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Supplementary appendix

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Supplement to: Arora P, Kempf A, Nehlmeier I, et al. Augmented neutralisation resistance of emerging omicron subvariants BA.2.12.1, BA.4, and BA.5. *Lancet Infect Dis* 2022; published online June 28. https://doi.org/10.1016/S1473-3099(22)00422-4.

Appendix

Augmented neutralisation resistance of emerging Omicron subvariants BA.2.12.1, BA.4 and BA.5

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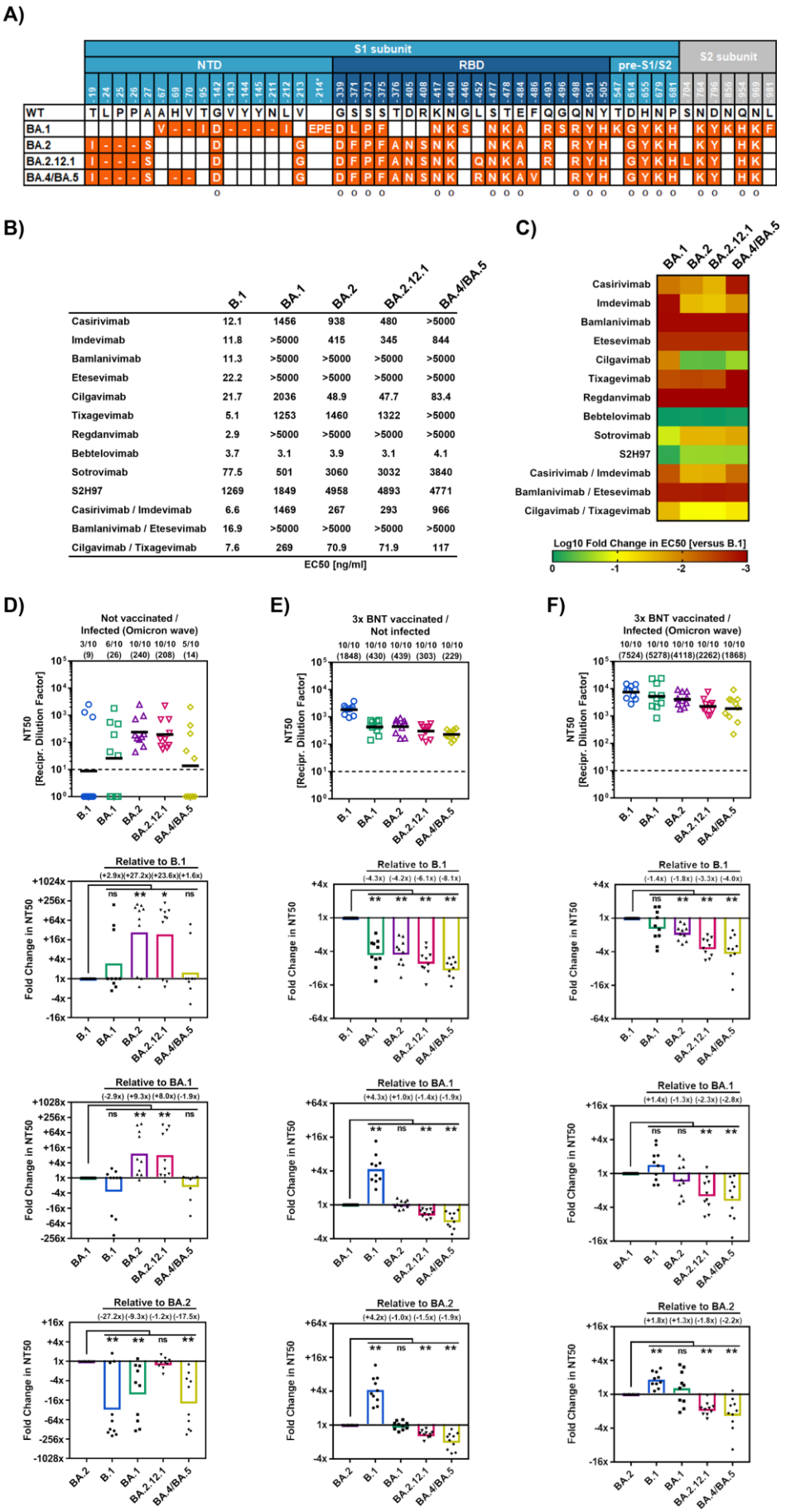


Figure: Neutralisation escape by Omicron subvariants.

