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Supplementary appendix

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Supplement to: Arora P, Cossmann A, Schulz SR, et al. Neutralisation sensitivity of the SARS-CoV-2 XBB.1 lineage. *Lancet Infect Dis* 2023; published online Jan 5. https://doi.org/10.1016/S1473-3099(22)00831-3.

Appendix

Neutralisation sensitivity of the SARS-CoV-2 XBB.1 lineage

Content

Table

Table: Plasma information

Cohort	ID	Gender	Age (years)	V1/V2/V3	Post V3 BTI (yes/no)	V4	Post V4 BTI (yes/no)	Time between sampling and last vaccination (days)	Time between sampling and positive PCR test last vaccination (days)	Anti-SARS-CoV-2 S1 IgG (BAU/ml)[b]
V1/V2/V3[a]	7423	Male	33	BNT/BNT/BNT	no	no	no	19	n.a.	6019
	7531	Female	27	BNT/BNT/BNT	no	no	no	14	n.a.	3466
	7543	Female	53	BNT/BNT/BNT	no	no	no	41	n.a.	4243
	7551	Male	59	BNT/BNT/BNT	no	no	no	13	n.a.	4297
	7871	Male	42	AZ/AZ/BNT	no	no	no	14	n.a.	5483
	7884	Female	34	BNT/BNT/BNT	no	no	no	39	n.a.	4577
	8741	Female	36	BNT/BNT/BNT	no	no	no	119	n.a.	2529
	7960	Male	39	BNT/BNT/BNT	no	no	no	24	n.a.	4202
	7973	Female	53	BNT/BNT/BNT	no	no	no	24	n.a.	6003
	8792	Female	53	BNT/BNT/BNT	no	no	no	164	n.a.	3002
V1/V2/V3 + BTI(BA.5)	9004	Male	36	BNT/BNT/MOD	yes (BA.5 wave)	no	no	223	25	6567
	9027	Female	24	AZ/BNT/BNT	yes (BA.5 wave)	no	no	219	10	4662
	9029	Female	29	BNT/BNT/BNT	yes (BA.5 wave)	no	no	214	22	6618
	9061	Female	55	AZ/AZ/BNT	yes (BA.5 wave)	no	no	263	31	5452
	9063	Female	62	AZ/BNT/BNT	yes (BA.5 wave)	no	no	273	41	4891
	9071	Female	46	AZ/AZ/MOD	yes (BA.5 wave)	no	no	249	49	5499
	9072	Female	31	AZ/BNT/BNT	yes (BA.5 wave)	no	no	274	36	2392
	9092	Male	43	AZ/BNT/BNT	yes (BA.5 wave)	no	no	261	16	5495
	9099	Female	39	AZ/AZ/BNT	yes (BA.5 wave)	no	no	279	33	6023
	9108	Female	53	BNT/BNT/MOD	yes (BA.5 wave)	no	no	253	15	4515
V1/V2/V3/V4$_{monovalent}$[a]	8221	Male	48	AZ/AZ/BNT	no	yes (BNT)	no	30	n.a.	1826
	8383	Male	44	BNT/BNT/BNT	no	yes (BNT)	no	37	n.a.	8018
	8391	Female	38	no information	no	yes (BNT)	no	40	n.a.	2341
	8808	Female	59	AZ/BNT/BNT	no	yes (BNT)	no	7	n.a.	4709

	8830	**Male**	**65**	**AZ/AZ/BNT**	**no**	**yes (BNT)**	**no**	**9**	**n.a.**	**1931**
	8864	**Female**	**49**	**BNT/BNT/BNT**	**no**	**yes (BNT)**	**no**	**46**	**n.a.**	**7836**
	9292	**Female**	**31**	**BNT/BNT/BNT**	**no**	**yes (BNT)**	**no**	**51**	**n.a.**	**5424**
	9310	**Male**	**53**	**BNT/BNT/BNT**	**no**	**yes (BNT)**	**no**	**2**	**n.a.**	**4990**
	9351	**Female**	**27**	**BNT/BNT/BNT**	**no**	**yes (BNT)**	**no**	**29**	**n.a.**	**11368**
	9357	**Female**	**58**	**BNT/BNT/BNT**	**no**	**yes (BNT)**	**no**	**33**	**n.a.**	**1668**
V1/V2/V3/V4$_{bivalent}$[a]	**9387**	**Female**	**59**	**BNT/BNT/BNT**	**no**	**yes (BNT$_{bivalent}$)**	**no**	**27**	**n.a.**	**6161**
	9445	**Male**	**65**	**AZ/AZ/BNT**	**no**	**yes (BNT$_{bivalent}$)**	**no**	**27**	**n.a.**	**7177**
	9446	**Female**	**61**	**BNT/BNT/BNT**	**no**	**yes (BNT$_{bivalent}$)**	**no**	**27**	**n.a.**	**5104**
	9448	**Female**	**56**	**no information**	**no**	**yes (BNT$_{bivalent}$)**	**no**	**11**	**n.a.**	**7945**
	9452	**Male**	**44**	**no information**	**no**	**yes (BNT$_{bivalent}$)**	**no**	**27**	**n.a.**	**12697**
	9479	**Male**	**62**	**no information**	**no**	**yes (BNT$_{bivalent}$)**	**no**	**33**	**n.a.**	**5576**
	9481	**Female**	**58**	**no information**	**no**	**yes (BNT$_{bivalent}$)**	**no**	**23**	**n.a.**	**8758**
	9484	**Male**	**45**	**no information**	**no**	**yes (BNT$_{bivalent}$)**	**no**	**33**	**n.a.**	**7828**
	9494	**Female**	**50**	**no information**	**no**	**yes (BNT$_{bivalent}$)**	**no**	**33**	**n.a.**	**3610**
	9496	**Male**	**40**	**BNT/BNT/MOD**	**no**	**yes (BNT$_{bivalent}$)**	**no**	**33**	**n.a.**	**8194**

