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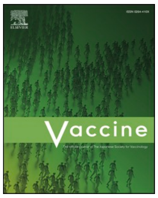

Long-term neutralization capacity of vaccine and breakthrough infection induced SARS-CoV-2 specific antibodies against omicron subvariants BA.2, XBB.1.5, and JN.1

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ABSTRACT

The long-term effectiveness of COVID-19 vaccines remains a critical area of study, especially with the emergence of new SARS-CoV-2 subvariants. In this study, we investigated the long-term neutralization capacity of SARS-CoV-2 -specific antibodies induced by the third, fourth and fifth vaccine doses, and by SARS-CoV-2 breakthrough infections. Spike (*S*)-specific antibodies decline relatively rapidly after each vaccine dose, with an estimated half-life of 3–4 months. However, after the third vaccine dose, S-specific serum antibody levels remained comparable at 12 and 24 months post-vaccination. Antibody levels induced by the fourth and fifth vaccine doses were higher, and the decay was slower than after the third vaccine dose. Additionally, nucleocapsid (N)-specific antibody levels increased significantly following multiple breakthrough infections. The neutralization capacity of the antibodies against Omicron XBB.1.5 and JN.1 subvariants were significantly increased by the fifth, XBB.1.5 subvariant specific, mRNA vaccine dose. Our findings strongly indicate that updated booster vaccines based on the latest s of concern are necessary to sustain high neutralizing antibody levels against emerging variants. No exhaustion of vaccine-induced antibody response was observed after repeated COVID-19 vaccinations.

1. Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent of coronavirus disease-19 (COVID-19) and long-COVID, appears to have become endemic worldwide. Omicron subvariants that became globally dominant from November 2021 onwards have infected millions of people and continue to do so [1]. Despite the efficacy of vaccines and booster doses in preventing severe COVID-19, breakthrough infections by Omicron subvariants are common [2,3]. While SARS-CoV-2 infections are no longer a public health emergency of international concern (PHEIC), COVID-19 and long-COVID still continue to impose a substantial burden on the economy and healthcare services worldwide [4].

Vaccinations against SARS-CoV-2 have been effective in preventing hospitalizations, healthcare burden, serious complications from COVID-19, and the occurrence of long-COVID [5]. Current vaccination

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Abbreviations: SARS-CoV-2, Severe acute respiratory syndrome coronavirus-2; COVID-19, Coronavirus disease-19; PHEIC, Public health emergency of international concern; S1, Spike1; MNT, Microneutralization test; EIA, Enzyme immunoassay; HCW, Healthcare workers.

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programs in Finland have proven effective at inducing neutralizing antibodies and raising the levels of SARS-CoV-2 spike (S) -specific antibodies, but the enhanced immune evasion ability gained due to the rapid mutation rate of the virus remains a significant concern [6,7]. Recent vaccines encoding for Omicron BA.4, BA.5, and XBB.1.5 specific spike proteins have been effective against these subvariants [8,9]. However, the long term efficiency and cross-neutralization capabilities of these recent vaccines against past and emerging variants has remained poorly characterized. In this study, we investigated the long-term durability and neutralization capacity of SARS-CoV-2-specific antibodies induced by the third, fourth and fifth booster vaccine doses and breakthrough infections against SARS-CoV-2 Omicron subvariants BA.2, XBB.1.5, and JN.1.

2. Material and methods

2.1. Ethical statement

The study was approved by Southwest Finland health district (ETMK 19/1801/2020, EudraCT 2021–004419-14) and Helsinki-Uusimaa health district (HUS/1238/2020, EudraCT 2021–004016-26) ethical review boards. All study participants took voluntarily part in the study and they signed a written informed consent before the first sampling.

2.2. Serum samples

Serum samples were obtained from 225 healthcare workers (HCW) at Turku University Hospital and Helsinki University Hospital. Samples were collected up to 24 months after the third, 12 months after the fourth, and 3 months after the fifth vaccine dose (Table 1.). HCWs received either mRNA-1273 (Moderna) or BNT162b2 (Pfizer BioNTech) as the third dose, and mRNA-1273 (Moderna), XBB.1.5 monovalent (Moderna), bivalent Original/Omicron BA.1 (Pfizer BioNTech) or bivalent Original/Omicron BA.4/5 (Pfizer BioNTech) as the fourth dose, and monovalent Original/Omicron XBB.1.5 (Pfizer BioNTech) or bivalent Original/Omicron BA.4/5 (Pfizer BioNTech) as the fifth dose. Breakthrough infections were identified with positive SARS-CoV-2 antigen or RT-qPCR test result reported by the study participants, or as an increase greater than the cut-off value in anti-S1 (4.8 EIA units) or anti-N (8.8 EIA units) IgG antibody levels between consecutive samples.

Table 1
Demographics of the study population.

	All	3D	4D	5D
N	225	175	31	19
Female	200	153	29	18
Male	25	22	2	1
Once infected	115	92	14	9
Double infected	75	65	6	4
Triple infected	8	6	1	1
Total infected	198	163	21	14
Age				
Mean	46	43	54	56
Median	47	44	60	61
Range	20–67	23–65	20–66	30–67
Number of breakthrough infections by timepoint				
non	27	12	10	5
pre3Dpre	2	2	0	0
3Dpre-3D3wk	4	3	1	0
3D3wk-3D3mo	8	8	0	0
3D3mo-3D6mo	68	68	0	0
3D6mo-3D9mo	14	14	0	0
3D6mo-3D12mo	68	67	1	0
3D12mo-3D24mo	78	78	0	0
pre4Dpre	5	–	4	1
4Dpre-4D3wk	7	–	4	3
4D3wk-4D3mo	7	–	5	2
4D3mo-4D6mo	6	–	4	2
4D6mo-4D9mo	7	–	3	4
4D9mo-4D12mo	10	–	7	3
pre5Dpre	1	–	–	1
pre5D-5D3wk	2	–	–	2
5D3wk-5D3mo	2	–	–	2

2.3. SARS-CoV-2 variants

In this study four SARS-CoV-2 variants were used: D614G (MW717675.1 and EPI_ISL_412971), Omicron BA.2 (OP199045 and EPI_ISL_9695067), Omicron XBB.1.5 (OQ509907 and EPI_ISL_16526646), and Omicron JN.1 (EPI_ISL_19193868). To isolate the SARS-CoV-2 strains, SARS-CoV-2 PCR-positive nasopharyngeal samples were inoculated to VeroE6 (for D614G) or VeroE6-TMPRSS2-H10 cells [10] (for Omicron BA.2, XBB.1.5, and JN.1) and then passaged in VeroE6-TMPRSS2-H10 cells in DMEM (EuroClone) supplemented with 2 % fetal calf serum (FCS; Gibco), 2 mM L-glutamine (Gibco), and penicillin-streptomycin. The isolation and full-length sequencing of SARS-CoV-2 variant D614G, and Omicron subvariants BA.2, and XBB.1.5 has been described previously [7,11,12]. For the analysis of antibody responses against one of the latest circulating SARS-CoV-2 subvariants, Omicron JN.1 was isolated and the full length sequence was analyzed as follows: Nucleic acid extraction was performed using the Nuclisens® EasyMAG® platform and reagents (Biomérieux, France). The presence of SARS-CoV-2 RNA was confirmed with an *in-house* real-time multiplex RT-PCR. The Bioline SensiFAST™ Probe No-ROX One-Step Kit based multiplex-PCR was used to amplify human β-actin RNA (IC), E gene of SARS-CoV-1 and SARS-CoV-2 viruses and S gene of SARS-CoV-2 spike protein. In addition, S gene P and B117_P probes were used to detect wild-type SARS-CoV-2 and H69-V70 deletion in S gene, respectively. Multiplex-PCR was performed using the MIC qPCR cyclers (Bio Molecular Systems, Australia). The NGS library preparation was performed with QIAseq DIRECT SARS-CoV-2 Library kit and QIAseq DIRECT SARS-CoV-2 Enhancer (QIAGEN, USA), the quality of the library was determined with Qubit™ dsDNA HS Assay Kit (Invitrogen™) and Bioanalyzer Agilent High Sensitivity DNA Kit (Agilent, USA). Sequencing was performed with Illumina™ MiSeq platform using MiSeq Reagent Micro kit v2 (300 cycles) (Illumina™, USA). Results were analyzed with Illumina BaseSpace application DRAGEN COVID Lineage version 4.0.6 and comparison was done with NextClade software version 3.6.0. CLC genomic Workbench Microbial Module (QIAGEN, USA) version 23.0.2, pipeline ‘Identify QIAseq SARS-CoV-2 Low Frequency and Shared Variants (Illumina)’ version 1.74 was used to produce consensus sequence for nCoV-19/Finland/TKU-3 (submitted to Gisaid).

