-19 vaccination. Serum infection neutralization capacity increased more than 10-fold for Delta and almost 18-fold for Omicron after a fourth vaccination indicating a better protection from infection with these highly infectious SARS-CoV-2 VoCs. The strength of our study is the examination of live-virus infection neutralization capacity of patients’ sera for the two most recent SARS-CoV-2 VoCs, Delta and Omicron. These two variants are also most distant from the original SARS-CoV-2 stain which was used to design the vaccines currently in use. Thus, the protective capacity against the new variants was hard to predict. Our observation is of high importance since hemodialysis patients show reduced immunological responses to vaccination compared to healthy controls which may be explained in the context of uremia. The hemodialysis patients in our study showed a significantly increased capacity to neutralize both SARS-CoV-2 VoCs, Delta and Omicron,
after the fourth vaccination. This translates into significantly higher percentages of vaccine responders. Our results are consistent with previous reports of significantly increasing anti-spike antibody titers after the fourth vaccination in hemodialysis patients \cite{15, 16}, but add an important quality as these antibody titers were determined against the original vaccine strain of SARS-CoV-2 but not against the currently circulating variants. Furthermore, in line with a previous work with a pseudovirus assay we found a reduced neutralization capacity for VoC Omicron compared to Delta \cite{17}.

Patients with a breakthrough infection between the first and the second blood sampling had a lower neutralization capacity for Omicron only slightly missing significance. This was not seen for the Delta neutralization capacity. This might be at least partly explained by the fact, that the analysis was performed between February and March 2022 when the Omicron wave peaked in Germany. Hence, with over 99.3% the majority of COVID-19 cases were Omicron infections at that time \cite{10}. Logistic regression was not able to predict a SARS-CoV-2 breakthrough infection possibly due to the low infection rate after the first blood collection. In French hemodialysis patients a response towards wild type virus neutralization two weeks after the third vaccination was present in approximately 54% of patients \cite{5}. Another study in a British cohort found response rates of 97% and 72% for Delta and Omicron, respectively, in hemodialysis patients one month after the third BNT162b2 vaccination when applying an IC50 cut-off at 40 \cite{18}. We found response rates of 57% for Omicron and 79% for Delta four months after the third vaccination. Methodological differences in the neutralization assays \cite{5, 18} as well as time interval differences associated with reduced immune responses to vaccination \cite{19} might explain these variations.

In line with previous reports \cite{19-21}, we identified immunosuppressive agents as a predictor for lower neutralization capacity which were primarily prescribed to patients with a history of kidney transplantation. Patients on immunosuppressive medication had significantly lower neutralization capacity for Delta and, by trend, for Omicron. Other studies, however, did not identify immunosuppressive drugs as a predictor of neutralization capacity in hemodialysis patients \cite{5}. Discrepancies might be explained due to the specific immunosuppressive agents prescribed, as a previous study showed significantly reduced seroconversion rates in patients on anti-CD20 therapy regimes or mycophenolate mofetil, especially in the combination with glucocorticoids \cite{21}, substances which were also prescribed in our patients.

Interestingly, a positive hepatitis B vaccination response was by trend associated with an improved neutralization capacity. This was, however, only seen for the Delta VoC. It thus
needs to be determined by further studies if hepatitis B vaccination response might serve as a surrogate for COVID-19 vaccination response or vice versa.

In clinical routine only NAb or anti-S antibody levels are readily and widely available. These, however, only detect the response against the original SARS-CoV-2 strains and not against the VoCs. Before the fourth vaccination, NAb were present in 96% of the study population and response rates did not further increase after the fourth vaccination. However, when looking at the absolute change of NAb titers, NAb increased significantly after the fourth vaccination. This increase was less pronounced than the increase in IC50 values in infection neutralization due to the limited range of the assay although the SARS-CoV-2 stain used for vaccination and in the NAb assay were identical. Although, NAb levels are regarded highly predictive of immune protection \textsuperscript{22}, this further demonstrates the limitation of the assays routinely available in the clinics.

We do not have outcome data of our cohort after the fourth vaccination with regard to infection prevention, but decreased COVID-19 incidence and severity in vaccinated hemodialysis patients has been observed by others \textsuperscript{5}. Thus, the presence of increasing NAb levels might still be a good indicator of vaccine response after the fourth vaccination and therefore useful in clinical routine. Nevertheless, further prospective studies have to evaluate how well a fourth vaccination protects hemodialysis patients from SARS-CoV-2 infection and COVID-19, respectively.