[a]: SARS-CoV-2 infection-free status of V1/V2/V3, V1/V2/V3/V4$_{monovalent}$ and V1/V2/V3/V4$_{bivalent}$ cohorts was confirmed by ELISA (= anti-NCP IgG-negative).

[b]: Anti-SARS-CoV-2 S1 IgG titres were determined for ancestral SARS-CoV-2.

Abbreviations: AZ, AZD1222/Vaxzevria; BNT, BNT162b2/Comirnaty; MOD, mRNA-1273/Spikevax; BNT$_{bivalent}$, Comirnaty Original/Omicron BA.4-5; BAU, binding antibody units; BTI, breakthrough infection; IgG, immunoglobulin G.

Methods

Cell culture

293T (human, female, kidney; ACC-635, DSMZ; RRID: CVCL 0063) and Vero cells (African green monkey kidney, female, kidney; CRL-1586, ATCC; RRID: CVCL 0574, kindly provided by Andrea Maisner) were cultivated at 37 °C in a humidified atmosphere containing 5% CO_2 using Dulbecco's modified Eagle medium (PAN-Biotech), supplemented with 10% fetal bovine serum (FBS, Biochrom), 1% penicillin/streptomycin solution (pen/strep, PAN-Biotech). Calu-3 (human, male, lung; HTB-55, ATCC; RRID: CVCL_0609, kindly provided by Stephan Ludwig) were cultured in minimum essential medium (Thermo Fisher Scientific) supplemented with 10% FBS, 1% pen/strep solution, 1% non-essential amino acid solution (PAA) and 1 mM sodium pyruvate (PAN-Biotech). Cell lines were validated by STR analysis, amplification and sequencing of a cytochrome c oxidase gene fragment, microscopic examination, and/or their specific growth characteristics. In addition, cell lines were routinely screened for mycoplasma contamination. Transfection of 293T cells was performed by calcium phosphate precipitation.

Expression plasmids and sequence analysis

Expression plasmids pCAGGS-DsRed[1], pCG1-SARS-CoV-2 B.1 SΔ18 (codon-optimised, C-terminal truncation of 18 amino acid residues, GISAID Accession ID: EPI_ISL_425259)[2] and pCG1-SARS-CoV-2 BA.5 SΔ18 (codon-optimised, C-terminal truncation of 18 amino acid residues, GISAID Accession ID: EPI_ISL_12029894)[3] have been described before. Gibson assembly was employed to generate the expression plasmid for SARS-CoV-2 XBB.1 SΔ18 (GISAID Accession ID: EPI_ISL_15384151). In brief, overlapping DNA strings (commercially purchased, Thermo Fisher Scientific, sequences available upon request) were mixed with BamHI/XbaI-digested pCG1 plasmid (a kind gift of Roberto Cattaneo, Mayo Clinic College of Medicine, Rochester, MN, USA) and GeneArt™ Gibson Assembly HiFi Master Mix (Thermo Fisher Scientific), and the sample was incubated for 45 min at 50 °C. Thereafter, the complete sample was transformed into one-shot OmniMAX 2 T1 competent *Escherichia coli* bacteria (Thermo Fisher Scientific). The next day, colonies were screened for presence of the insert by PCR, and positive clones were expanded for plasmid preparation. Integrity

of S protein sequences was confirmed by Sanger sequencing (Microsynth SeqLab). Information on S protein sequences was obtained from GISAID (Global Initiative on Sharing All Influenza Data) (https://gisaid.org/) and CoV-Spectrum (https://cov-spectrum.org/) databases.