Median Infectious Dose (ID_{50}) assay was used to determine virus stock titers as described previously [11,13]. The structural model (PDB ID: 7WK2) of Omicron spike protein was used to highlight the subvariant-specific amino acid changes of the SARS-CoV-2 Omicron subvariants with UCSF Chimera 1.15.

2.4. SARS-CoV-2 S1 and N protein-specific enzyme immunoassay

SARS-CoV-2 S1 and N-specific antibodies present in sera were detected and analyzed with an in-house enzyme immunoassay (EIA) as previously described [11,13]. Briefly, 96-well plates (Nunc Maxisorp) were coated with SARS-CoV-2 S1 (3.5 μg/ml) or N (2 μg/ml) proteins diluted in PBS. Serum samples (50 μl/well) were diluted 1:1000 for S1-specific EIA and 1:300 for N-specific EIA in PBS supplemented with 5 % swine serum, and were incubated for 2 h at 37 °C. Bound antibodies were detected with anti-human IgG antibodies conjugated with horse radish peroxidase (Dako) diluted 1:8000 for S1-specific EIA and 1:16000 for N-specific EIA, followed by colorimetric detection with TMB (Kementec Solutions) and the measurement of the absorbance at 450 nm (Victor Nivo, Revvity).

The measured optical density (OD) values were converted to EIA units using a linear interpolation between OD-values of known positive (100 EIA units) and negative (0 EIA units) serum specimens. The thresholds to determine seropositivity were established as described previously [7].

2.5. Microneutralization tests

The titers of neutralizing antibodies in the sera were determined with a microneutralization test (MNT) as described previously [11,13]. Briefly, 50 ID_{50} of virus was added to serum sample dilutions, resulting in two-fold dilutions from 1:10 up to 1:40,960 (D614G) or 1:2560 (Omicron subvariants). Virus-serum dilution mixtures were incubated for 1 h and VeroE6-TMPRSS2-H10 cells (50,000 cells per well in 96-well plate) were added for four days. The cells were fixed with 4 % formaldehyde, stained with crystal violet, and visualized for cell death. Reciprocal of the serum dilution able to inhibit 50 % of cell death was determined as the neutralization titer (half-maximal inhibitory dose, ID_{50}). $ID_{50} > 10$ was considered positive for neutralizing antibodies. Serum with neutralizing antibodies, cells alone, and virus without serum were used as controls in every MNT plate.

2.6. Statistics and reproducibility

Data was arranged in Excel 2016 (Microsoft 365) and analyzed with

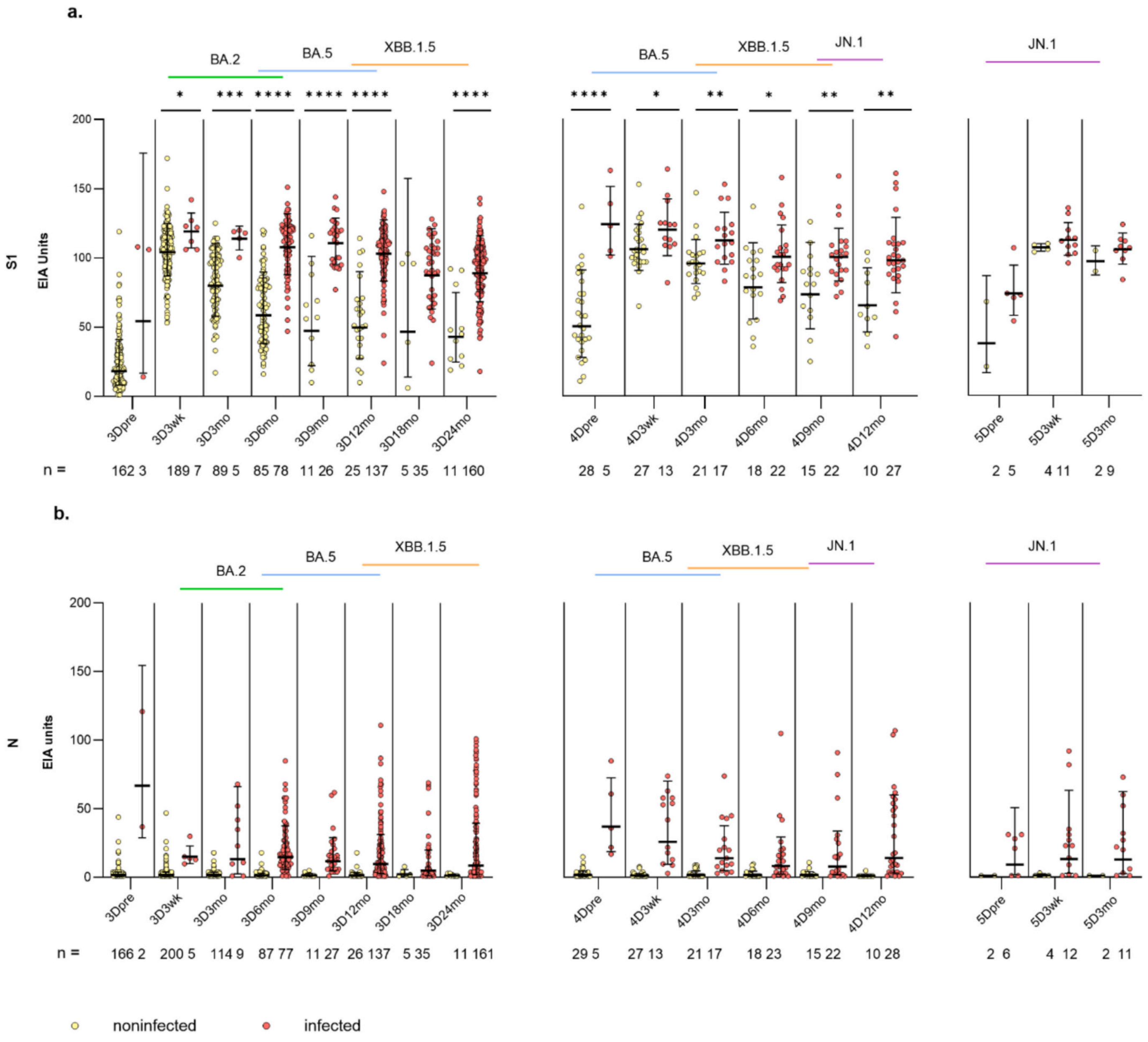

Fig. 1. SARS-COV-2 S1 and N protein-specific IgG antibody levels in sera from uninfected and infected vaccinees. Serum samples collected from voluntary health care workers (HCWs) five months after the second vaccine dose (3Dpre), three weeks (3D3wk) and three (3D3mo), six (3D6mo), nine (3D9mo), twelve (3D12mo), eighteen (3D18mo), and twenty-four months (3D24mo) after the third dose, and three weeks (4D3wk), and three (4D3mo), six (4D6mo), nine (4D9mo), and twelve months (4D12mo) after the fourth dose, and three weeks (5D3wk) and three months (5D3mo) after the fifth dose were analyzed for A. S1- specific and B. N-specific IgG antibodies determined by EIA. Before fourth dose (4Dpre) and before fifth dose (5Dpre) samples were selected as close as possible to the fourth and fifth vaccine dose administration. Yellow dots indicate uninfected vaccinees and red dots indicate SARS-CoV-2 infected vaccinees. Colored lines indicate the dominant subvariant at the time of serum sampling. Geometric means and geometric standard deviations of the IgG antibody levels are shown. Mann-Whitney *U* test was used to analyze the statistical significance between the samples. Two-tailed *p*-values <0.05 were considered statistically significant. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

GraphPad Prism (version 10.1.2). To test the statistical significance, Wilcoxon signed-rank test or Friedman test followed by Dunn's multiple comparisons was used for paired samples, and Mann Whitney *U* test was used for unpaired samples or time points where multiple participants lacked samples. Tests were two-sided, and *p*-value <0.05 was considered statistically significant. The correlation between the values was analyzed using the Spearman's correlation test. One phase decay analysis with 95 % confidence interval was used to analyze antibody half-lives.

