

SUPPLEMENTARY MATERIALS

Vikström et al., Vaccine-induced correlate of protection against fatal COVID-19 in older and frail adults during waves of neutralization-resistant variants of concern: an observational study.

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SUPPLEMENTAL METHODS

Study design, population, and ethical permits

All managers at all nursing homes in the regions Västerbotten, Jämtland-Härjedalen and Örebro were informed about the study through clinical networks set up for local healthcare governance. Nursing home managers in the metropolitan area of Stockholm and Malmö were informed through the healthcare provider Familjeläkarna AB or the research network Clinical Studies Sweden (Forum Söder), respectively. The managers agreed to help in distributing study information and allowed employed care workers to assist study subjects if needed. A legal representative of a study subject could consent to participate in the study in cases where the individual could not do this due to dementia or for other reasons. Donating capillary blood samples and enrollment also provided that the individual did not express concerns verbally or with body language. To enable comparison of immunological responses in nursing home residents, we also enrolled community living study subjects under and over the age of 65 through an open cohort study of covid-19 vaccination¹. A flow diagram shows key elements of the nursing home cohort enrollment and key outcomes (Figure S6). Study subjects under the age of 65 were enrolled via an on-going open cohort study of SARS-CoV-2 infection and vaccination of health care workers (the COMMUNITY study¹). All study subjects were enrolled by informed consent. The study was approved by the Swedish Ethical Review Authority (decisions 2020-01653 or 2021-00055 including amendments 2021-01397, 2021-02328, 2021-03937 and 2022-00564-02).

Sampling strategies

Capillary blood was added to a Dried Blood Spot sample card (qDBS, Capitainer AB) that comprised two filter paper discs, each containing 10µl of blood after successful sampling. The sample cards were returned to the laboratory via postal service. Blood was eluted in PBS from the filter paper disc and stored at +4°C until analyzed for SARS-CoV-2 S-directed IgG as previously described². Venous blood sampling was performed as previously described^{1,3}.

S-binding antibody responses, nucleocapsid antibodies, and ACE-competition

We used a 5-fold dilution series of serum or capillary blood to detect anti-IgG SARS-CoV-2 S-protein was Briefly, serum or eluates from capillary blood sampling cassettes were added to S-protein coated wells and incubated over-night at +4°C. After washing the wells, goat-anti human IgG alkaline phosphate conjugated antibody (#A18814, Thermo Scientific) diluted 1/8000 was added 1 hour at +37°C. After washing, 100 µl of Phosphatase Substrate 1mg/ml (#S0942, Sigma-Aldrich) was added to

each well and incubated for 30 min at +37°C and then the colorimetric reaction was stopped with 50 µl 3M NaOH per well and absorbance was measured spectrophotometrically at 405 nm with microplate reader Tecan Sunrise. Specific IgG levels were assessed as the area under the curve (AUC) from the titers, with cut off OD value at 0.15 (Graphpad Prism 9.4.1). For nucleocapsid (N)-binding measurements, extracted qDBS samples were diluted 1:2500 and detected using a SULFO-TAG conjugated mouse monoclonal anti-human IgG antibody. For inhibition of ACE-2 spike binding, the samples were diluted 1:5 to allow competition with the recombinant human ACE2 conjugated with SULFO-TAG for binding to the spike antigen. The plates were analyzed on a MESO QuickPlex SQ 120 instrument (Meso Scale Diagnostics), an electrochemiluminescence (ECL) reader measuring the light emitted from the SULFO-TAG. Results for the V-PLEX SARS-CoV-2 panel 2 are reported in AU/ml and derived from back fitting the measured signals for samples to a calibration curve generated for each plate. The cut-offs for binding antibodies were provided by the manufacturer and applies for venous serum samples. ACE-2 results are reported as percent inhibition calculated according to: $(1 - (\text{average sample ECL signal}) / (\text{average ECL signal of diluent only})) \times 100$. Highly positive samples show high percent inhibition whereas negative or low samples show low percent inhibition.

In vitro pseudovirus neutralization assay

SARS-CoV-2 spike-pseudotyped lentiviruses were generated by the co-transfection of HEK293T cells with a spike-encoding plasmid (with a 19 amino acid C-terminal truncation), a lentiviral packaging plasmid (Addgene #8455), and a firefly luciferase-encoding transfer plasmid (Addgene #170674) using polyethylenimine (PEI). Culture media was replaced 12-16 h after transfection, and pseudotyped viruses were harvested from the supernatant at 48- and 72-h post transfection, clarified by centrifugation, and stored at -80C until use. Pseudovirus titrated to generate ~100,000 relative light units (RLUs) were incubated with serial 3-fold dilutions of heat-inactivated serum for 60 min at 37C, and then ~10,000 HEK293T-hACE2 cells were added to each well. Plates were incubated at 37C for 44-48 h and luminescence was then measured using Bright-Glo (Promega) on a GM-2000 luminometer (Promega) per the manufacturer's instructions. Neutralization was calculated relative to the mean of eight control wells infected in the absence of antibody and fit using a four-parameter logistic curve in Prism v9 (GraphPad Software).