Pseudovirus particle production and cell entry

We employed a previously published protocol to generate pseudovirus particles carrying SARS-CoV-2 S protein[4]. First, 293T cells transfected to express the respective S protein or DsRed (control) were inoculated with VSV-G-transcomplemented VSV*ΔG(FLuc) (kindly provided by Gert Zimmer)[5] at a multiplicity of infection of 3. After 1 h of incubation, the inoculum was aspirated and cells were washed with PBS, before medium containing anti-VSV-G antibody (culture supernatant from I1-hybridoma cells; ATCC no. CRL-2700) was added and cells were further incubated for 16-18 h. Next, cell culture supernatants were collected, clarified by centrifugation (4,000 x g, 10 min), and stored at -80 °C until further use. In order to study cell tropism of S protein-bearing pseudovirus particles, target cells were seeded into 96-well plates. On the next day, equal volumes of pseudovirus particles were added onto target cells. At 16-18 h postinoculation, the culture medium was aspirated and cells were lysed using PBS containing 0.5% Triton X-100 (Carl Roth). After an incubation phase of 30 min, cell lysates were transferred into white 96-well plates and luciferase substrate (Beetle-Juice, PJK) was added, before luminescence was recorded using a Hidex Sense plate luminometer (Hidex).

Neutralisation assay

Neutralisation assays were performed according to a previously published protocol[6]. Vero cells were seeded into 96-well plates and allowed to reach confluency the next day. In order to assess the ability of monoclonal antibodies (mAbs) to neutralise S protein-driven cell entry, pseudovirus particles bearing the respective S proteins were pre-incubated (30 min at 37 °C) with different concentrations (5, 0.5, 0.05, 0.005, 0.0005 µg/ml) of individual mAb (Casirivimab, Imdevimab, Bamlanivimab, Etesevimab, Cilgavimab, Tixagevimab, S2H97, Amubarvimab, Romlusevimab, Regdanvimab, Bebtelovimab, Sotrovimab, or an unrelated human control antibody) or mAb cocktails (Casirivimab-Imdevimab, Bamlanivimab-Etesevimab, Cilgavimab-Tixagevimab, Amubarvimab-Romlusevimab; in case of mAb cocktails, each antibody was used at half the concentration to keep total antibody concentrations

constant). For experiments addressing SARS-CoV-2 neutralisation by antibodies that are present in the plasma of vaccinated individuals with or without breakthrough infection, pseudovirus particles were pre-incubated (30 min at 37 °C) with four-fold serial dilutions of heat-inactivated blood plasma, starting at a plasma dilution of 1:50. Subsequently, the mixtures were added to confluent Vero cell layers. At 16-18 h postinoculation, luminescence was measured as described above. Neutralisation efficiency was calculated based on the relative inhibition of pseudovirus entry, for which pseudovirus particles incubated in the absence of mAb or plasma served as reference (= 0% inhibition). A non-linear regression model was used to calculate antibody concentrations (effective concentration 50, EC50) and plasma dilutions (neutralising titre 50, NT50) that lead to half-maximal inhibition. Of note, plasma samples that yielded an NT50 value lower than 12.5 were considered negative and were assigned an NT50 value of 1.

Ethics committee approval and enrolment of study participants

Collection of plasma samples was performed after approval by the research ethics committee of the Institutional Review Board of Hannover Medical School (8973 BO K 2020). Each participant provided written informed consent prior to the use of plasma samples for research.

Plasma samples

All plasma samples were pre-screened for SARS-CoV-2 S1-specific IgG using the Anti-SARS-CoV-2-QuantiVac-ELISA (IgG) (EUROIMMUN) and heat-inactivated (56 °C, 30 min) prior to neutralisation assays. A total of four cohorts were tested; cohort 1 (V1/V2/V3): triple vaccinated individuals that either received three doses of BNT162b2/Comirnaty (BNT) or two doses of AZD1222/Vaxzevria (AZ) followed by one dose of BNT (n = 10; median age = 40.5 years; male to female ratio 4:6); cohort 2 (V1/V2/V3 + $BTI_{BA.5}$): individuals with a history of three vaccinations that experienced a breakthrough infection (BTI) during the BA.5 wave (June 2022 to present [November 2022] in Germany) (n = 10; median age = 41 years; male to female ratio 2:8); cohort 3 (V1/V2/V3/$V4_{monovalent}$): individuals with a history of three vaccinations that received a fourth vaccination with the monovalent BNT vaccine (n = 10; median age = 48.5 years; male to female ratio 4:6); cohort 4 (V1/V2/V3/$V4_{bivalent}$): individuals with a history of three vaccinations that received a fourth vaccination with the B.1/BA.4-5 bivalent

BNT/Omicron BA.4-5 vaccine (n = 10; median age = 57 years; male to female ratio 5:5). Individual information on the plasma samples can be found in the Appendix Table. SARS-CoV-2 infection-free status of V1/V2/V3, V1/V2/V3/$V4_{monovalent}$ and V1/V2/V3/$V4_{bivalent}$ cohorts was confirmed by absence of anti-SARS-CoV-2 nucleocapsid protein (NCP) IgG using the Anti-SARS-CoV-2 NCP ELISA (IgG) (EUROIMMUN).