In a study by Espi \textit{et al.}, a third vaccination did not improve the immune response in patients that had already shown a high response after the second vaccination and was associated with more side effects \textsuperscript{5}. In our cohort, we did not record side effects but observed even in NAb high-responder a further significant increase of neutralization capacity and, more importantly, a very strong increase in infection-neutralization capacity of the two most prevalent SARS-CoV-2 VoCs. Differences worth mentioning to the work of Espi \textit{et al.} might be the application of a third dose three months after the second dose. Whereas, the fourth vaccination was administered at least four months after the third dose in our cohort. Nevertheless, reports of increased side effects in high-responders may argue for an individual decision-making process depending on routinely available antibody levels.

Finally, some limitations have to be mentioned. We examined the neutralization capacity of the Omicron sub lineage BA.1. The question remains if these results are generalizable to other Omicron subvariants which are currently becoming predominant. Further studies have to show if improved neutralization capacity after the fourth vaccination is associated with COVID-19 incidence and severity.
Conclusion

In conclusion, a fourth vaccination against SARS-CoV-2 significantly improves the antibody-mediated immune response in hemodialysis patients. A routinely applied four-time vaccination regimen therefore seems reasonable in hemodialysis patients. NAbs might be a good clinical surrogate of vaccination response. However, the presence of neutralization antibody titers above the upper limit of quantification should not hinder a fourth vaccination as this further improves and broadens live-virus infection neutralization. Further outcome data is necessary to evaluate the effect of a fourth vaccination towards SARS-CoV-2 breakthrough infection incidence and COVID-19 severity.
Key learning points
(3 bullet points per topic with max 50 words per point)

What is already known about this subject?

- Hemodialysis patients are a vulnerable patient group when infected with SARS-CoV-2, especially when also exposed to immunosuppressive medication.
- Four vaccinations increase neutralization antibody titers in hemodialysis patients.
- After a fourth vaccination, neutralization of the SARS-CoV-2 Omicron variant remained lower compared to the Delta variant in a pseudovirus assay.

What this study adds?

- A fourth vaccination in hemodialysis patients largely (10- and 18-fold) improves the live-virus infection neutralization capacity for the most prevalent variants of concern, Delta and Omicron, respectively.
- Even in patients with high anti-S or neutralizing antibody titers binding the original virus, a parameter that is available in clinical routine, a further increase in neutralization capacity was demonstrated.
- Immunosuppressive medication was associated with reduced neutralization capacity for the Delta, but not the Omicron variant of concern.

What impact this may have on practice

- A routinely applied four-time vaccination regimen seems reasonable in hemodialysis patients.
- A significantly improved neutralization of SARS-CoV-2 variants might warrant a fourth vaccination also in patients with high antibody titers in routine assays.
Significance statement

120 words

Hemodialysis patients are a vulnerable patient group when infected with SARS-CoV-2 especially when also exposed to immunosuppressive medication. It is unclear if hemodialysis patients benefit from four vaccinations in terms of virus neutralization. We found a significantly improved infection neutralization capacity after the fourth vaccination in hemodialysis patients for both SARS-CoV-2 variants of concern, Delta and Omicron. Furthermore, also neutralization antibody high responder showed an improved and broadened virus neutralization capacity. Our findings suggest that hemodialysis patients benefit from a fourth vaccination against SARS-CoV-2, even when high neutralization antibody titers are already present. A routinely applied four-time vaccination regimen might be reasonable in hemodialysis patients.
Authors’ Contributions
CCC, LP, LR, UP, and MCB wrote the first draft of the manuscript. LP and MCB performed the statistical analysis. LP, MT, VK, CHL, MW, ES, EP, PE, LT, CK, CS contributed to blood sampling and data acquisition. CCC, CC, RB, BHL performed in vitro virus neutralization assays and measurement of neutralizing antibodies. Project supervision was done by LR, UH, UP, and MCB. Each author contributed important intellectual content during manuscript drafting or revision and accepts accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved.

Acknowledgements
We would like to thank all patients for their participation in the study. We thank Prof. Oliver Keppler’s group from the Institute of Virology and the Max von Pettenkofer Institute, Ludwig-Maximilians University of Munich for kindly providing the SARS-CoV-2 isolates.

Disclosures, Conflict of Interest Statement
All authors declare no conflict of interest.

Data sharing statement
The datasets for this manuscript are not publicly available because written informed consent did not include wording on data sharing (German data protection laws). Reasonable requests to access the datasets should be directed to the corresponding author.