Definition of effective vaccination and accounting for infections before study start

Based on the antibody responses observed in this study, a booster vaccine dose was considered effective at day six after administration. To account for viral RNA persistence, only the first SARS-CoV-2-positive specimen was counted per 90-day period. To account for early infections that were missed in the early pandemic phase before PCR-diagnostics was widely available, we assessed antibodies directed against the SARS-CoV-2 viral nucleocapsid in the first capillary blood sample donated by a study subject. An individual with N-directed SARS-CoV-2 antibody titer corresponding to >5000 in a venous sample was considered to have been infected.

Data processing packages

The R-packages used were readr (version 2.1.1), tidyverse (version 1.3.1), lubridate (version 1.8.0), magrittr (version 2.0.2), xlsx (version 0.6.5), scales (version 1.1.1), survival (version 3.2 – 13), survminer (version 0.4.9), mgcv (version 1.8 – 38), boot (version 1.3-28), and car (version 3.0 – 13).

Statistical analyses

The generalized additive model with cubic splines was based on 404 paired samples from 202 infection-naïve individuals and were taken between day six after vaccine dose 3 up to the administration date of dose 4. Residual analysis showed good performance of the model and four knots produced the best model with regards to fit and flexibility (Figure S7). We used 1010 observations of AUC in 1010

infection-naïve study subjects from day 20 to day 80 after dose 3 of in the nursing home cohort to approximate the decline in S-directed antibodies. We applied logarithmic regression with $\log(\text{AUC})$ as dependent variable and time in days as explanatory variable. We observed a linear decline in scatterplots of $\log(\text{AUC})$ and residual analysis verified normality of residual errors and homogeneity of residual variance (Figure S8).

For the evaluation of SARS-CoV-2-associated mortality, we included study subjects with at least one valid laboratory result from a blood sample taken at day six or later after vaccine dose 3. This allowed us to project antibody levels to day 60 under the assumption of the same antibody half-life for all individuals. We included all PCR-verified first-time infections at day six post dose 3 or later. These restrictions defined SARS-CoV-2 cases and a control population. We randomly sampled two infection-naïve controls per case at the day of positive PCR sampling for each case. Controls were eligible if alive at the case infection date, they had received an effective vaccine dose 3, and were not infected by SARS-CoV-2 the following 30 days. We used the log-rank test to compare cases and controls under the null hypothesis that both populations have identical hazard-functions. In the Cox proportional hazard model interaction between responder and covid was tested and no individual was censored. Confidence intervals were estimated by bias corrected bootstrapping with 2000 iterations and the function `boot.ci` of the `boot` package in R.

REFERENCES

1. Havervall S, Marking U, Svensson J, et al. Anti-Spike Mucosal IgA Protection against SARS-CoV-2 Omicron Infection. *N Engl J Med* 2022;387(14):1333-1336. DOI: 10.1056/NEJMc2209651.
2. Byström JW, Vikström L, Rosendal E, et al. Serological assessment of SARS-CoV-2 exposure in northern Sweden by the use of at-home sampling to meet geographical challenges in rural regions. *medRxiv* 2022. DOI: 10.1101/2020.06.02.20120477.
3. Normark J, Vikström L, Gwon YD, et al. Heterologous ChAdOx1 nCoV-19 and mRNA-1273 Vaccination. *N Engl J Med* 2021;385(11):1049-1051. DOI: 10.1056/NEJMc2110716.

SUPPLEMENTAL TABLES

	Nursing home residents	Individuals <65 years	COVID-19 experienced
Number	96	53	28
Male (%)	29 (30)	18 (34)	11 (39)
Female (%)	67 (70)	35 (66)	17 (61)
Age, median (range)	85 (71–100)	50 (21–60)	80 (27–97)
Days between dose 3 and sampling, median (range)	30 (25–35)	32 (26–56)	28 (25–35)
Days between dose 2 and 3, (median, IQR)	242 (218–259)	192 (185–204)	(87–242)

Table S1: Characteristics of cohorts sampled at approximately 30 days post dose 3.

	Nursing Home residents	Community living >65	Community living <65
Number	16	27	5
Male (%)	3 (19)	15 (54)	0 (0)
Female (%)	13 (81)	13 (46)	5 (100)
Age, median (range)	84 (41-93)	81,5 (76-91)	57 (51-61)
Days between dose 2 and 3, median (IQR)	219 (194-225)	199 (190-203)	218 (199-309)
Days between dose 3 and 4, median (IQR)	143 (143-144)	123 (120-125)	268 (268-271)

Table S2: Characteristics of individuals that were sampled at dose 4 and up to 30 days post dose 4.