Data analysis

For data analysis Microsoft Excel (part of Microsoft Office Professional Plus, version 2016, Microsoft Corporation) and GraphPad Prism version 8.3.0 (GraphPad Software) were used. Statistical significance was assessed by either two-tailed Student's t-test with Welch correction (cell line tropism) or Wilcoxon matched-pairs signed rank test (neutralisation). Only p values of 0.05 or lower were considered statistically significant (ns [not significant], $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

Limitations of the study

This study has limitations. First, we utilised pseudovirus particles bearing SARS-CoV-2 S protein to study SARS-CoV-2 neutralisation. Although pseudovirus particles were shown to faithfully recapitulate SARS-CoV-2 host cell entry and its neutralisation, our data await formal confirmation with clinical SARS-CoV-2 isolates. Second, as our study represents only a brief virological assessment of the SARS-CoV-2 XBB.1 lineage, sample size for each cohort is relatively small and precludes investigation of potential differences in SARS-CoV-2 XBB.1 neutralisation as a result of biological factors such as age and gender. Therefore, future studies with larger cohorts are needed to address this topic. Third, due to lack of donor-matched samples, we could only indirectly assess the impact of mono- and bivalent vaccine boosters on SARS-CoV-2 XBB.1 neutralisation. Fourth, because of insufficient sample numbers, we were not able to investigate the impact of B.1/BA.1-based bivalent booster vaccination on SARS-CoV-2 XBB.1 neutralisation. Fifth, all but two plasma samples were collected within 2 months post vaccination/infection, which is why we cannot make any statement on SARS-CoV-2 XBB.1 neutralisation after extended time periods post vaccination/BTI.

Acknowledgements

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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. Arora P, Kempf A, Nehlmeier I, et al. Augmented neutralisation resistance of emerging omicron subvariants BA.2.12.1, BA.4, and BA.5. *The Lancet Infectious diseases* 2022; **22**(8): 1117-8.
4. 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. *J Virol* 2019; **93**(2).
5. 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.
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Supplementary figures

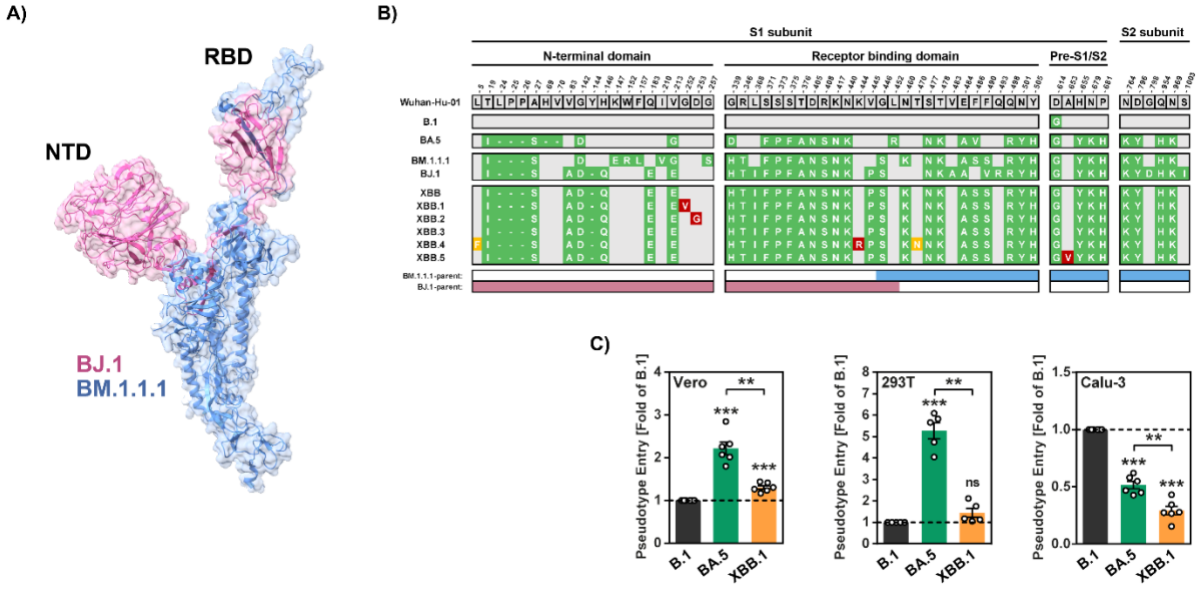

Figure S1: Genetic characteristics and host cell entry properties for the S protein of the SARS-CoV-2 XBB.1 lineage.