3. Results

3.1. Demographics of the study population

Serum samples were collected from HCWs ($n = 225$) working at the Turku University Hospital (TYKS) and Helsinki University Hospital (HUS), of whom 200 (89 %) were female and 25 (11 %) male, aged 20 to 67 years (median 47 years) (Table 1.).

Of the study population, 175 (77.8 %) had received three vaccine doses with the original Covid-19 vaccines (BNT162b2 and mRNA-1293), and 50 (22.2 %) had received four ($n = 31$) or five ($n = 19$) vaccine doses. The median age of the three times vaccinated HCWs was 44 years, while the median age of the fourth and fifth dose recipients was 60 years. The fourth booster doses were mRNA-1273, monovalent XBB.1.5, bivalent Omicron BA.1, or bivalent Original/Omicron BA.4/5. The fifth booster dose was a monovalent Omicron XBB.1.5 or a bivalent Original/ Omicron BA.4/5 vaccine. Individual data on vaccines administered was not available. Of all the participants, 198 (88 %) were infected at least once. Of the third dose, fourth dose and fifth dose participants, 163 (93 %) 21 (68 %) and 14 (74 %) respectively, were infected at least once.

3.2. S1-specific antibodies boosted by the fourth and fifth vaccinations continued to decay, but remained detectable 24 months post-vaccinations

We have previously analyzed the sera obtained up to 12 months post the third dose and 6 months post the fourth dose from the vaccinated HCWs [2]. In this study, to analyze the long-term antibody levels and the effect of the fifth vaccine dose, we included samples collected up to 24 months post the third vaccination, 12 months post the fourth vaccination, and 3 months post the fifth vaccination, and analyzed both S1 and N protein-specific antibodies with EIA (Fig. 1).

More specifically, serum samples analyzed in this study were collected before the third (0–105, median 9 days), fourth (0–162, median 3 days), and fifth dose (0–97, median 19 days), and 3 weeks, and 3, 6, 9, 12, 18, and 24 months after the third dose (3Dpre, 3D3wk, 3D3mo, 3D6mo, 3D9mo, 3D12mo, 3D18mo, 3D24mo), and 3 weeks, and 3, 6, 9, and 12 months after the fourth dose (4Dpre, 4D3wk, 4D3mo, 4D6mo, 4D9mo, 4D12mo), and 3 weeks and 3 months after the fifth dose (5Dpre, 5D3wk, 5D3mo).

In uninfected vaccinees, S1-specific antibody levels remained at the same level at 9 to 24 months post the third dose (Fig. 1) and all uninfected vaccinees had detectable S1-specific antibodies 24 months post the third vaccine dose. The fourth and fifth vaccine doses boosted antibody levels to those seen after the third dose (Fig. 1). Again, at 12 months post the fourth vaccine dose all uninfected vaccinees had detectable S1-specific antibodies.

Out of 175 vaccinees who received three vaccine doses, 163 (93 %) had experienced at least one SARS-CoV-2 breakthrough infection by 3D24mo time point, with 78 vaccinees contracting infection between 3D12mo and 3D24mo time points. Breakthrough infection-induced S1-specific antibody levels were significantly higher than vaccine-induced antibody levels up to 24 months post the third vaccine dose (Fig. 1). In addition, out of 50 vaccinees with four or five vaccine doses, 35 (72 %) had contracted at least one SARS-CoV-2 infection by 4D12mo or 5D3mo time points (red dots in Fig. 1). Compared to uninfected vaccinees, the breakthrough infection-induced S1-specific antibody levels were significantly higher at all time points after the fourth vaccine doses. Out of all vaccinees with SARS-CoV-2 breakthrough infection ($n = 198$), 83 (43 %) experienced more than one breakthrough infection. Multiple breakthrough infections did not induce higher S1-specific antibody levels compared to those seen after the first SARS-CoV-2 infection (Fig. 2), although on average a small x0.9 fold difference ($p = 0.0027$) in levels could be observed between 1st and 2nd breakthrough infection.

N protein-specific antibody levels remained very low/undetectable among uninfected vaccinees, with the exception of 2/25 (8 %) vaccinees with high background levels (Fig. 1). Out of the SARS-CoV-2 infected vaccinees, N-specific antibody levels remained low in 32/198 (16 %) individuals. Vaccinees with more than one SARS-CoV-2 breakthrough infection had 2.4× ($p < 0.0001$) to 2.9× ($p = 0.0045$) fold higher N-specific antibody levels compared to participants with only one breakthrough infection (Fig. 2). The difference in N-specific antibody levels between the second and the third SARS-CoV-2 breakthrough infection was non-significant.

In summary, although booster doses induced high S1 specific antibody levels, SARS-CoV-2 infections induced higher levels. The number of breakthrough infections increased at later time points and multiple breakthrough infections did not further increase S1-specific antibody levels, while there was a clear increase in N-specific antibody levels.

3.3. S1-specific antibody levels of uninfected vaccinees decay slower post the fourth and the fifth vaccine dose

S1-specific antibody levels of uninfected vaccinees were lower before the third vaccine dose compared to the levels seen before the fourth vaccine dose (Fig. 1). At three weeks post the fourth and fifth booster doses, S1-specific antibody levels were at comparable levels to those seen at three weeks post the third vaccine dose (Fig. 3).

From three months up to 12 months post vaccination, S1-specific antibody levels were higher after the fourth and fifth doses compared to the levels after three vaccine doses (fold change 1.2× for 3D3mo vs. 4D3mo and 3D3mo vs. 5D3mo, 1.3× for 3D6mo vs. 4D6mo, 1.5× for 3D9mo vs. 4D9mo, and 1.3× for 3D12mo vs. 4D12mo), and the average half-life of S1-specific antibodies was 84.66 days (range 55.20–130.4) after the third dose and 344.9 days after the fourth dose (range 66.3–732.3), indicating that the decay of S1-specific antibody levels is slowed down after the fourth and fifth vaccine doses (Fig. 3, Extended Fig. 1). As expected, N-specific antibody levels remained low in uninfected vaccinees. In summary, S1-spesific antibodies were boosted by all vaccinations to similar peak levels. The third vaccination induced S-specific antibody levels decayed faster than fourth and fifth vaccine dose induced antibody levels.

3.4. Neutralizing antibodies followed up to 24 months after the third vaccine dose continued to decline but were increased by breakthrough infections

In order to select relevant variants of concern for microneutralization tests to analyze the neutralizing antibodies, we surveyed the appearance of different Omicron subvariants in the Finnish population at the time of serum sampling of our vaccinees. Omicron subvariants BA.2, and later XBB.1.5 and JN.1 subvariants, were prevalent during the time after the third and subsequent vaccine doses. During the evolution of Omicron subvariants the strains used in this study had harbored 27 (BA.2), 37 (XBB.1.5) and 46 (JN.1) amino acid mutations in the spike protein. These changes are visualized in the 3-dimensional structure of the trimeric S protein (Fig. 4).

To assess the long-term neutralization capacity of SARS-CoV-2 specific antibodies, randomly selected serum samples collected from HCWs ($n = 47$) were analyzed up to 24 months post the third vaccine ($n = 20$), 12 months post the fourth vaccine ($n = 15$), and three months post the fifth vaccine ($n = 12$) dose with a microneutralization test (MNT) against D614G variant and Omicron subvariants BA.2, XBB.1.5, or JN.1 (Fig. 5).