(A) Summary of SARS-CoV-2 BA.1, BA.2 BA.2.12.1 and BA.4/BA.5 S protein mutations compared to the S protein of the SARS-CoV-2 Wuhan-01 isolate (wildtype = WT). Mutations that coincide in BA.1, BA.2, BA.2.12.1 and BA.4/BA.5 are indicated by circles. Of note, the BA.1 S protein contains an insertion between amino acid residues 214 and 215 and S proteins of BA.4 and BA.5 harbour identical mutations.

(B) Summary of the effective concentration 50 (EC50) values of monoclonal antibodies. Please see also supplementary figure 1.

(C) Fold change in EC50 of monoclonal antibodies (compared to SARS-CoV-2 B.1). Please see also supplementary figure 1.

(D) Top: Pseudovirus particles harbouring the indicated S proteins were pre-incubated with serial dilutions of plasma from non-vaccinated individuals that were infected during the "Omicron wave" in Germany (dominated by BA.1 and BA.2) before being added to Vero cells. Pseudovirus cell entry was analysed and normalised to samples without plasma (= 0% inhibition) and used for calculation of the neutralising titre 50 (NT50) values. Presented are the combined data for ten sera and each data point represents the mean of four technical replicates (dashed line indicates the limit of detection = 10; lowest serum/plasma dilution tested, 1:50). NT50 values below the limit of detection were set as 1. Black lines and numerical values in brackets show the geometric mean NT50. Further, the proportion of sera with reactivity (NT50 $\geq$ 10) against the respective S protein-bearing pseudovirus particles is indicated. Bottom: The fold change in NT50 was calculated using either B.1, BA.1, BA.2, BA.2.12.1 or BA.4/BA.5 as reference (set as 1). Statistical significance of differences between individual groups was assessed by Wilcoxon matched-pairs signed rank test ($p > 0.05$, not significant [ns]; $p \leq 0.05$, *; $p \leq 0.01$, **; $p \leq 0.001$, ***). Please see also supplementary figure 2 and Table 1.

(E) The experiment was conducted as described in panel A with the exception that sera from ten individuals that received three doses of the Comirnaty/BNT162b2 vaccine were used. Please see also supplementary figure 2 and Table 1.

(F) The experiment was conducted as described in panel A with the exception that sera from ten individuals that received three doses of the Comirnaty/BNT162b2 vaccine and were infected during the "Omicron wave" in Germany were used. Please see also supplementary figure 2 and Table 1.