(A) Protein model of the XBB.1 S protein (monomeric form) in which the regions derived from either BJ.1 (pink) or BM.1.1.1 (blue) S proteins are highlighted. Abbreviations: NTD, N-terminal domain; RBD, receptor binding domain. (B) Mutations in the S proteins of SARS-CoV-2 lineages B.1, BA.5, BM.1.1.1, BJ.1, XBB (including sublineages XBB.1-5) compared to the S protein of the Wuhan-Hu-01 isolate. Mutations highlighted in red (high frequency, > 90%) and orange (low frequency, < 50%) indicate unique mutations associated with individual XBB sublineages. Further, XBB S protein regions originating from either BM.1.1.1 or BJ.1 are indicated (the breaking point is located between amino acid residues 446 and 459). (C) Pseudovirus particles bearing the indicated S proteins were inoculated onto 293T (human, kidney), Vero (African green monkey, kidney) and Calu-3 (human, lung) cells. Cell entry was analysed at 16-18 h postinoculation by measuring luciferase activity in cell lysates. Presented are the normalised mean data from five to six biological replicates (performed with four technical replicates) in which cell entry was normalised against that of particles carrying B.1 S (set as 1). Error bars represent the standard error of the mean (SEM). Statistical significance was assessed by two-tailed Student's t-test with Welch correction (not significant [ns], $p > 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