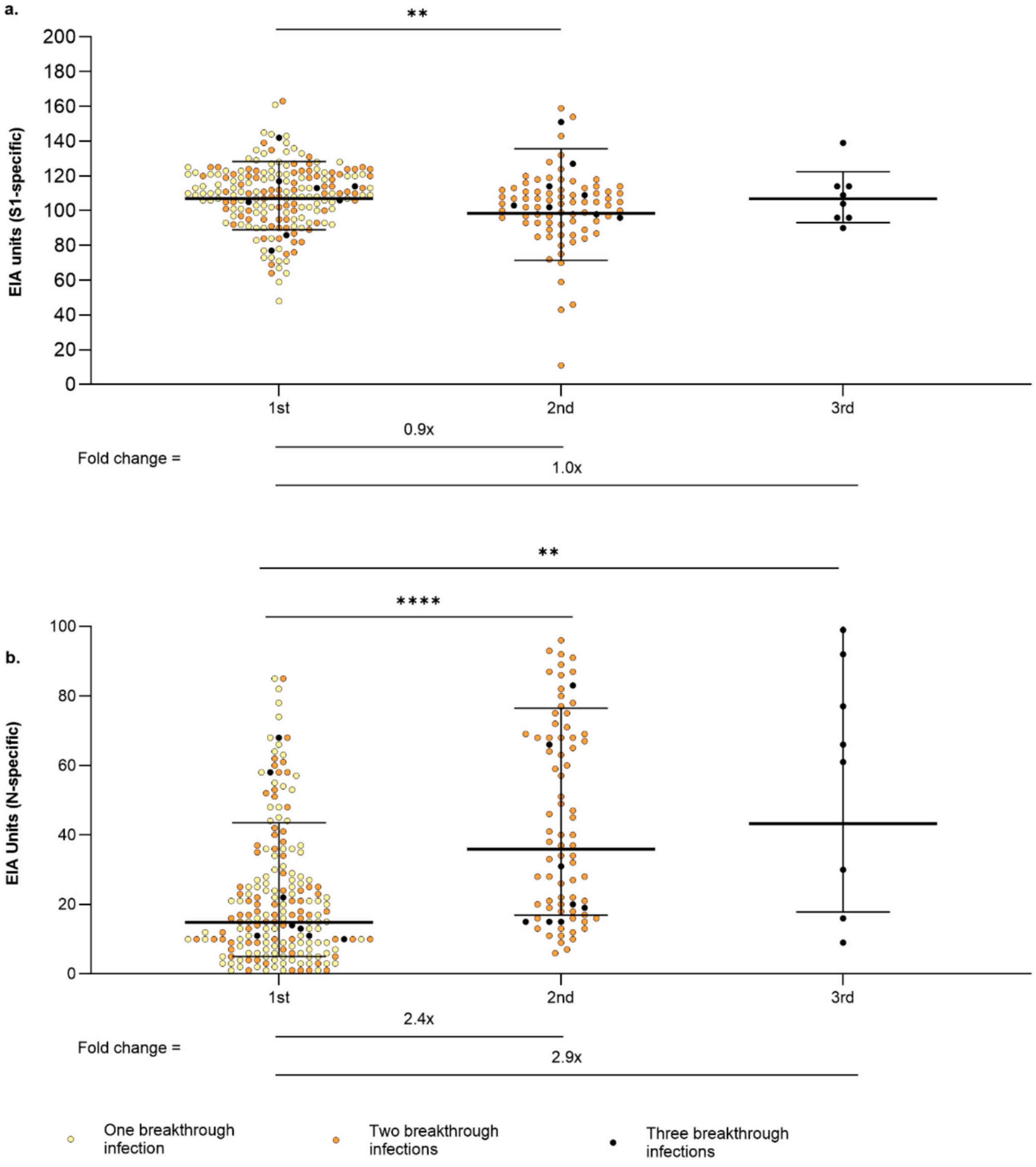

Fig. 2. S1 and N-specific IgG antibody levels in vaccinees with multiple breakthrough infections. SARS-COV-2 A. S1-specific and B. N-specific IgG antibody levels in sera of vaccinated HCWs with one (1st), two (2nd), and three or more (3rd) breakthrough infections were compared. Yellow dots indicate vaccinees who contracted only one breakthrough infection, orange dots indicate vaccinees who contracted two breakthrough infections, and black dots indicate vaccinees who contracted three or more breakthrough infections during the study period. Geometric means and geometric standard deviations are shown. Mann-Whitney *U* test was used to analyze the statistical significance between the samples. Two-tailed *p*-values <0.05 were considered statistically significant. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

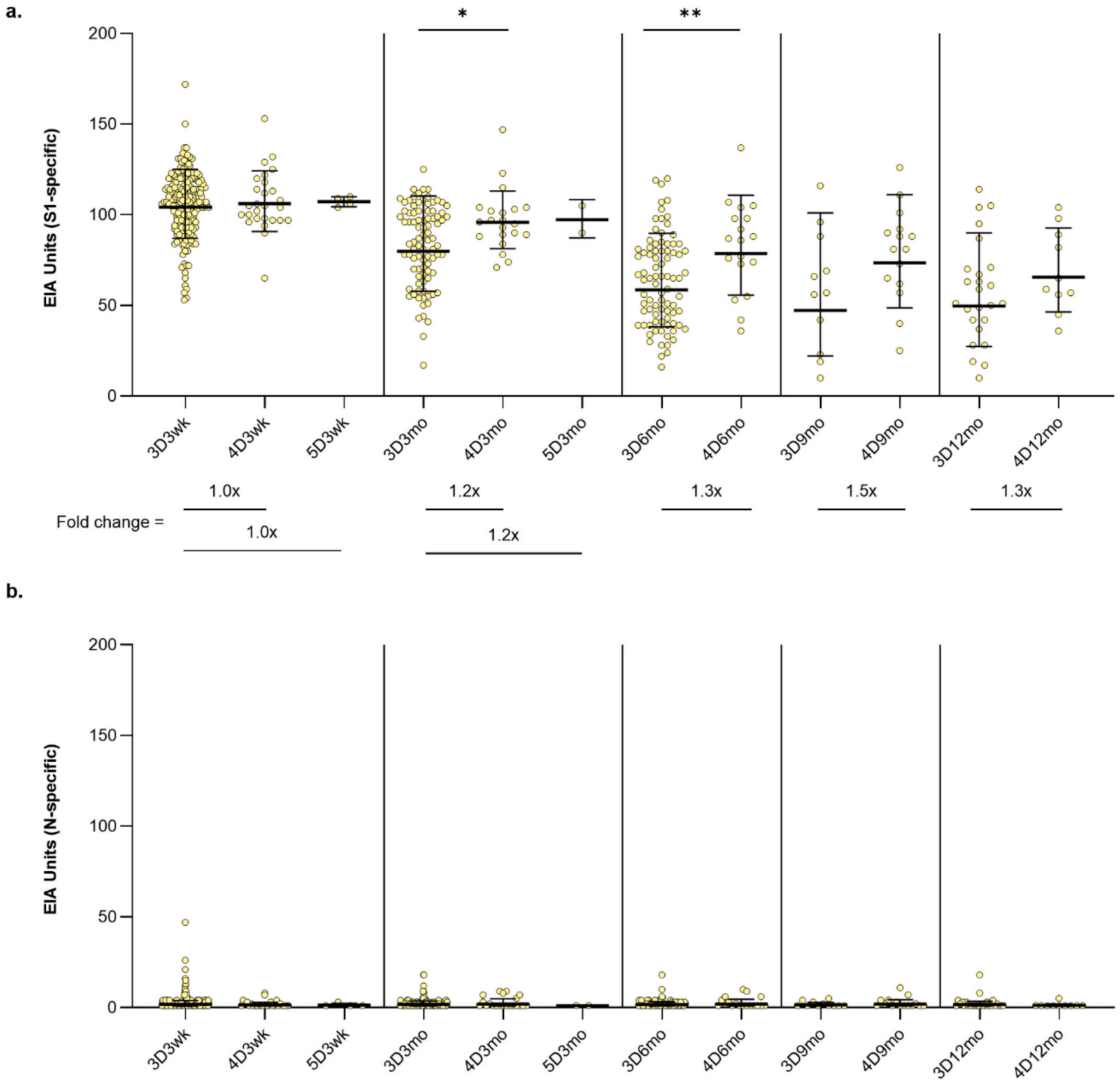

Fig. 3. Comparison of S1 and N-specific IgG antibody levels at the same time points post the third, fourth and fifth vaccine doses. SARS-COV-2 A. S1-specific and B. N-specific IgG antibody levels in sera obtained from uninfected HCWs with three, four, or five vaccine doses were compared at same time points post vaccinations. Geometric means and geometric standard deviations of the means of IgG antibody levels are shown. Mann-Whitney U test was used to analyze the statistical significance between the samples. Two-tailed p-values <0.05 were considered statistically significant. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

In uninfected vaccinees (yellow dots in Fig. 5), the neutralization capacity continued to decline from the previously analyzed 3D12mo time point to the 3D24mo time point. At 24 months post the third vaccine dose the neutralization capacity was below the detection limit against recent Omicron subvariants XBB.1.5 and JN.1, whereas against D614G and Omicron BA.2 2/2 and 1/2 vaccinees, respectively, still had neutralizing antibodies.