Table

ID	Gender	Age group (years)	Sample group	Vaccination	Time since last vaccination and sampling (days)	Documented SARS-CoV-2 infection? (yes/no, period, variant)	Time since first positive test and sampling (days)
8674	Female	25-34	Infected	No	n.a.	Yes[1], 5th wave[2], unknown	22
8699	Male	55-64		No	n.a.	Yes[1], 5th wave[2], unknown	10
8729	Female	55-64		No	n.a.	Yes[1], 5th wave[2], unknown	18
8730	Male	25-34		No	n.a.	Yes[1], 5th wave[2], unknown	20
8731	Male	18-24		No	n.a.	Yes[1], 5th wave[2], unknown	21
8732	Female	18-24		No	n.a.	Yes[1], 5th wave[2], unknown	22
8733	Male	18-24		No	n.a.	Yes[1], 5th wave[2], unknown	150
8735	Male	25-34		No	n.a.	Yes[1], 5th wave[2], unknown	21
8786	Female	35-44		No	n.a.	Yes[1], 5th wave[2], unknown	unknown
8801	Female	65-74		No	n.a.	Yes[1], 5th wave[2], unknown	10
7543	Female	45-54	Vaccinated	Yes (BNT/BNT/BNT)	41	No[3], n.a., n.a.	n.a.
7551	Male	55-64		Yes (BNT/BNT/BNT)	13	No[3], n.a., n.a.	n.a.
7690	Female	35-44		Yes (BNT/BNT/BNT)	32	No[3], n.a., n.a.	n.a.
7884	Female	25-34		Yes (BNT/BNT/BNT)	39	No[3], n.a., n.a.	n.a.
7960	Male	35-44		Yes (BNT/BNT/BNT)	24	No[3], n.a., n.a.	n.a.
7973	Female	45-54		Yes (BNT/BNT/BNT)	24	No[3], n.a., n.a.	n.a.
7974	Female	55-64		Yes (BNT/BNT/BNT)	45	No[3], n.a., n.a.	n.a.
8046	Female	45-54		Yes (BNT/BNT/BNT)	47	No[3], n.a., n.a.	n.a.
8047	Female	55-64		Yes (BNT/BNT/BNT)	16	No[3], n.a., n.a.	n.a.
8049	Female	55-64		Yes (BNT/BNT/BNT)	39	No[3], n.a., n.a.	n.a.
8577	Female	45-54	Vaccinated & Infected	Yes (BNT/BNT/BNT)	99	Yes[1], 5th wave[2], unknown	65
8618	Female	25-34		Yes (BNT/BNT/BNT)	149	Yes[1], 5th wave[2], unknown	25
8619	Male	25-34		Yes (BNT/BNT/BNT)	139	Yes[1], 5th wave[2], unknown	23
8648	Male	35-44		Yes (BNT/BNT/BNT)	146	Yes[1], 5th wave[2], unknown	22
8680	Female	35-44		Yes (BNT/BNT/BNT)	123	Yes[1], 5th wave[2], unknown	52
8693	Female	45-44		Yes (BNT/BNT/BNT)	149	Yes[1], 5th wave[2], unknown	48
8742	Female	45-44		Yes (BNT/BNT/BNT)	174	Yes[1], 5th wave[2], unknown	19

8744	Male	25-34		Yes (BNT/BNT/BNT)	187	Yes[1], 5th wave[2], unknown	32
8765	Female	35-44		Yes (BNT/BNT/BNT)	152	Yes[1], 5th wave[2], unknown	22
8770	Female	35-44		Yes (BNT/BNT/BNT)	167	Yes[1], 5th wave[2], unknown	26

[1]: SARS-CoV-2 infection was confirmed by real-time reverse transcriptase polymerase chain reaction.

[2]: The fifth SARS-CoV-2 wave in Germany started in January 2022, reached its peak around mid of March and is presently waning (as of 26.05.2022).

[3]: Vaccinees were anti-Spike 1 IgG negative before vaccination and tested negative for anti-NCP IgG after their last vaccination.

Abbreviations: ID, identifier; n.a., not applicable; BNT, BNT162b2/Comirnaty.

Methods

Cell culture

All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM, PAN-Biotech), supplemented with 10% fetal bovine serum (Biochrom), 100 U/ml penicillin and 0.1 mg/ml streptomycin (PAN-Biotech). Incubation was performed at 37 °C in a humidified atmosphere containing 5% CO_2. 293T (human, female, kidney; ACC-635, DSMZ; RRID: CVCL_0063) were used for pseudovirus particle production, while Vero cells (African green monkey kidney, female, kidney; CRL-1586, ATCC; RRID: CVCL_0574, kindly provided by Andrea Maisner) served as target cells for neutralisation assays. Validated of cell lines was performed by STR analysis, amplification and sequencing of a fragment of the cytochrome c oxidase gene, microscopic examination and/or according to their growth characteristics.

Expression plasmids and sequence analysis

Expression plasmids pCAGGS-DsRed [1], pCG1-SARS-CoV-2 B.1 SΔ18 (codon-optimized, C-terminal truncation of 18 amino acid residues, GISAID Accession ID: EPI_ISL_425259) [2], pCG1-SARS-CoV-2 BA.1 SΔ18 (codon-optimized, C-terminal truncation of 18 amino acid residues, GISAID Accession ID: EPI_ISL_6640919) [3] and pCG1-SARS-CoV-2 BA.2 SΔ18 (codon-optimized, C-terminal truncation of 18 amino acid residues, GISAID Accession ID: EPI_ISL_8738174) [4] have been described elsewhere. Gibson assembly was employed to generate expression plasmids for SARS-CoV-2 BA.2.12.1 S Δ18 (based on GISAID Accession ID: EPI_ISL_12028907) and SARS-CoV-2 BA.4/BA.5 SΔ18 (based on GISAID Accession ID: EPI_ISL_11550739 and EPI_ISL_12029894) were using five overlapping DNA strings (Thermo Fisher Scientific), BamHI/XbaI-digested pCG1 plasmid and GeneArt™ Gibson Assembly HiFi Master Mix (Thermo Fisher Scientific). Reactions were prepared according to the manufacturer's instructions. The pCG1 expression plasmid was a kind gift from Roberto Cattaneo, Mayo Clinic College of Medicine, Rochester, MN, USA. All PCR-amplified sequences were verified using a commercial sequencing service (Microsynth SeqLab). S protein sequences and the underlying information (collection date, location) were obtained from the GISAID (global initiative on sharing all influenza data) database (https://www.gisaid.org/).