Analysis model	Factor	Hazard ratio	95% boot-strap CI	p-value
Complete case analysis	S IgG AUC <479 at 60 days post dose 3	3.61	1.92 – 6.90	<0.0001
	Primary infection with SARS-CoV-2	2.70	1.43 – 4.90	<0.0001
	Male sex	2.19	1.85 – 5.85	<0.0086
	One year increase in age	1.04	1.01 – 1.08	0.0216
Missing S IgG values replaced based on the total population distribution among cases and controls	S IgG AUC <479 at 60 days post dose 3	3.15	1.84 – 5.17	<0.0001
	Primary infection with SARS-CoV-2	3.09	1.87 – 5.26	<0.0001
	Male sex	3.05	1.93 – 5.85	<0.0001
	One year increase in age	1.04	1.00 – 1.05	0.152
Missing S IgG values replaced based on that all values would be <479	S IgG AUC <479 at 60 days post dose 3	1.98	1.19 – 3.85	0.00648
	Primary infection with SARS-CoV-2	2.95	1.86 – 5.00	<0.0001
	Male sex	2.98	1.88 – 4.77	<0.0001
	One year increase in age	1.04	0.99 – 1.05	0.174
Missing S IgG values replaced based on that all values would be ≥479	S IgG AUC <479 at 60 days post dose 3	2.99	1.46 – 5.52	0.0005
	Primary infection with SARS-CoV-2	4.40	2.77 – 7.65	<0.0001
	Male sex	3.05	1.93 – 4.99	<0.0001
	One year increase in age	1.02	1.00 – 1.05	0.108

Table S3: Cox proportional-hazards model to investigate factors associated with 30-day mortality. Complete case analysis and sensitivity analyses using replacement of missing values.

SUPPLEMENTAL FIGURES

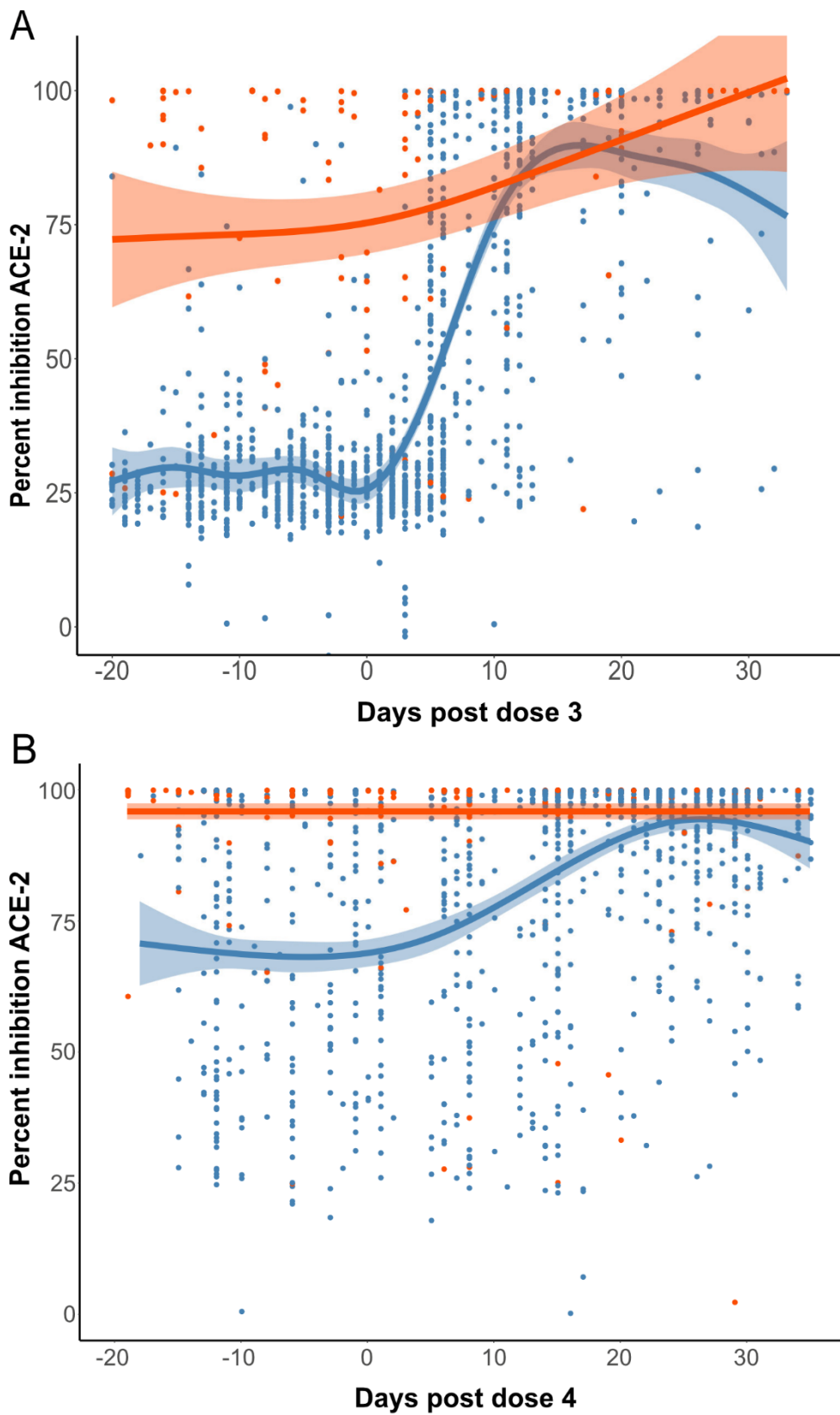


Figure S1. Pseudo neutralization after mRNA vaccine dose 3 (panel A) and dose 4 (panel B). Day 0 indicates the day of vaccination. Dots represent individual observations. Regression lines with 95% confidence intervals are shown. Blue color indicates no previous SARS-CoV-2 infection. Red color indicates that there was a history of previous PCR-verified infection.