Funding
None.
References

1. El Karoui K, Hourmant M, Ayav C, et al. Vaccination and COVID-19 Dynamics in Dialysis Patients. *Clin J Am Soc Nephrol* 2022; 17: 395-402.

2. Ahmed N, Khderat AH, Sarsour A, et al. The vulnerability of maintenance dialysis patients with COVID-19: mortality and risk factors from a developing country. *Ann Med* 2022; 54: 1511-1519.

3. Hilbrands LB, Duivenvoorden R, Vart P, et al. COVID-19-related mortality in kidney transplant and dialysis patients: results of the ERACODA collaboration. *Nephrol Dial Transplant* 2020; 35: 1973-1983.

4. Erber J, Kappler V, Haller B, et al. Infection Control Measures and Prevalence of SARS-CoV-2 IgG among 4,554 University Hospital Employees, Munich, Germany. *Emerg Infect Dis* 2022; 28: 572-581.

5. Espi M, Charmetant X, Barba T, et al. A prospective observational study for justification, safety, and efficacy of a third dose of mRNA vaccine in patients receiving maintenance hemodialysis. *Kidney international* 2022; 101: 390-402.

6. Wand O, Nacasz N, Fadeela A, et al. Humoral response and breakthrough infections with SARS-CoV-2 B.1.617.2 variant in vaccinated maintenance hemodialysis patients. *J Nephrol* 2022: 1-9.

7. Garcia-Beltran WF, St Denis KJ, Hoelzemer A, et al. mRNA-based COVID-19 vaccine boosters induce neutralizing immunity against SARS-CoV-2 Omicron variant. *Cell* 2022; 185: 457-466.e454.

8. Wratil PR, Stern M, Priller A, et al. Three exposures to the spike protein of SARS-CoV-2 by either infection or vaccination elicit superior neutralizing immunity to all variants of concern. *Nature medicine* 2022; 28: 496-503.

9. Verdier JF, Boyer S, Chalmin F, et al. Response to three doses of the Pfizer/BioNTech BNT162b2 COVID-19 vaccine: a retrospective study of a cohort of haemodialysis patients in France. *BMC Nephrol* 2022; 23: 189.

10. Robert-Koch-Institute. Wöchentlicher Lagebericht des RKI zur Coronavirus-Krankheit-2019 (COVID-19). 2022.

11. Parshina E, Zulkarnaev A, Tolkach A, et al. Patients receiving hemodialysis do not lose SARS-CoV-2 antibodies more rapidly than non-renal controls: a prospective cohort study. *Ren Fail* 2022; 44: 392-398.

12. Koerber N, Priller A, Yazici S, et al. Dynamics of spike-and nucleocapsid specific immunity during long-term follow-up and vaccination of SARS-CoV-2 convalescents. *Nat Commun* 2022; 13: 153.

13. Chan KH, Leung KY, Zhang RR, et al. Performance of a Surrogate SARS-CoV-2-Neutralizing Antibody Assay in Natural Infection and Vaccination Samples. *Diagnostics (Basel)* 2021; 11.
14. Espi M, Koppe L, Fouque D, et al. Chronic Kidney Disease-Associated Immune Dysfunctions: Impact of Protein-Bound Uremic Retention Solutes on Immune Cells. *Toxins (Basel)* 2020; 12.

15. Housset P, Kubab S, Hanafi L, et al. Humoral response after a fourth "booster" dose of a coronavirus disease 2019 vaccine following a 3-dose regimen of mRNA-based vaccination in dialysis patients. *Kidney international* 2022.

16. Cinkilic O, Anft M, Blazquez-Navarro A, et al. Inferior humoral and sustained cellular immunity against wild-type and omicron variant of concern in hemodialysis patients immunized with 3 SARS-CoV-2 vaccine doses compared with 4 doses. *Kidney international* 2022; 101: 1287-1289.

17. Anft M, Blazquez-Navarro A, Frahnert M, et al. Inferior cellular and humoral immunity against Omicron and Delta variants of concern compared with SARS-CoV-2 wild type in hemodialysis patients immunized with 4 SARS-CoV-2 vaccine doses. *Kidney international* 2022.

18. Carr EJ, Wu M, Harvey R, et al. Omicron neutralising antibodies after COVID-19 vaccination in haemodialysis patients. *The Lancet* 2022; 399: 800-802.

19. Quiroga B, Soler MJ, Ortiz A, et al. Humoral Response to Third Dose of SARS-CoV-2 Vaccines in the CKD Spectrum. *Clin J Am Soc Nephrol* 2022.