Pseudovirus particle production

Pseudovirus particles bearing SARS-CoV-2 S proteins were produced as described before [5]. 293T cells expressing the respective S protein or DsRed (control) following transfection via the calcium phosphate method were inoculated with VSV-G-transcomplemented VSV*ΔG(FLuc) (kindly provided by Gert Zimmer) [6]. After an incubation period of 1 h, the inoculum was removed and cells were washed with phosphate-buffered saline (PBS). Next, DMEM medium containing anti-VSV-G antibody (culture supernatant from I1-hybridoma cells; ATCC no. CRL-2700) was added and cells were further incubation for 16-18 h before the pseudovirus particle-containing culture supernatants were harvested, clarified from cellular debris by centrifugation (4,000 x g, 10 min) and used for neutralisation assays.

Plasma collection and neutralisation assay

Plasma from (i) non-vaccinated individuals that were infected during the fifth SARS-CoV-2 wave in Germany ("Omicron wave", dominated by SARS-CoV-2 BA.1 and BA.2 lineages)

SARS-CoV-2, individuals vaccinated with three doses of the BNT162b2/Comirnaty vaccine without breakthrough infection (n=10), or (iii) individuals vaccinated with three doses of the BNT162b2/Comirnaty vaccine with breakthrough infection during the "Omicron wave" in Germany (n=10) were collected at the Hannover Medical School (Medizinische Hochschule Hannover, MHH) as part of the COVID-19 Contact (CoCo) Study (German Clinical Trial Registry DRKS00021152). Collection of samples was approved by the Institutional Review Board of MHH (8973_BO_K_2020). Patient information is provided in Table 1. All plasma samples were heat-inactivated at 56 °C for 30 min.

Neutralisation assays were performed according to an established protocol [7,8]. Briefly,

S protein containing pseudovirus particles were pre-incubated for 30 min at 37 °C with different concentrations of individual monoclonal antibodies or antibody cocktails, or different plasma dilutions. Thereafter, mixtures were added to Vero cells (96-well format) and incubated for 16-18 h. Transduction efficiency was analysed by measuring the activity of virus-encoded firefly luciferase. For this, cells were lysed in PBS containing 0.5% Triton X-100 (Carl Roth) for 30 min at room

temperature and lysates were transferred into white 96-well plates. Next, lysates were mixed with luciferase substrate (Beetle-Juice, PJK) and luminescence was recorded using a Hidex Sense plate luminometer (Hidex). For data normalisation, pseudovirus particles incubated with medium alone served as reference (= 0% inhibition).

Statistical analysis

Data were analysed using Microsoft Excel (as part of the Microsoft Office Professional Plus, version 2016, Microsoft Corporation) and GraphPad Prism version 8.3.0 (GraphPad Software). Statistical significance was assessed by two-way analysis of variance with Dunnett's post-hoc test (antibody neutralisation) or Wilcoxon signed-rank test (plasma neutralisation). Only p-values 0.05 or lower were considered statistically significant ($p > 0.05$, not significant [ns]; $p \leq 0.05$, *; $p \leq 0.01$, **; $p \leq 0.001$, ***). Antibody concentrations (effective concentration 50, EC50) and plasma dilutions (neutralising titre 50, NT50) leading to a half-maximal inhibition were calculated by a non-linear regression model.

Ethics committee approval

Collection of samples was approved by the Institutional Review Board of MHH (8973_BO_K_2020). All of the participants provided written informed consent for usage of plasma samples for research.