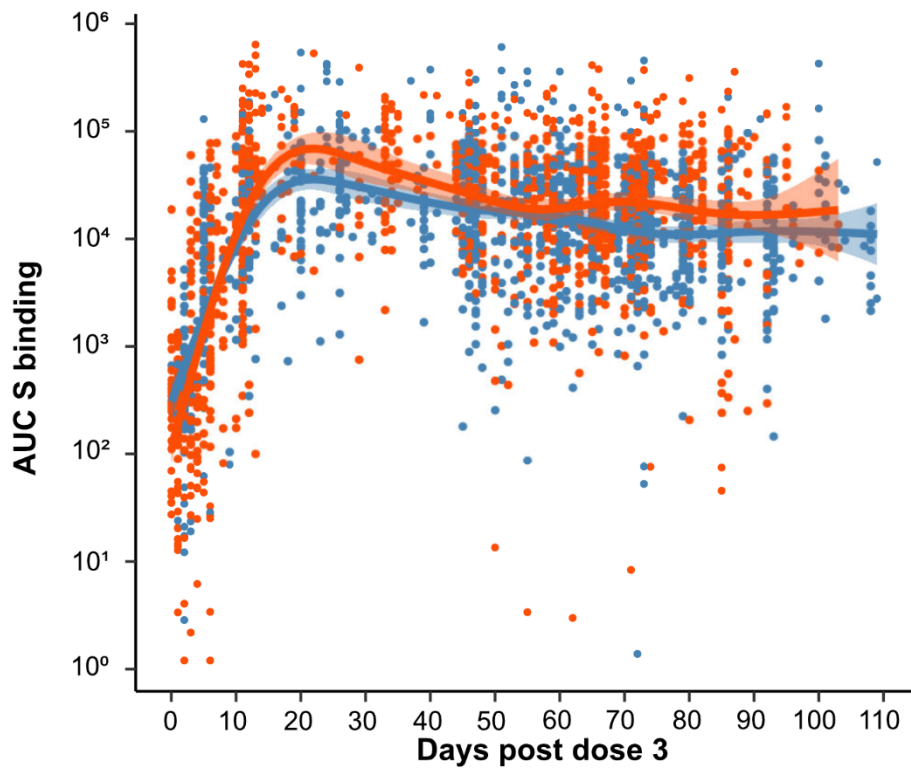


Figure S2. Trends of S-directed IgG in sera by vaccine type. A third vaccination by a half-dose of mRNA-1273 (red) was different from a dose of BNT162b2 (blue). Observations are shown as dots with colored lines representing a cubic spline model with 8 knots to capture trends, 95% confidence intervals are shown as shaded areas. Day 0 indicates the day of vaccination.