During the follow-up period until 24 months post the third vaccine dose, breakthrough infections among the vaccinees accumulated from 3D6mo onward (red dots in Fig. 5), coinciding with the emergence of Omicron subvariants BA.2, BA.5, and XBB.1.5 (colored lines in Fig. 5). Breakthrough infections increased the neutralizing antibodies against all four subvariants, with the highest levels against D614G and lower levels against Omicron subvariants (BA.2 > XBB.1.5 > JN.1). All HCWs with a breakthrough infection had high levels of neutralizing antibodies against D614G and Omicron BA.2 at 3D24 time point, whereas two vaccinees with a breakthrough infection showed no neutralizing antibodies against Omicron XBB.1.5 or JN.1 subvariants at 3D12mo and 3D24mo time points.

3.5. Neutralizing antibodies against omicron subvariants were increased after the fourth and fifth vaccine doses

Neutralizing antibodies induced by mono- or bivalent fourth vaccine

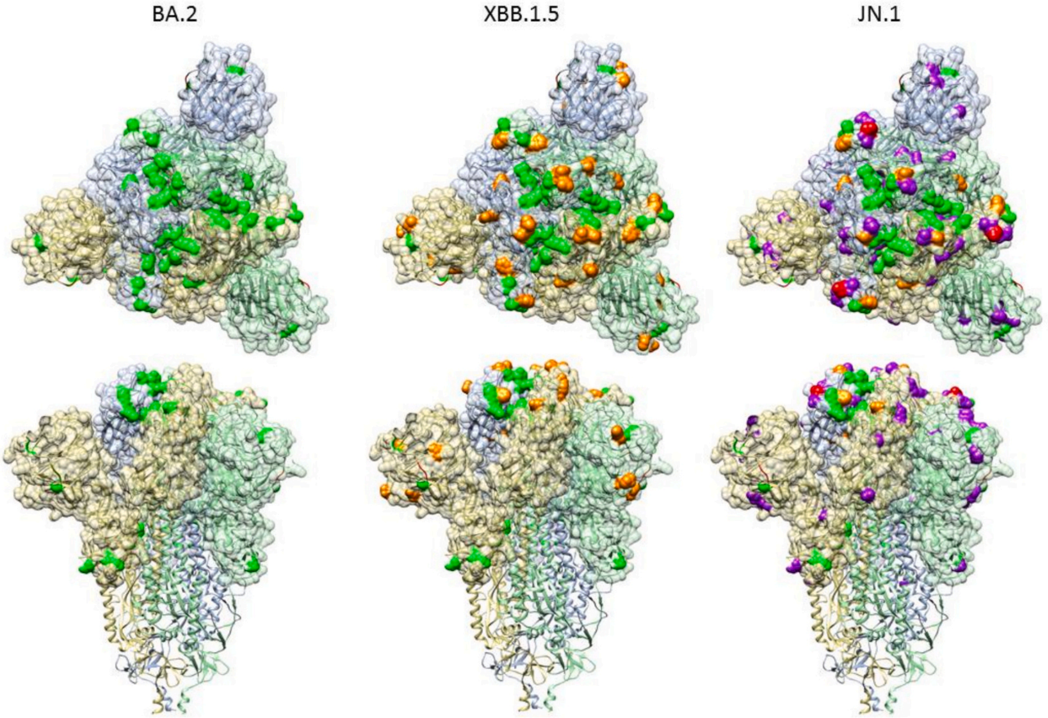

Fig. 4. Three-dimensional structure of SARS-CoV-2 spike protein and Omicron BA.2, XBB.1.5, and JN.1 spike-specific mutations. The 3-dimensional structure of the trimeric S-protein of Omicron strain (PDB ID 7WK2) is shown. The monomers are shown in different colors (light green, yellow, and blue). Green indicates amino acid substitutions present in BA.2, orange indicates amino acid substitutions present in XBB.1.5, purple indicates amino acid substitutions present in JN.1, and red indicates deletions compared to the original Wuhan subvariant spike protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

doses and by mono- or bivalent fifth vaccine doses were assessed with MNTs against D614G and Omicron subvariants BA.2, XBB.1.5, or JN.1 (Fig. 5). In uninfected vaccinees (yellow dots in Fig. 5), the fourth vaccine dose restored high levels of neutralizing antibodies against D614G variant and Omicron BA.2 subvariant, and all vaccinees had detectable neutralizing antibodies against these subvariants at 12 months post the fourth vaccine dose. The levels of neutralizing antibodies increased but remained at low levels against Omicron subvariants XBB.1.5 and JN.1. One vaccinee had no detectable neutralizing antibodies against XBB.1.5 and JN.1 (1/20 and 1/19, respectively) at 3 weeks post the fourth vaccine dose. At 12 months post the fourth dose, two vaccinees had no neutralizing antibodies against Omicron subvariants XBB.1.5 or JN.1 (2/6 and 2/7, respectively). However, in uninfected vaccinees, the fifth vaccine dose induced high levels of neutralizing antibodies against all four subvariants, with detectable neutralizing antibody levels still at 3 months post the fifth vaccine dose. In vaccinees with breakthrough infections (red dots in Fig. 5), the levels of neutralizing antibodies were above the detection limit against all four subvariants in samples collected after the fourth and fifth vaccine dose.

3.6. *The fourth and fifth vaccine doses increased the peak neutralization capacity at each time point compared to that after the third vaccine dose*

The levels of neutralizing subvariant-specific antibodies post each booster vaccine dose were compared in uninfected vaccinees. At 3 weeks post the third and the fourth vaccine dose, the levels of neutralizing antibodies were at similar against D614G variant, and Omicron BA.2 and JN.1 subvariants (Fig. 6).

However, there was a significant 2.5-fold increase in the neutralizing antibody levels against the XBB.1.5 subvariant after the fourth vaccine dose (3D3wk vs. 4D3wk, $p = 0.006$). Interestingly, compared to the third or fourth dose-induced antibody levels 3 weeks post vaccination, the fifth vaccine dose induced significantly higher neutralizing antibody levels against BA.2 ($p = 0.020$), XBB.1.5 ($p < 0.0001$) and JN.1 ($p = 0.0145$). At 3 months post booster doses, neutralizing antibody levels induced by the fifth dose remained significantly higher against all subvariants compared to those after the third dose (D614G 3.3-fold, $p = 0.0095$; BA.2 4.0-fold, $p = 0.0446$; XBB.1.5 13.7-fold, $p < 0.0001$; and JN.1 5.5-fold, $p = 0.0046$). Highest fold difference observed in neutralizing antibodies against XBB.1.5 is likely due to antibodies induced by monovalent XBB.1.5 vaccine, which interestingly also seem to boost neutralizing antibodies against BA.2 and JN.1. The difference in the levels of neutralizing antibodies against Omicron subvariants at 12 months post the third and fourth doses remained similar, (2.6 fold change for D614G, 2.9 for BA.2, 2.3 for XBB.1.5, and 1.6 for JN.1). The half-life of the neutralizing capability of the antibodies against D614G variant and all subvariants, except subvariant JN.1, was increased after the fourth dose (D614G 55.17 days post 3rd dose vs 77.46 days post 4th dose, BA.2 80.31 vs 281, XBB.1.5 71.62 vs 94.81, JN.1 56.07 vs 53.49) (Fig. 6 and Extended Fig. 2).

3.7. *The differences in subvariant-specific neutralization capacities were equalized in infected vaccinees post the fifth vaccine dose*

The neutralization capacity of sera from HCWs was compared between D614G variant and Omicron subvariants BA.2, XBB.1.5, and JN.1 (Fig. 7).