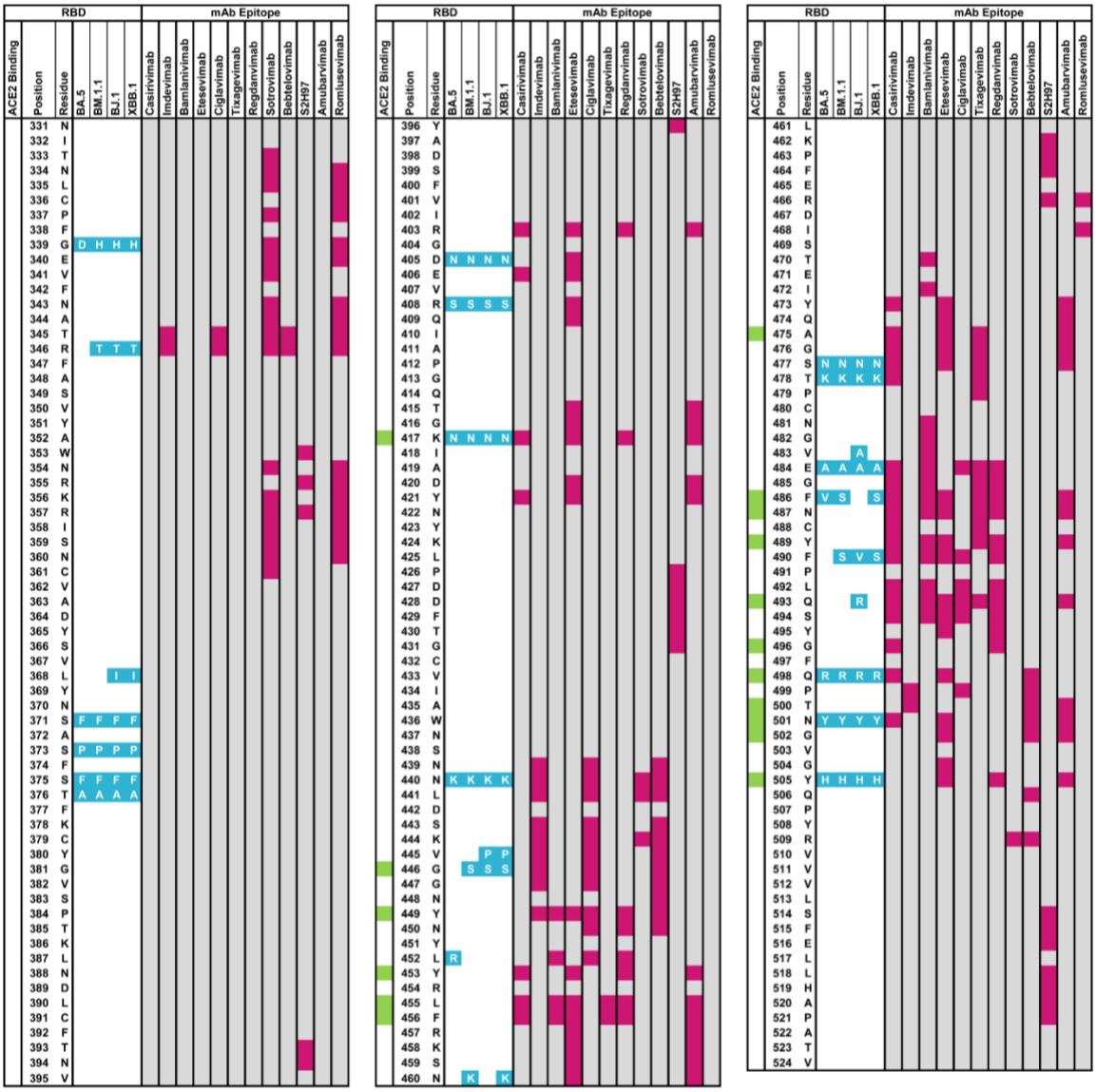

Figure S2: Location of XBB.1-specific RBD mutations in the context of mAb epitopes.

XBB.1 recombinant lineage-specific RBD mutations are highlighted in blue (numbering according to SARS-CoV-2 Wuhan-Hu-01). RBD residues that interact with ACE2 (green) or that form the binding interface of mAbs (pink) are indicated.

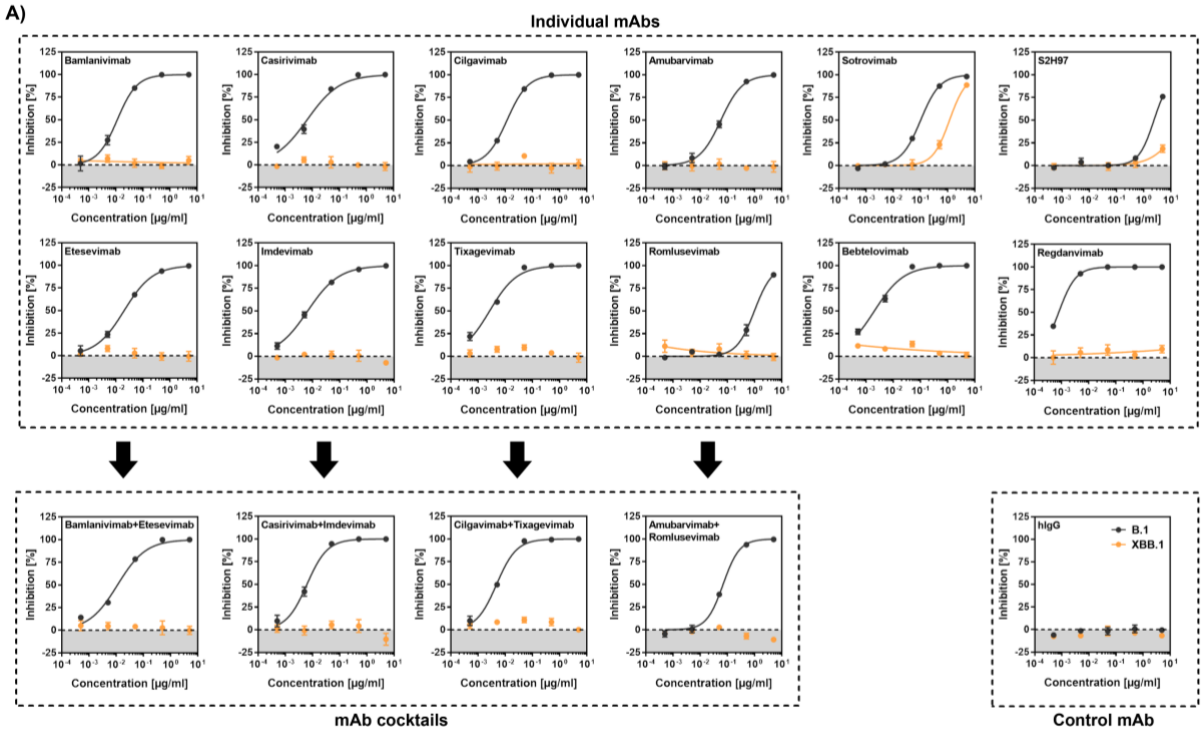

Figure S3: Individual mAb neutralisation data.

Pseudovirus particles harbouring the indicated S proteins were preincubated with different concentrations of single mAb or mAb cocktails before being inoculated onto Vero cells (of note, for mAb cocktails, each antibody was used at half concentration to keep total antibody concentrations identical). Pseudovirus entry was analysed and normalised to samples containing no antibody (= 0% inhibition). Further, data for a human control antibody that does not target the S protein (hIgG) are shown. Presented are the mean data of three biological replicates (performed with four technical replicates). Error bars represent the standard error of the mean.