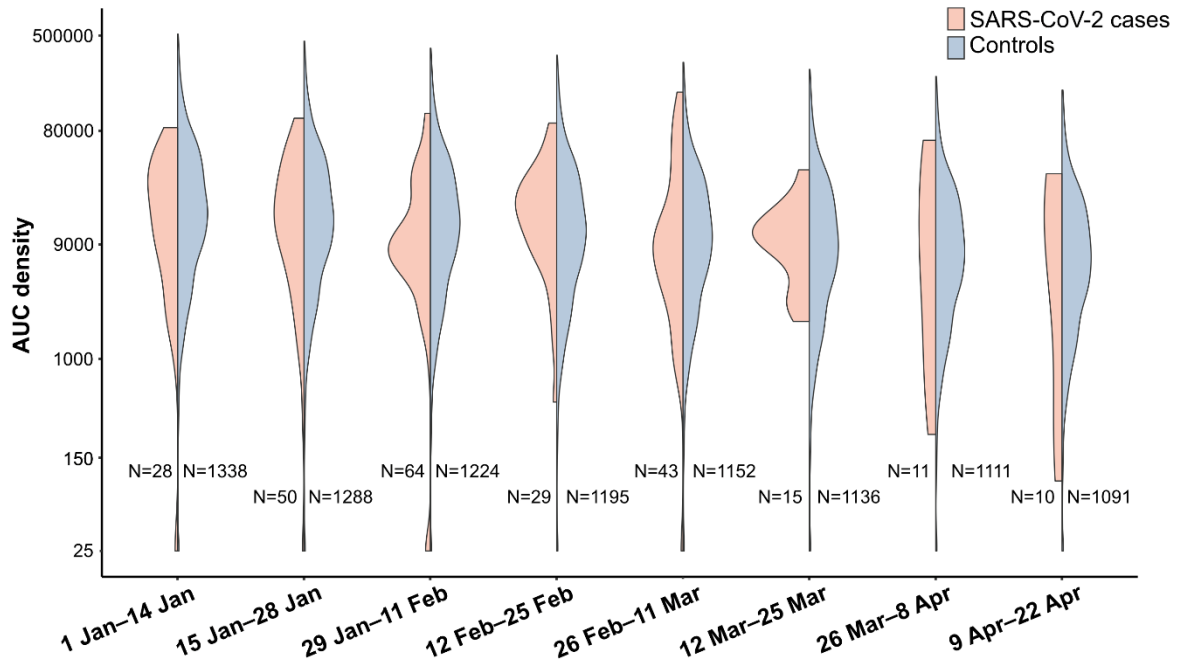


Figure S3. Projected population distribution of S-binding IgG among SARS-CoV-2 infected (red) and non-infected (blue) nursing home residents during the Omicron waves. Antibody levels are shown as density plots per 14-day period starting 1 January 2022. All included study subjects fulfilled criteria of being previously SARS-CoV-2 infection-naïve and had received the third but not the fourth mRNA vaccine dose.

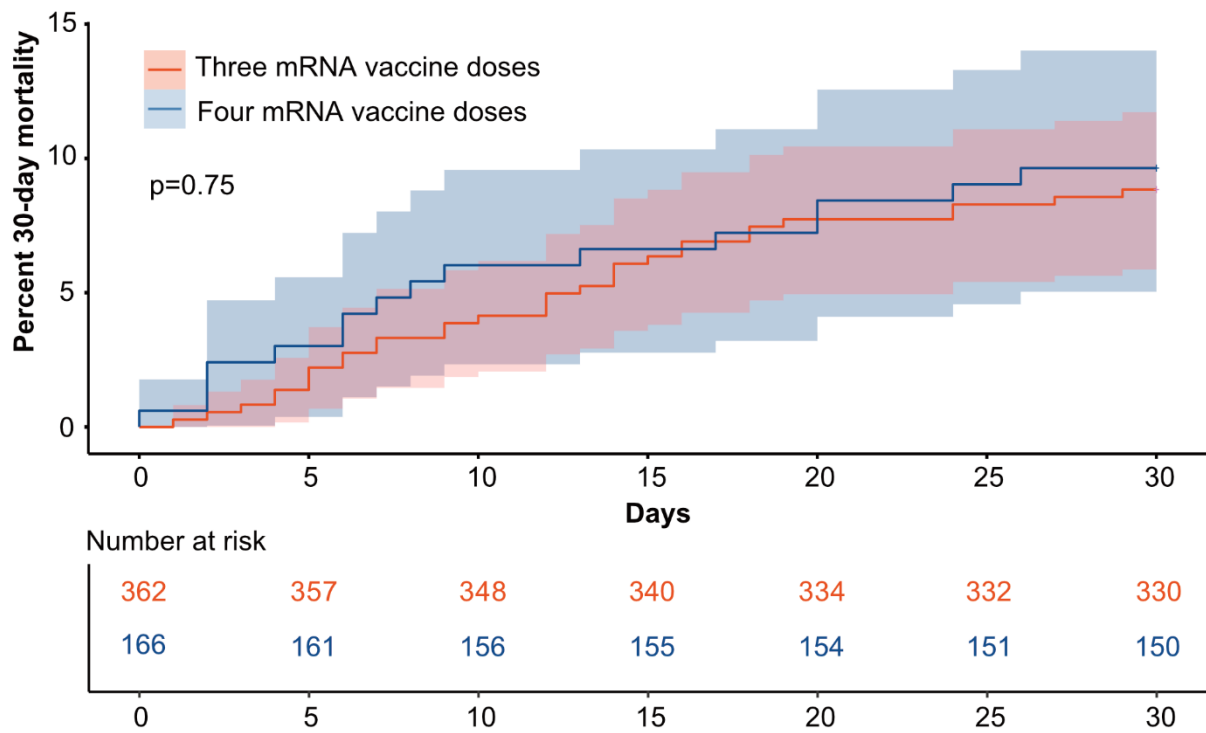


Figure S4. Deaths among SARS-CoV-2-infected study subjects at nursing homes. Cumulative 30-day SARS-CoV-2-associated mortality among 528 previously infection naïve nursing home residents that were vaccinated with three (red) of four (blue) mRNA vaccine doses.