In uninfected vaccinees, neutralization capacity was the highest against D614G throughout the follow-up period of 24 months post the third vaccine dose. The difference between neutralizing capacity of Omicron subvariants compared to D614G decreased from 3 weeks to 24 months post the third dose: fold difference of D614G vs. BA.2 declined from 4.3 to 2.3, fold difference of D614G vs. XBB.1.5 declined from 32.6 to 4.0, and fold difference of D614G vs. JN.1 declined from 21.2 to 4.0. However, this can be explained by the fact that, at later time points, most serum samples lack neutralizing antibodies against XBB.1.5 and JN.1,

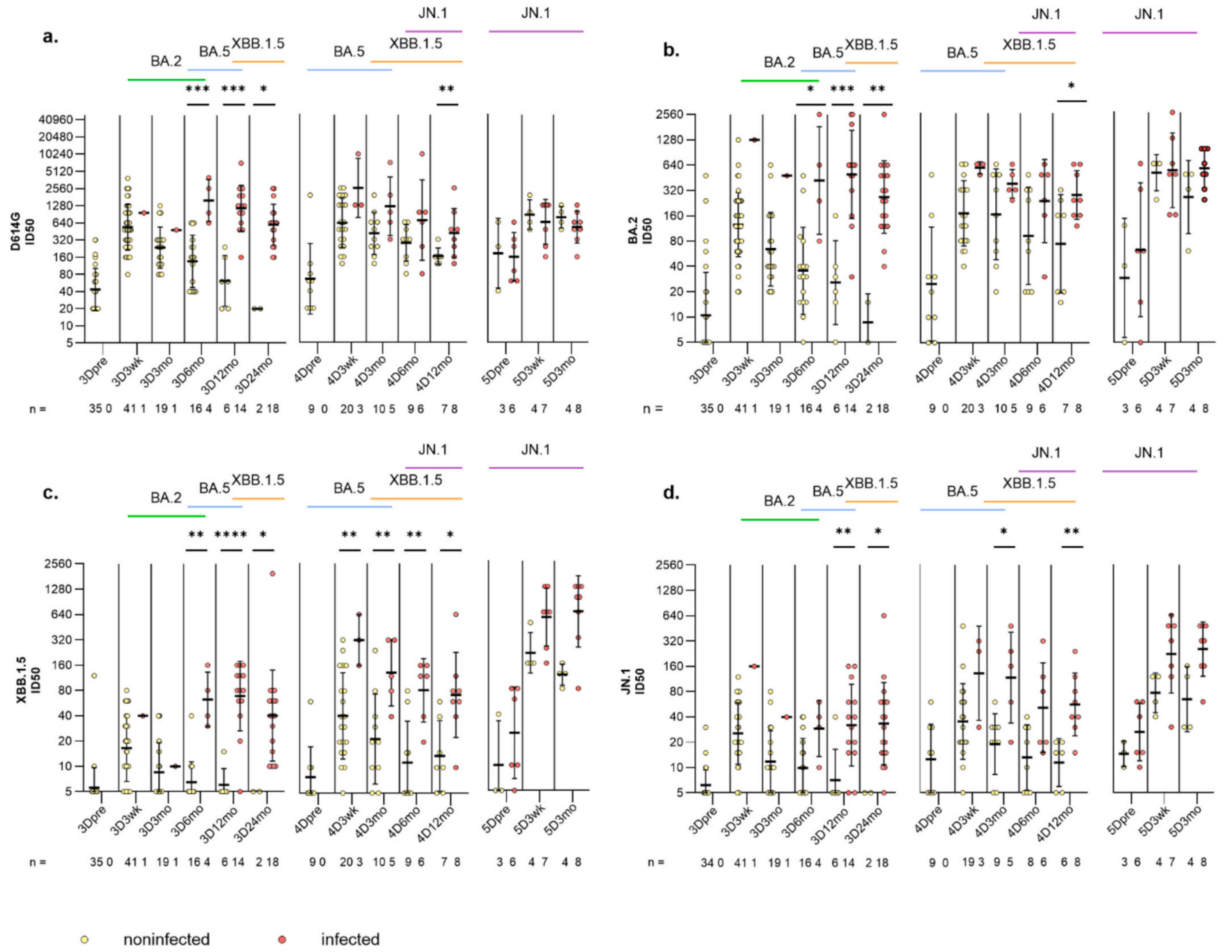

Fig. 5. The levels of neutralizing antibodies against SARS-CoV-2 D614G, BA.2, XBB.1.5, and JN.1 subvariants. Serum samples were collected five months after the second vaccine dose (3Dpre), three weeks (3D3wk) and three (3D3mo), six (3D6mo), eight (3D8mo), twelve (3D12mo), and twenty-four (3D24mo) months after the third dose, and three weeks (4D3wk), and three (4D3mo), six (4D6mo), and twelve months (4D12mo) after the fourth dose, and three weeks (5D3wk) and three months (5D3mo) after the fifth dose (n denotes the number of samples analyzed per time point). Before fourth dose (4Dpre) and before fifth dose (5Dpre) samples were selected as close as possible to the fourth and fifth vaccine dose administration. Yellow dots indicate uninfected vaccinees and red dots indicate SARS-CoV-2 infected vaccinees. Colored lines indicate the dominant subvariant at the time of sampling. The neutralization capacity of antibodies against SARS-CoV-2 subvariants A. D614G B. BA.2C. XBB.1.5 D. JN.1 is indicated as median infectious dose (ID_{50}). Geometric means and geometric standard deviations of the ID_{50} levels are shown. Note the different y-axis values. Mann-Whitney *U* test was used to analyze the statistical significance within time points between the samples collected from uninfected and infected vaccinees. Two-tailed *p*-values <0.05 were considered statistically significant. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rather than by a faster decline of D614G-specific antibodies compared to Omicron-specific antibodies. Differences in neutralizing capacities between Omicron subvariants were significant up to 3 months after the third vaccine dose, with the exception of XBB.1.5 and JN.1 subvariants. At 6 months after the third vaccine dose, only the differences between BA.2 and XBB.1.5 were significant.

In uninfected vaccinees who received the fourth vaccine dose (XBB.1.5 monovalent Spikevax or bivalent Comirnaty Original/Omicron BA.1), the trend of the neutralization capacity followed the same trend as seen after the third vaccine dose. Neutralization capacity was the highest against D614G and lower against the three Omicron subvariants throughout the follow-up period of 12 months post the fourth dose. In contrast to time points post three vaccine doses, the fold differences of neutralizing antibodies against D614G vs. Omicron XBB.1.5 or JN.1 increased up to 6 and 3 months post the fourth dose, respectively, and decreased thereafter. Fold differences between D614G and Omicron BA.2 decreased at all time points, except at 6 months when fold change was slightly increased., Differences in neutralizing capacities between Omicron subvariants BA.2 and XBB.1.5 or JN.1 were significant up to 3 months post fourth vaccine dose, while differences between XBB.1.5 and JN.1 were not. In response to the fifth vaccine dose (monovalent Comirnaty original/Omicron XBB.1.5 or bivalent Comirnaty original/Omicron BA.4/5), the trend of the neutralizing capacity in uninfected vaccinees was similar to the trend post the fourth vaccine dose, although differences were insignificant, with the exception of D614G vs. JN.1 at three weeks after fifth dose.