Limitations of the study

Our study has limitations. We utilised pseudotyped particles as a surrogate model for the analysis of SARS-CoV-2 variant neutralisation. Although these particles adequately model SARS-CoV-2 neutralisation by antibodies [9], our results formally await confirmation with authentic SARS-CoV-2. Further, the majority of convalescent and vaccinee sera/plasma were collected within the first two months after the positive test result or the last vaccination. We thus cannot exclude that Omicron sublineage-specific differences in the extent of antibody evasion may become more or less pronounced at later time points. Finally, since only sera/plasma from BNT vaccinated individuals were tested we cannot rule out that other vaccines or vaccination regimens might result in differential neutralisation of the Omicron sublineages.

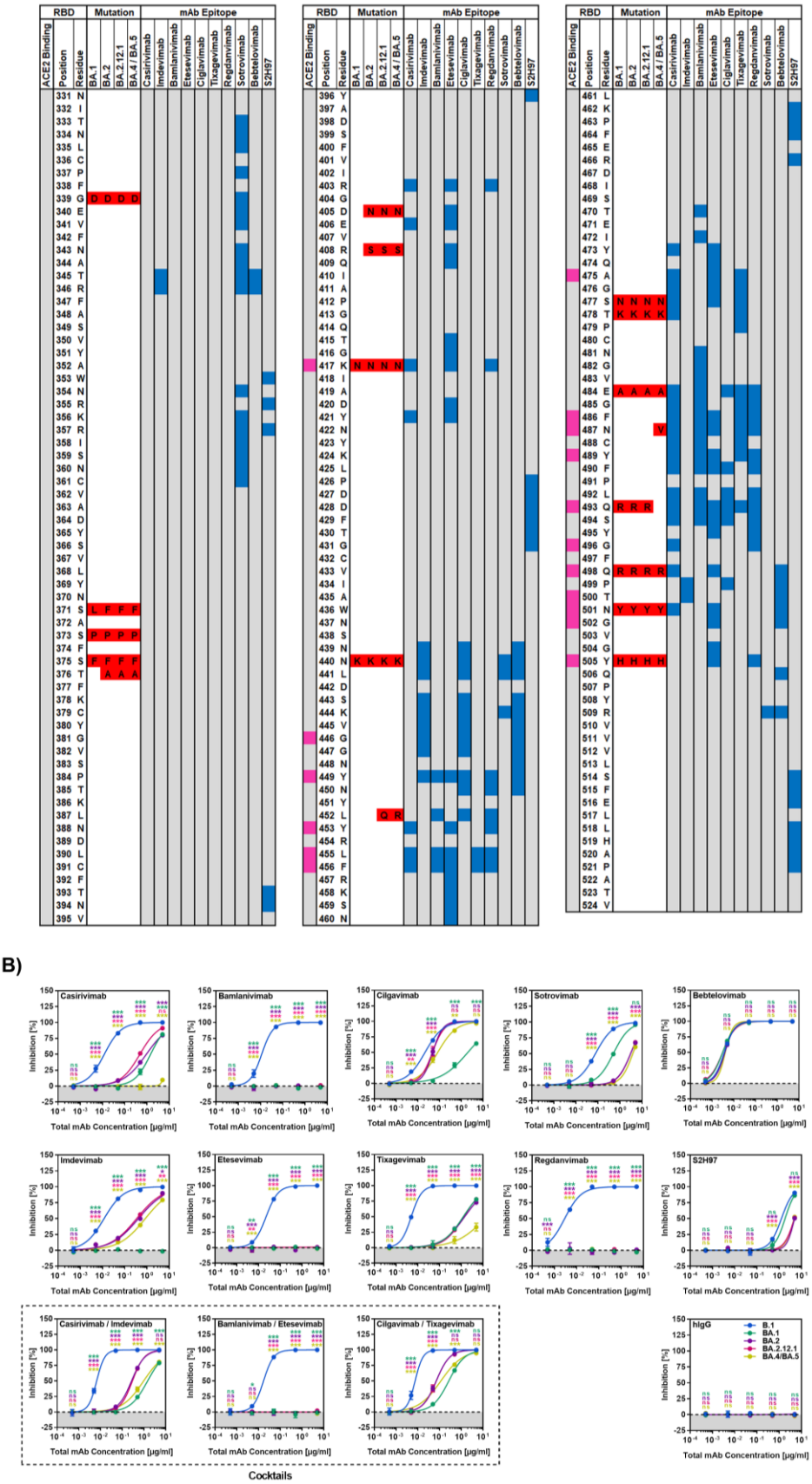
Acknowledgements

We thank Laura Hetzel for technical assistance. Further, we gratefully acknowledge the originating laboratories responsible for obtaining the specimens, as well as the submitting laboratories where the genome data were generated and shared via GISAID, on which this research is based.