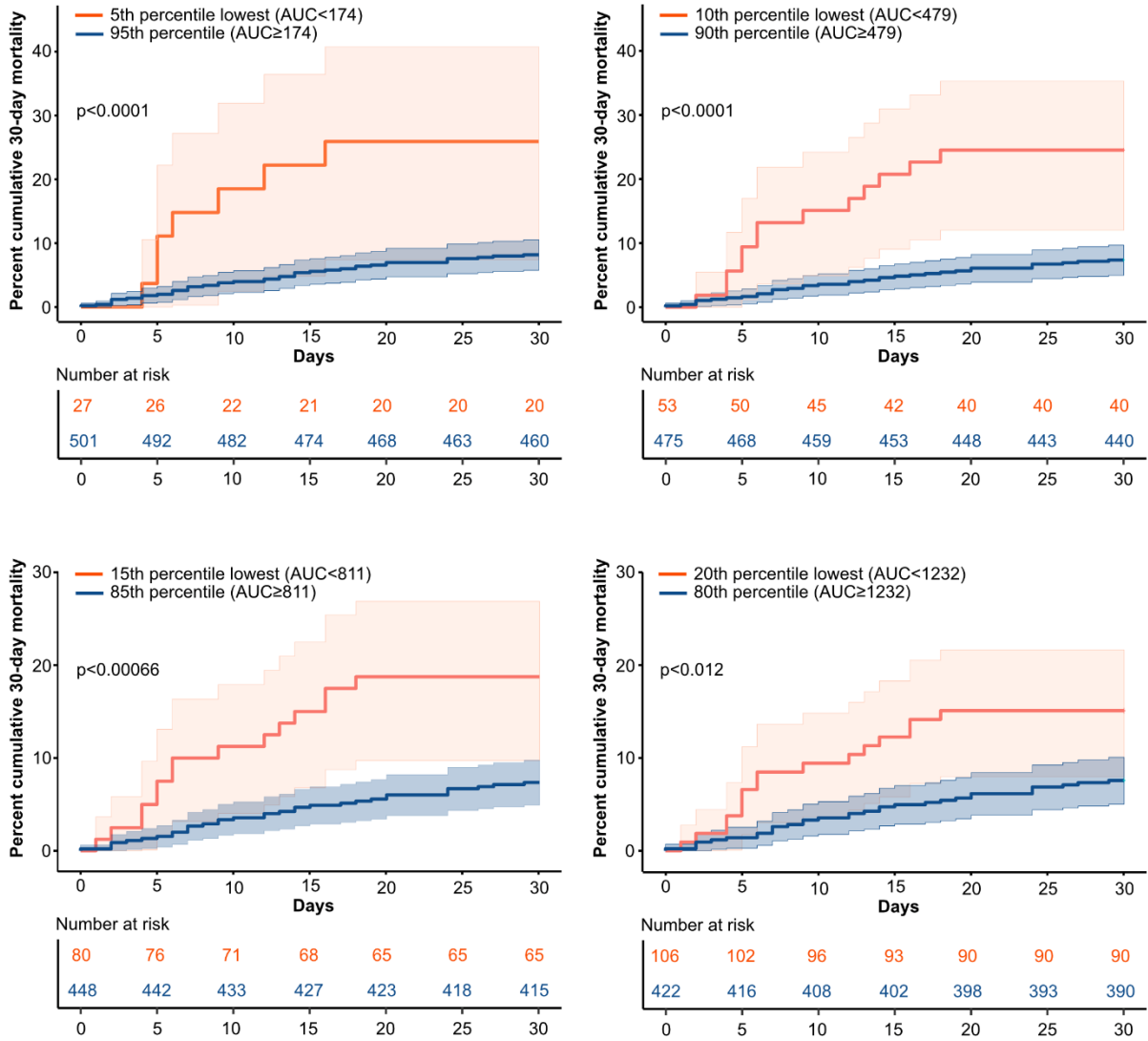


Figure S5. Relationship between S-directed IgG antibody levels at day 60 after vaccine dose 3, and 30-day mortality among 528 SARS-CoV-2-cases. The four panels show the infected population divided by the lowest percentile of antibody levels (5, 10, 15 and 20 percent lowest). Kaplan-Meier curves with 95% confidence intervals for the lowest percentile (red) and the remaining population (blue) are shown. Day 0 is the day of the PCR-verified SARS-CoV-2 infection.