Next, we assessed the effect of Omicron breakthrough infection to neutralization capacity. In infected vaccinees post three vaccine doses, the neutralization capacity was the highest against D614G, followed by BA.2. The differences were significant at 24 month time point for D614G and BA.2 vs. XBB.1.5 and JN.1. Thus, despite the BA.2 and XBB.1.5 being the dominant circulating subvariants at the time of samplings,

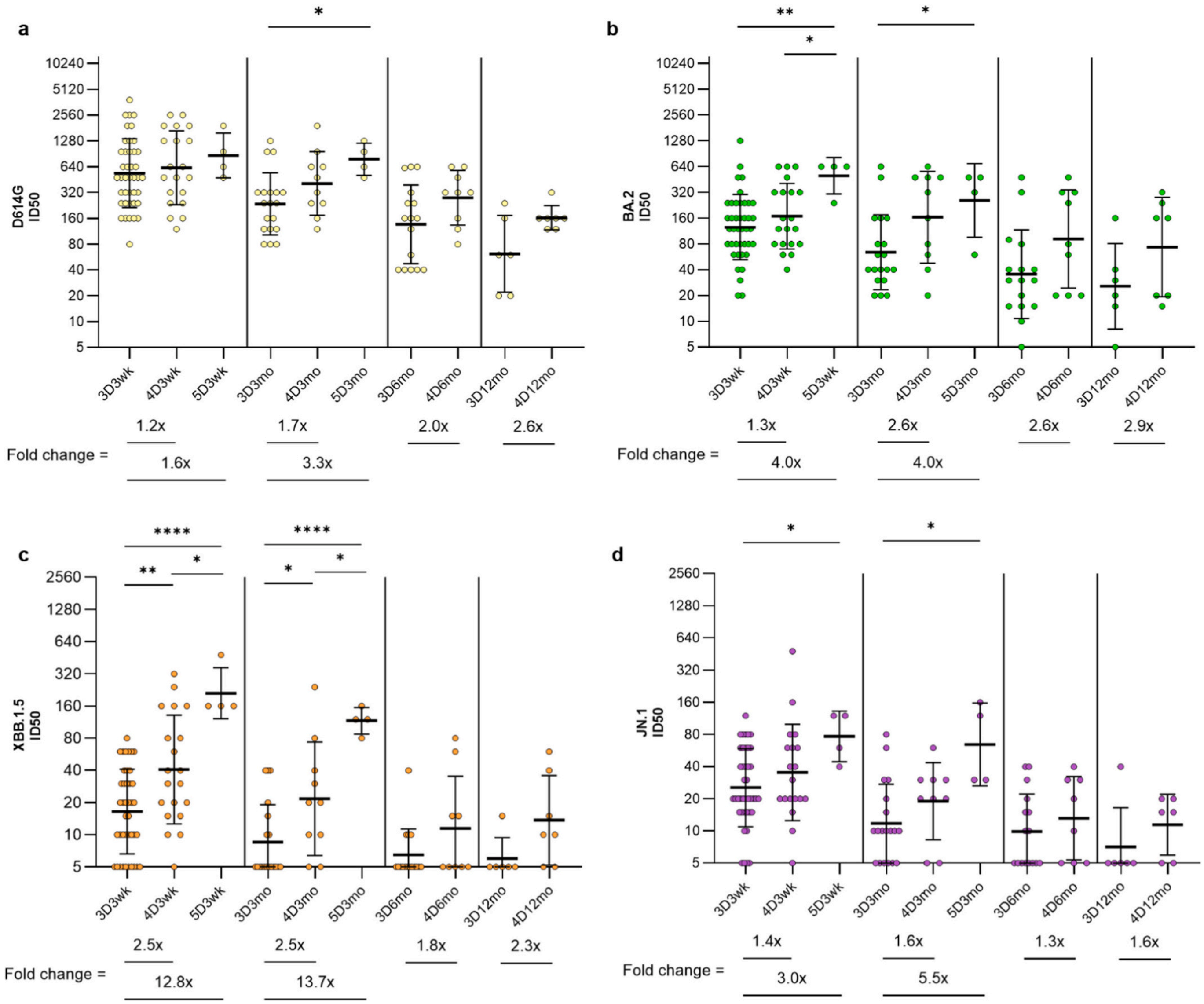

Fig. 6. The neutralizing antibody levels at the same time points post the third, fourth and fifth vaccine doses. Median infectious dose (ID_{50}) of serum samples of uninfected vaccinees collected at three weeks (3D3wk) and three (3D3mo), six (3D6mo), and twelve months (3D12mo) after the third dose, and three weeks (4D3wk), and three (4D3mo), six (4D6mo), and twelve months (4D12mo) after the fourth dose, and three weeks (5D3wk) and three months (5D3mo) after the fifth dose were compared at corresponding time points post third, fourth, and fifth vaccine dose. Yellow dots indicate ID_{50} values against A. D614G, green dots B. Omicron BA.2, orange dots Omicron C. XBB.1.5, and D. purple dots Omicron JN.1 subvariants. Geometric means and geometric standard deviations of the ID_{50} values are shown. Mann-Whitney U test was used to analyze the statistical significance between the samples. Fold changes were calculated by comparing the geometric means of D614G and the Omicron subvariants to each other at each time point. Two-tailed *p*-values <0.05 were considered statistically significant. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ancestral D614G variant was still most efficiently neutralized by the antibodies. Interestingly however, the difference in JN.1 vs. XBB.1.5 fold change at 6 months and 12 months post third vaccine dose was 2-times, but decreased to 1.2-times at 24 months post third vaccine dose, likely reflecting antibody responses obtained in XBB.1.5 infections.

In infected vaccinees post four vaccine doses, the fold differences between D614G and Omicron XBB.1.5 or JN.1 increased up to 6 month, and decreased at the 12 month time point. The fold differences between D614G and Omicron BA.2 decreased at all time points. Differences among Omicron subvariants were statistically significant at the 6 month time point. In infected vaccinees post five vaccine doses, the fold differences between D614G and BA.2 or XBB.1.5 were almost nonexistent. These results indicate that vaccination with a monovalent XBB.1.5 vaccine, combined with Omicron infection, induced the production of XBB.1.5-specific antibodies, whereas XBB.1.5 booster alone or a bivalent Omicron vaccine combined with Omicron infection did not.

3.8. The neutralization capacity of antibodies against all four SARS-CoV-2 variants decays at a similar rate post the fourth and fifth vaccine doses

To assess the rate of decay of neutralizing antibodies in the sera of uninfected vaccinees, the neutralizing capacity against D614G variant and Omicron subvariants BA.2, XBB.1.5, and JN.1 was analyzed by comparing the ID_{50} values post the third, fourth, and fifth vaccine doses to the subsequent time point (Fig. 8A-B).

With D614G and Omicron BA.2, the neutralization capacity declined faster post the third vaccine dose compared to that seen after the fourth dose (3D3wk vs 3D12mo 8.7× for D614G and 4.9× for BA.2 and 4D3wk vs. 4D12mo 3.8× for D614G and 2.3× for BA.2). Neutralizing antibodies