Supplementary references

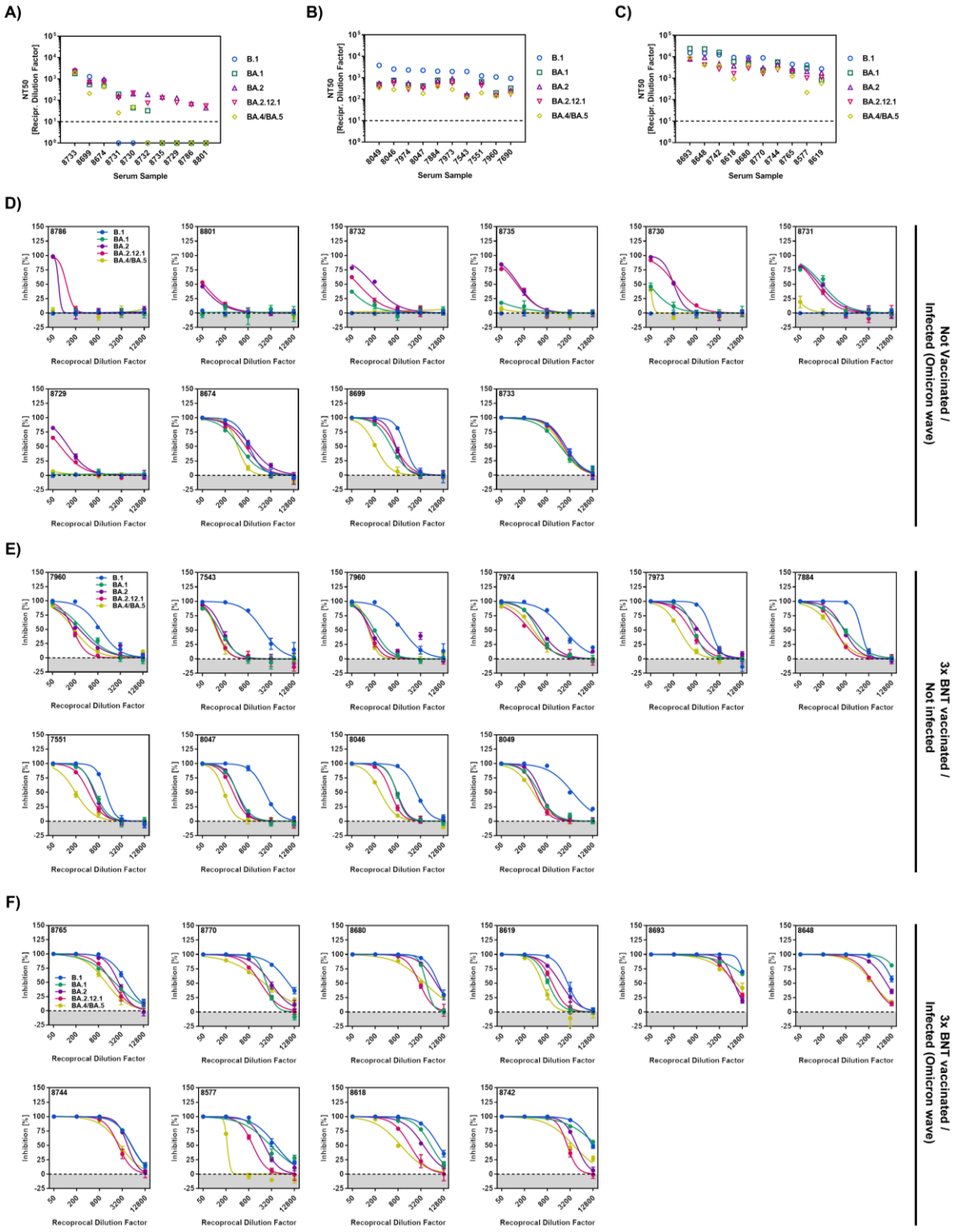
1. Hoffmann M, Kleine-Weber H, Schroeder S, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 2020; **181**(2): 271-80 e8.
2. Hoffmann M, Arora P, Gross R, et al. SARS-CoV-2 variants B.1.351 and P.1 escape from neutralizing antibodies. *Cell* 2021; **184**(9): 2384-93 e12.
3. Hoffmann M, Kruger N, Schulz S, et al. The Omicron variant is highly resistant against antibody-mediated neutralization: Implications for control of the COVID-19 pandemic. *Cell* 2022; **185**(3): 447-56 e11.
4. Arora P, Zhang L, Kruger N, et al. SARS-CoV-2 Omicron sublineages show comparable cell entry but differential neutralization by therapeutic antibodies. *Cell Host Microbe* 2022.
5. Kleine-Weber H, Elzayat MT, Wang L, et al. Mutations in the Spike Protein of Middle East Respiratory Syndrome Coronavirus Transmitted in Korea Increase Resistance to Antibody-Mediated Neutralization. *Journal of virology* 2019; **93**(2).
6. Berger Rentsch M, Zimmer G. A vesicular stomatitis virus replicon-based bioassay for the rapid and sensitive determination of multi-species type I interferon. *PloS one* 2011; **6**(10): e25858.
7. Arora P, Sidarovich A, Kruger N, et al. B.1.617.2 enters and fuses lung cells with increased efficiency and evades antibodies induced by infection and vaccination. *Cell reports* 2021; **37**(2): 109825.
8. Hoffmann M, Hofmann-Winkler H, Kruger N, et al. SARS-CoV-2 variant B.1.617 is resistant to bamlanivimab and evades antibodies induced by infection and vaccination. *Cell reports* 2021; **36**(3): 109415.
9. Schmidt F, Weisblum Y, Muecksch F, et al. Measuring SARS-CoV-2 neutralizing antibody activity using pseudotyped and chimeric viruses. *J Exp Med* 2020; **217**(11).