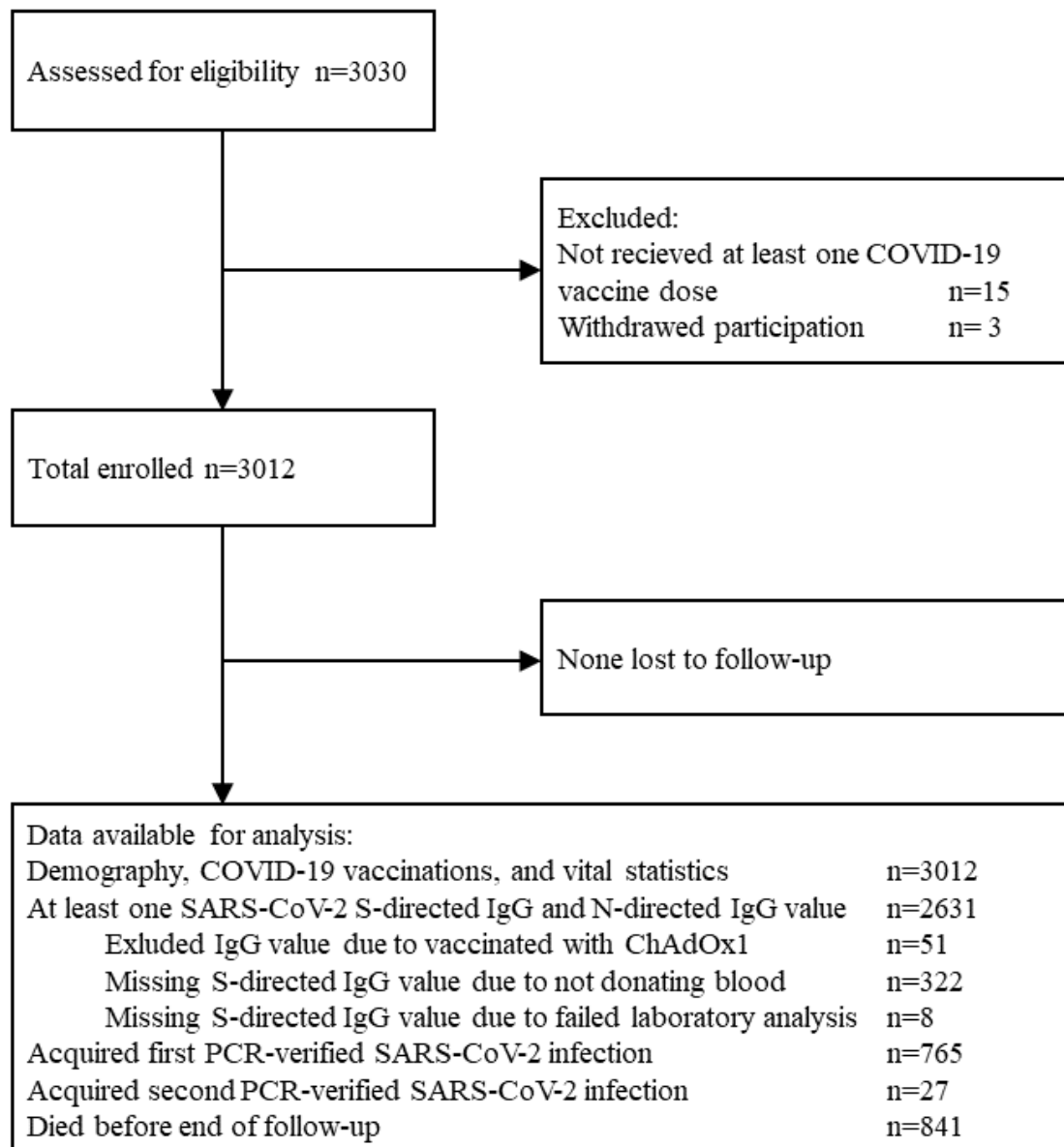


Figure S6. Flow diagram including key elements of enrollment and outcomes for the open cohort with members living in nursing homes.