20. Quiroga B, Soler MJ, Ortiz A, et al. Safety and immediate humoral response of COVID-19 vaccines in chronic kidney disease patients: the SENCOVAC study. *Nephrol Dial Transplant* 2021.

21. Wieske L, van Dam KPJ, Steenhuis M, et al. Humoral responses after second and third SARS-CoV-2 vaccination in patients with immune-mediated inflammatory disorders on immunosuppressants: a cohort study. *The Lancet Rheumatology* 2022; 4: e338-e350.

22. Khoury DS, Cromer D, Reynaldi A, et al. Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. *Nature medicine* 2021; 27: 1205-1211.
## Tables and Figures

### Table 1. Patient characteristics.

|                          | total n=142 | low/non-responder n=12 | responder n=130 | P   |
|--------------------------|-------------|------------------------|-----------------|-----|
| Age (years)              | 72.6 (61.5 - 80.6) | 77.1 (67.0 - 79.7) | 72.2 (60.5 - 80.6) | 0.47 |
| Female                   | 48 (33.8%) | 7 (58.3%)              | 41 (31.5%)      | 0.11 |
| Dialysis vintage (months)| 48.9 (21.3 - 83.7) | 38.7 (13.4 - 63.6) | 49.3 (21.9 - 84.0) | 0.34 |
| Vaccines                 |             |                        |                 |     |
| mRNA and vector          | 8 (5.6%)   | 0 (0.0%)               | 8 (6.2%)        | 1.0 |
| only mRNA                | 134 (94.4%)| 12 (100.0%)            | 122 (93.8%)     |     |
| COVID-19 infection before fourth blood examination | 22 (15.5%) | 2 (16.7%) | 20 (15.4%) | 1.0 |
| Time lap between infection and fourth blood examination (days) | 215.7 ±223.3 | 157.5 ±222.7 | 224.6 ±231.1 | 0.71 |
| Charlson Comorbidity Index | 5.0 (4.0 - 7.0) | 5.5 (4.0 - 6.2) | 5.0 (4.0 - 7.0) | 0.95 |
| History of kidney transplantation | 16 (11.3%) | 1 (8.1%) | 15 (11.5%) | 1.00 |
| Immunosuppressive medication | 16 (11.3%) | 4 (33.3%) | 12 (9.2%) | 0.031 |
| Hepatitis B vaccination non-response | 51 (36.4%) | 5 (41.7%) | 46 (35.4%) | 0.94 |
| Renal diagnosis           |             |                        |                 |     |
| Glomerulopathy            | 22 (16.1%) |                        |                 |     |
| Diabetic nephropathy      | 24 (17.5%) |                        |                 |     |
| Hypertensive nephropathy  | 17 (12.4%) |                        |                 |     |
| Congenital or cystic renal disease | 13 (9.5%) |                      |                 |     |
| Tubulointerstitial disease | 2 (1.5%) |                       |                 |     |
| Reflux nephropathy        | 3 (2.2%)   |                        |                 |     |
| Other                     | 18 (13.1%) |                        |                 |     |
| Nephropathy of unkown origin | 38 (27.7%) |                      |                 |     |