Supplementary figures



Supplementary figure 1: Location of receptor binding domain mutations in the context of antibody epitopes and individual antibody neutralisation data.

(A) Schematic representation of the SARS-CoV-2 S protein receptor binding domain (RBD, numbering according to the S protein of the SARS-CoV-2 Wuhan-01 isolate). Resides that contact ACE2 (pink) or that are recognized as epitopes for monoclonal antibodies (blue) are indicated. Mutations found in SARS-CoV-2 BA.1, BA.2, BA.2.12.1 and BA.4/BA.5 are highlighted in red. (B) Pseudovirus particles harbouring the indicated S proteins were pre-incubated with serial dilutions of individual monoclonal antibodies or antibody cocktails (an irrelevant antibody, hIgG, served as control) before being added to Vero cells. Pseudovirus cell entry was analysed and normalised to samples without antibody (= 0% inhibition). Data represent the mean of three biological replicates (each conducted with four technical replicates). Error bars indicate the SEM and statistical significance was assessed by two-way analysis of variance with Dunnett's post-hoc test ($p > 0.05$, not significant [ns]; $p \leq 0.05$, *; $p \leq 0.01$, **; $p \leq 0.001$, ***).



Supplementary figure 2: Individual plasma neutralisation data.

(A) NT50 data for plasma samples from non-vaccinated individuals that were infected during the "Omicron wave" in Germany (ranked according to their neutralising activity against B.1).

(B) NT50 data for plasma samples from individuals that received three doses of the Comirnaty/BNT162b2 vaccine (ranked according to their neutralising activity against B.1).

(C) NT50 data for plasma samples from individuals that received three doses of the Comirnaty/BNT162b2 vaccine and were infected during the “Omicron wave” in Germany (ranked according to their neutralising activity against B.1).

(D) Individual neutralisation data for plasma samples from non-vaccinated individuals that were infected during the “Omicron wave” in Germany.

(E) Individual neutralisation data for plasma samples from individuals that received three doses of the Comirnaty/BNT162b2 vaccine.

(E) Individual neutralisation data for plasma samples from individuals that received three doses of the Comirnaty/BNT162b2 vaccine and were infected during the “Omicron wave” in Germany.