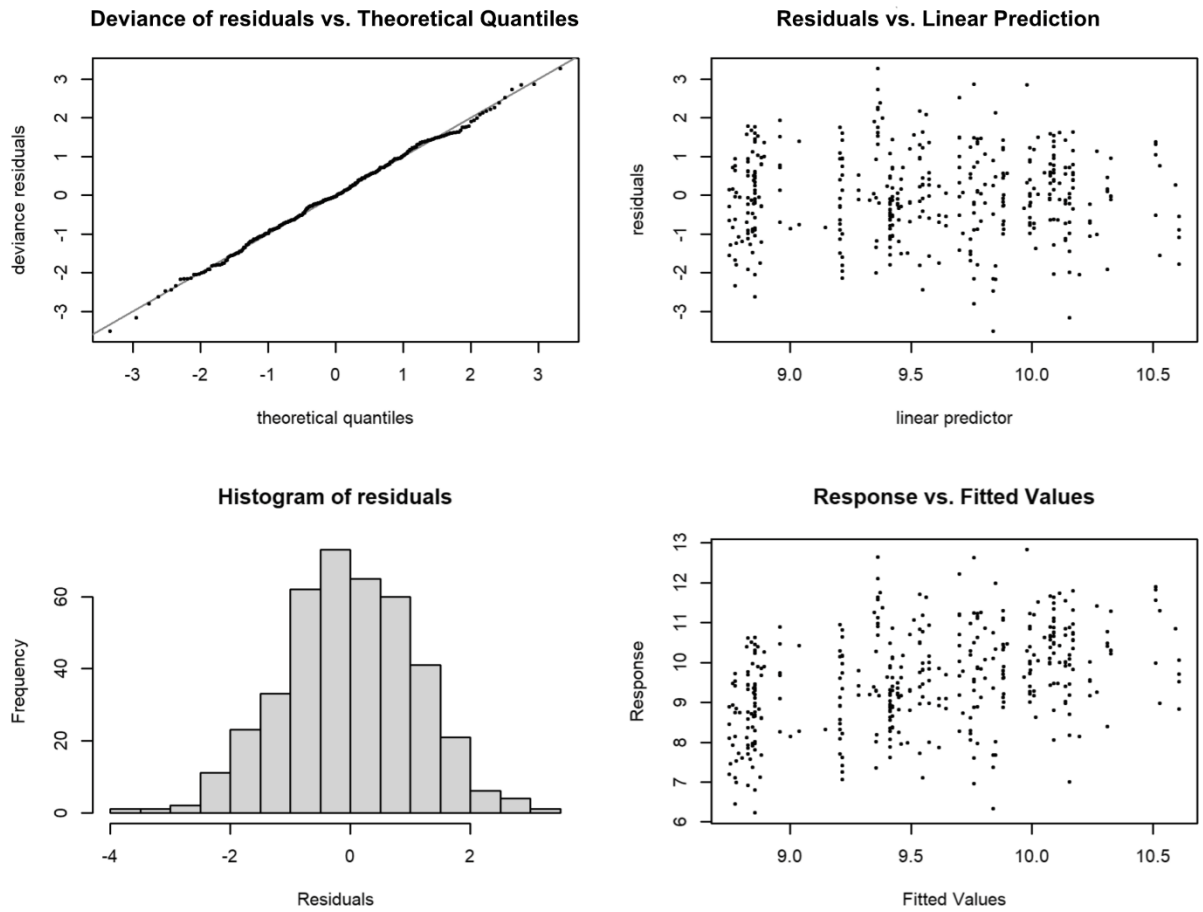


Figure S7. Model tests for the generalized additive model used for estimating differences between vaccine brands. Analyses tested for gender difference, interaction between vaccine brands and the pace of decline in AUC over time by analyzing S-directed antibody AUC-levels after dose 3 in paired samples from 202 study subjects with no SARS-CoV-2-infection between the samples and no history of previous infection.

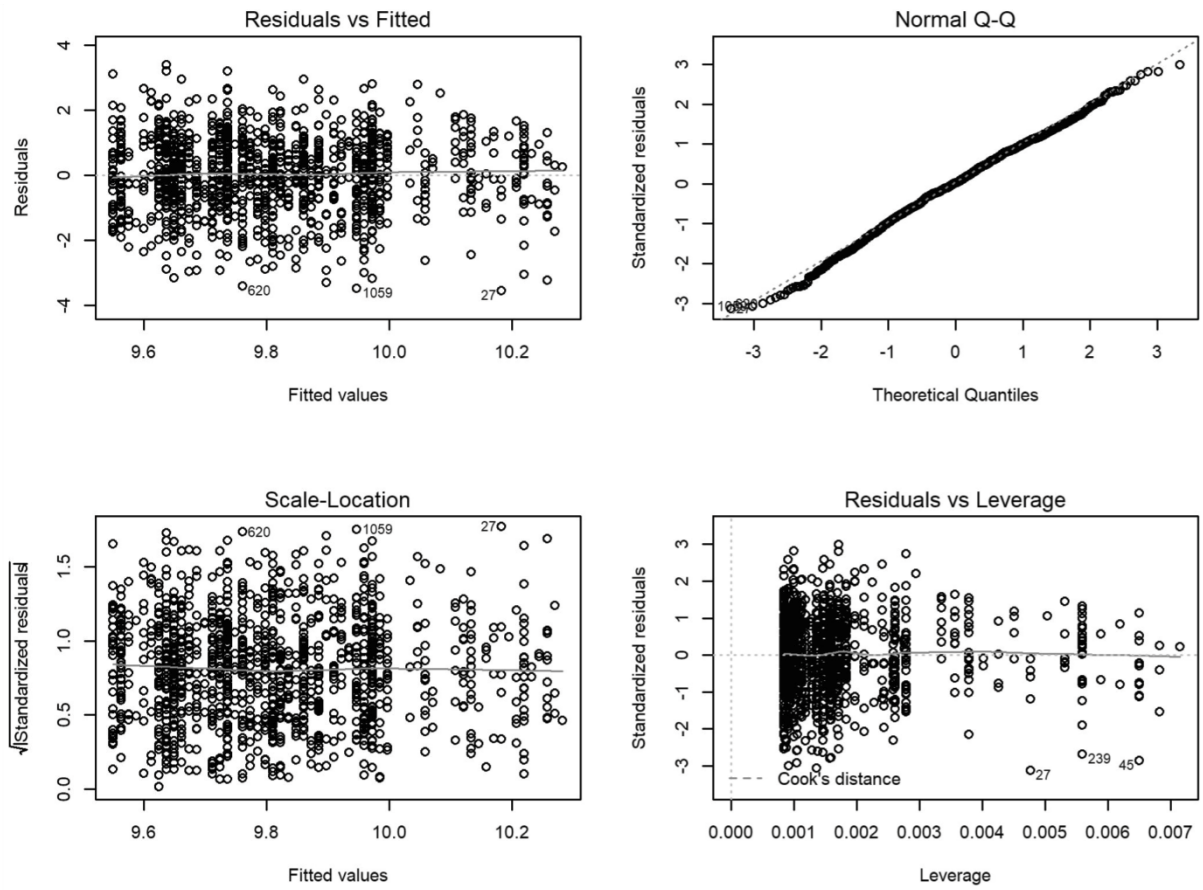


Figure S8. Model tests for a logarithmic regression model used for approximating the decline in AUC-levels. The analyses included 1010 observations of 1010 unique individuals from day 20 to day 80 after vaccine dose 3.