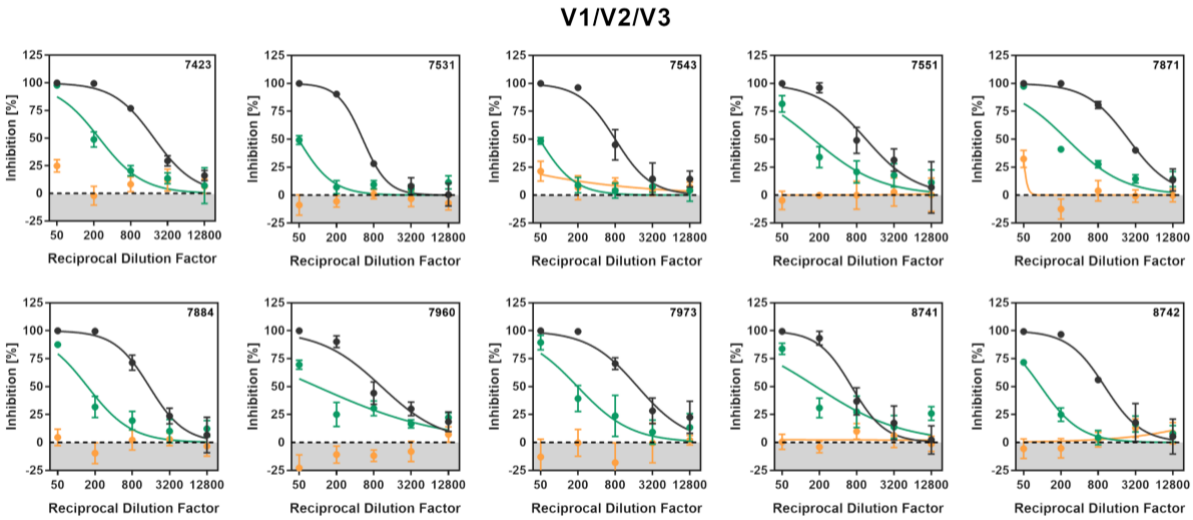

Figure S4: Individual neutralisation data for cohort V1/V2/V3.

Individual plasma neutralisation data for pseudovirus carrying the S protein of SARS-CoV-2 lineages B.1 (dark grey), BA.5 (green), or XBB.1 (yellow).

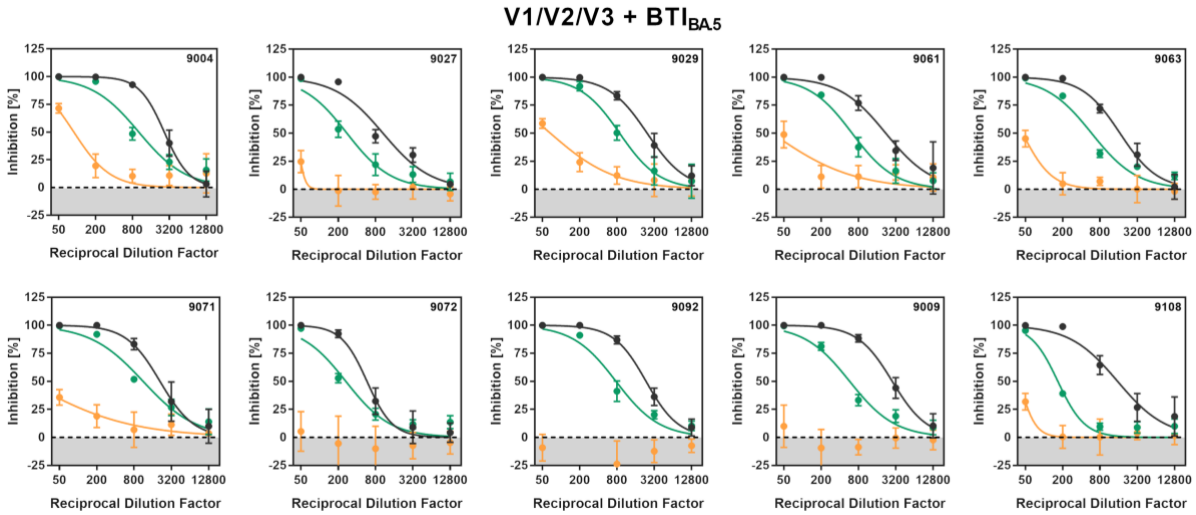

Figure S5: Individual neutralisation data for cohort V1/V2/V3 + BTI$_{BA.5}$.

Individual plasma neutralisation data for pseudovirus carrying the S protein of SARS-CoV-2 lineages B.1 (dark grey), BA.5 (green), or XBB.1 (yellow).