Results are presented as mean (±SD) and median (interquartile range) for normally and non-normally distributed data, respectively; categorical data as total number (percentage). P values present the results of group-wise comparisons of patients neutralizing Omicron after the fourth vaccination.
| Predictor | univariate | | multivariate | |
|-----------|------------|---------------------|---------------------|
| A. Delta  |            |                     |                     |
| Intercept | -          | -                   | 1918.2 (985.5, 2850.9) | <0.001 |
| Age (1 year) | -2.1 (-14.1, 10.0) | 0.74 | 2.6 (-14.5, 19.6) | 0.77 |
| Dialysis vintage (1 month) | 2.1 (-0.4, 4.6) | 0.10 | 0.05 (-0.04, 0.13) | 0.27 |
| Charlson comorbidity index | -28.4 (-102.6, 45.7) | 0.45 | -17.3 (-120.8, 86.1) | 0.74 |
| Immunosuppressive medication | -814.7 (-1293.8, -355.9) | 0.001 | -867.3 (-1356.7, -377.9) | <0.001 |
| Hepatitis B vaccination non-response | -331.9 (-658.1, -5.6) | 0.046 | -290.8 (-605.3, 23.7) | 0.070 |
| B. Omicron |            |                     |                     |
| Intercept | -          | -                   | 1167.7 (91.6, 2243.8) | 0.034 |
| Age (1 year) | -0.5 (-13.9, 12.9) | 0.94 | 0.2 (-19.4, 19.9) | 0.98 |
| Dialysis vintage (1 month) | 1.0 (-1.9, 3.8) | 0.50 | 0.02 (-0.07, 0.12) | 0.62 |
| Charlson comorbidity index | -7.9 (-90.5, 74.8) | 0.85 | -0.6 (119.9, 118.7) | 0.99 |
| Immunosuppressive medication | -382.7 (-933.3, 167.9) | 0.17 | -457.6 (-1031.3, 116.0) | 0.12 |
| Hepatitis B vaccination non-response | -228.1 (-590.7, 134.4) | 0.22 | -180.7 (-568.3, 206.8) | 0.36 |
| C. Neutralizing antibodies |            |                     |                     |
| Intercept | -          | -                   | 837.9 (661.4, 1014.4) | <0.001 |
| Age (1 year) | -1.2 (-3.6, 1.1) | 0.30 | -1.3 (-4.6, 1.9) | 0.41 |
| Dialysis vintage (1 month) | 0.4 (-0.1, 0.19) | 0.12 | 0.01 (-0.01, 0.02) | 0.32 |
| Charlson comorbidity index | -7.0 (-21.3, 7.4) | 0.34 | 2.8 (-16.7, 22.3) | 0.78 |
| Immunosuppressive medication | -209.6 (-302.1, -117.0) | <0.001 | -223.0 (319.9, -126.0) | <0.001 |
| Hepatitis B vaccination non-response | -228.1 (-590.7, 134.4) | 0.22 | -22.8 (-86.5, 40.9) | 0.48 |

Abbreviations: b, regression coefficient; CI, confidence interval.
Figures

Figure 1. Flow chart of the COVIIMP study (A). Study design and observed SARS-CoV-2 infection cases (B).

Abbreviations: vac., vaccination

COVIIMP study
all patients enrolled
n = 513

patients not on maintenance hemodialysis,
n = 272

patients on maintenance hemodialysis
n = 241

less than 3 vaccinations reported, n = 57
did not receive 4th vaccination, n = 38
death before second blood analysis, n = 1
loss of follow-up, n = 3

analysis of antibody-mediated immunity
before and after 4th vaccination
n = 142

1st blood analysis

2nd blood analysis

vac. 1 vac. 2 vac. 3 vac. 4

infection before, n = 12

infection between, n = 10

infection all, n = 22

Abbreviations: vac., vaccination
Figure 2. Changes of SARS-CoV-2 infection neutralization capacity before and after the fourth COVID-19 vaccination in hemodialysis patients.

Real virus neutralization assay was performed using (A) the SARS-CoV-2 Delta (B.1.617.2) and (B) the Omicron (B.1.1.529, sub lineage BA.1) variant of concern upon serial dilution of hemodialysis patient sera before and after the fourth vaccination. Inhibitory concentration (IC50) dilution values are given. (C) Change of neutralizing antibody titers given in AU/ml in a surrogate neutralization assay. Dots indicate the measurement of an individual patient with lines connecting individual patient values before and after fourth vaccination. Boxes indicate median and interquartile range. Statistical analysis was performed using paired-samples Wilcoxon test, p values indicate statistical significance between groups.
Figure 3. Percentage of responder before and after the fourth vaccination.

A responder was defined by a Delta or Omicron IC50 virus infection neutralization of >1:20 as well as neutralizing antibodies (NAb) ≥10 AU/ml. Green and red indicate the percentages classified as responder and non-responder, respectively. Statistical analysis was done using McNemar test for paired samples.
Figure 4. Influence of immunosuppressive medication, SARS-CoV-2 breakthrough infection and hepatitis B response status on COVID-19 vaccine responses.

Serum real-virus neutralization capacity for Delta (left column) and Omicron (right column) was analyzed after the fourth vaccination in subgroups. Comparison of immunosuppressive drug treatment (A, B), prevalence of SARS-CoV-2 infection before the second blood sampling (C, D), and hepatitis B vaccination non-response (E, F) on serum neutralization capacity. Statistical analysis was performed using Mann-Whitney-U test, p values indicate statistical significance between groups.
Figure 5. Serum neutralization capacity for Omicron variant of concern stratified by patients with SARS-CoV-2 breakthrough infections after the first blood sampling and before the fourth vaccination.

Statistical analysis was performed using Mann-Whitney-U test, p value indicates statistical significance between groups. The y-axis is interrupted between 500 and 2500 for better visibility.