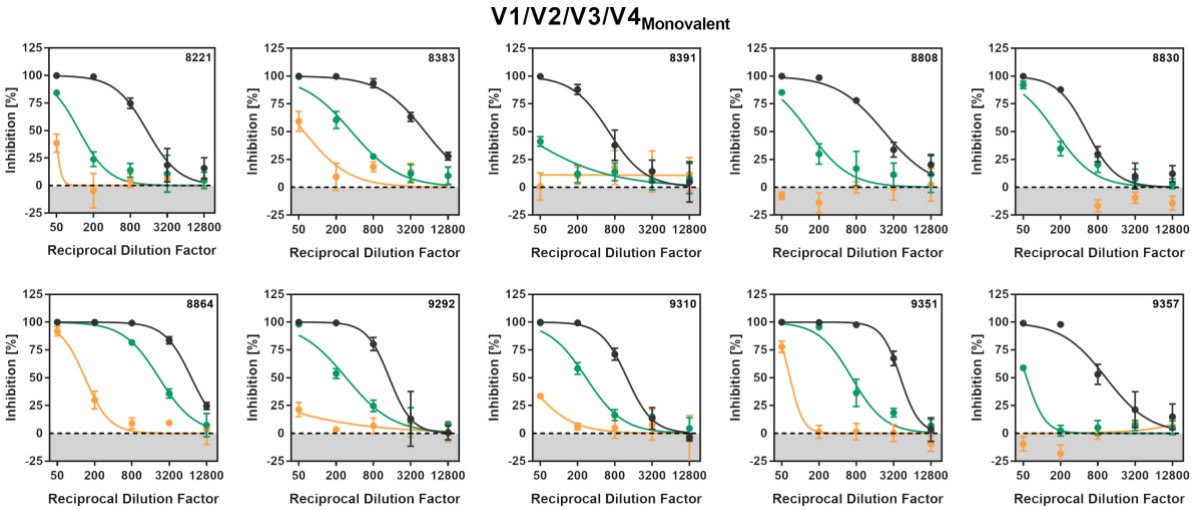

Figure S6: Individual neutralisation data for cohort V1/V2/V3/V4$_{Monovalent}$.

Individual plasma neutralisation data for pseudovirus carrying the S protein of SARS-CoV-2 lineages B.1 (dark grey), BA.5 (green), or XBB.1 (yellow).

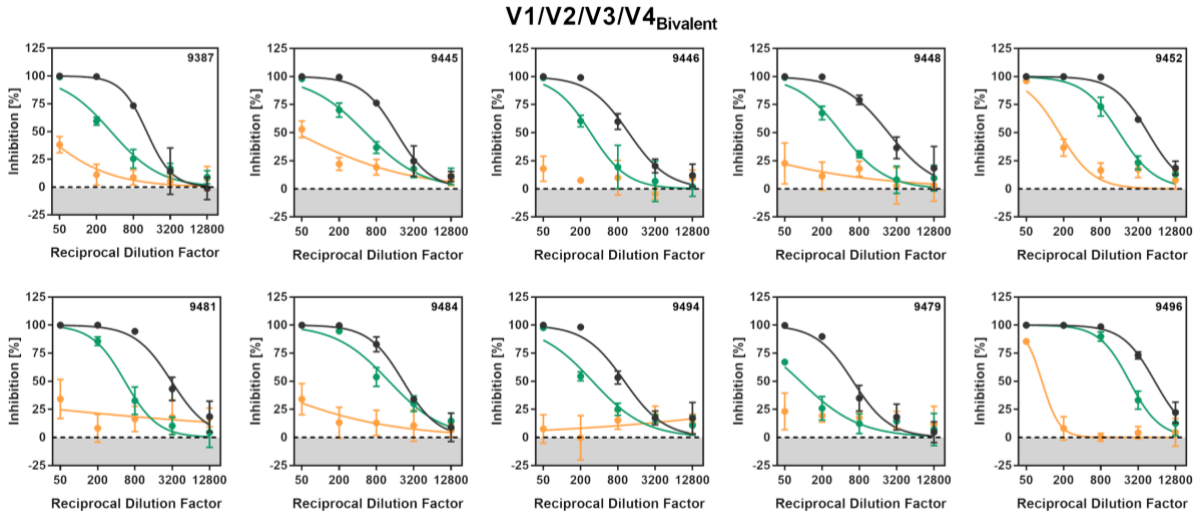

Figure S7: Individual neutralisation data for cohort V1/V2/V3/V4$_{Bivalent}$.

Individual plasma neutralisation data for pseudovirus carrying the S protein of SARS-CoV-2 lineages B.1 (dark grey), BA.5 (green), or XBB.1 (yellow).