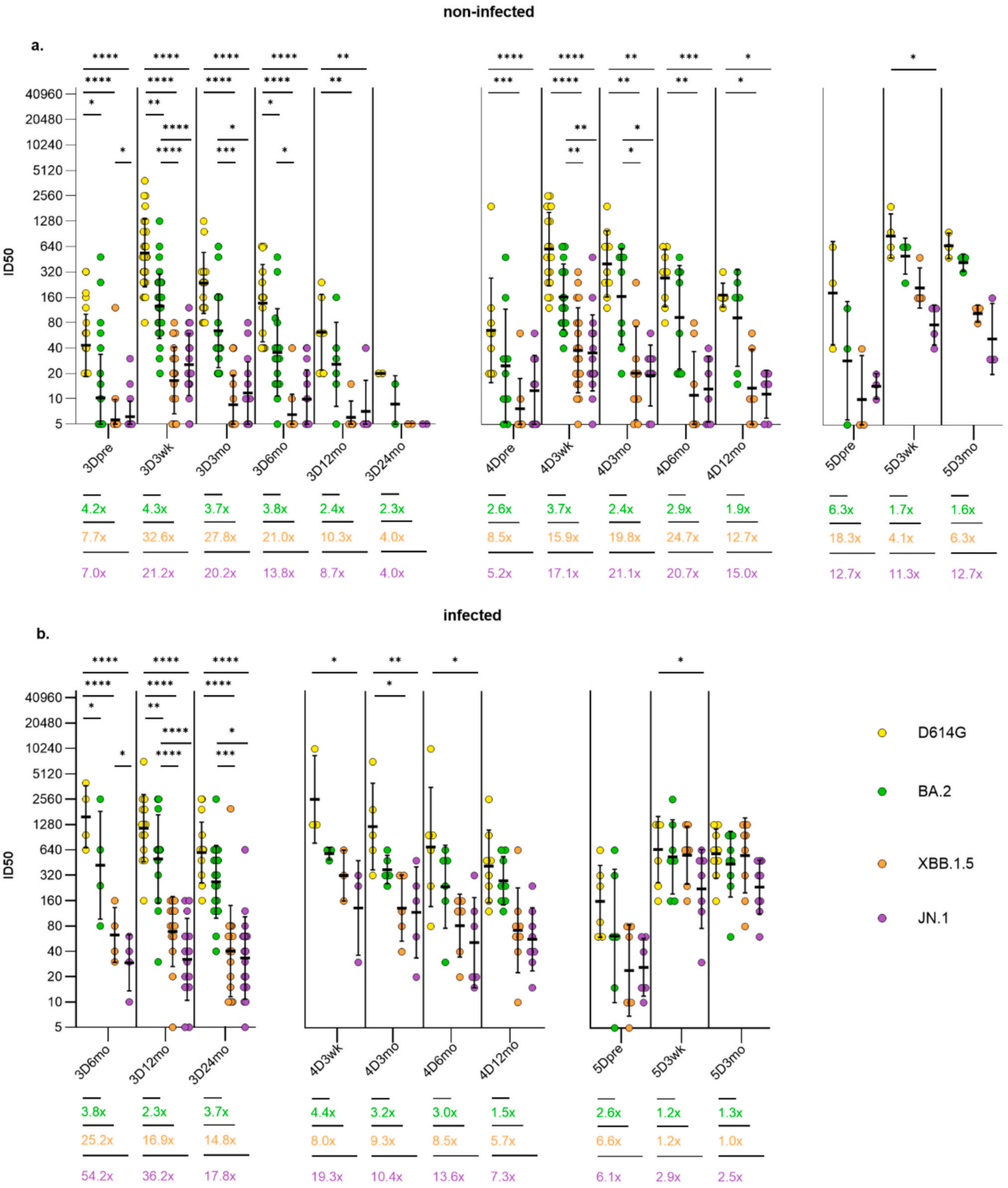

Fig. 7. Comparison of the differences in subvariant-specific neutralization capacities. Median infectious dose (ID_{50}) values of serum samples collected at five months after the second vaccine dose (3Dpre), three weeks (3D3wk) and three (3D3mo), six (3D6mo), twelve (3D12mo), and twenty-four (3D24mo) months after the third dose, and three weeks (4D3wk), and three (4D3mo), six (4D6mo), and twelve (4D12mo) months after the fourth dose, and three weeks (5D3wk) and three months (5D3mo) after the fifth dose from A. uninfected and B. infected vaccinees who had received three, four or five vaccine doses. Before fourth dose (4Dpre) and before fifth dose (5Dpre) samples were selected as close as possible to the fourth and fifth vaccine dose administration. The data presents the comparison of neutralizing antibodies between D614G and Omicron BA.2, XBB.1.5, and JN.1 subvariants. Yellow dots indicate D614G, green dots Omicron BA.2, orange dots Omicron XBB.1.5, and purple dots Omicron JN.1. Geometric means and geometric standard deviations of the ID_{50} values are shown. Friedmann test with Dunns' multiple comparison was used to analyze the statistical significance between the samples. Fold changes were calculated by comparing the geometric means of the D614G and Omicron subvariants to each other at each time point. Two-tailed *p*-values <0.05 were considered statistically significant. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

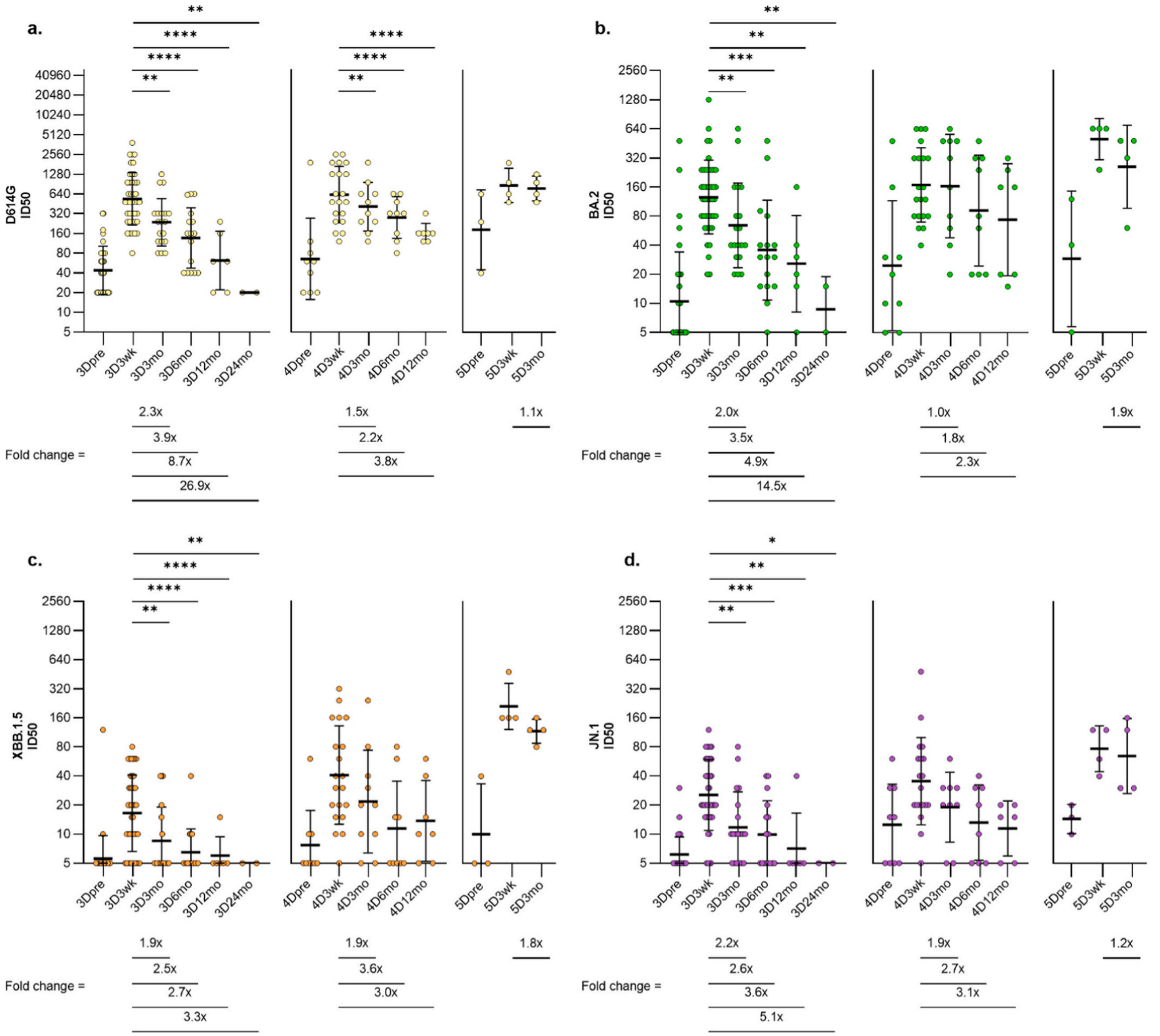

Fig. 8. The decay rate of the neutralizing antibodies against four SARS-CoV-2 subvariants. The kinetics of the Median Infectious Dose (ID_{50}) values were analyzed of the serum samples collected at five months after the second vaccine dose (3Dpre), three weeks (3D3wk) and three (3D3mo), six (3D6mo), twelve (3D12mo), and twenty-four (3D24mo) months after the third dose, and three weeks (4D3wk), and three (4D3mo), six (4D6mo), and twelve (4D12mo) months after the fourth dose, and three weeks (5D3wk) and three months (5D3mo) after the fifth dose from uninfected HCWs with three, four, or five vaccine doses. Before fourth dose (4Dpre) and before fifth dose (5Dpre) samples were selected as close as possible to the fourth and fifth vaccine dose administration. Analysis was done with SARS-CoV-2 subvariants A. D614G, B. BA.2C. XBB.1.5 D. JN.1. Geometric means and geometric standard deviations of the ID_{50} levels are shown. Mann-Whitney *U* test was used to analyze the statistical significance between the samples. Fold changes were calculated by comparing the geometric mean of three weeks post the third, fourth, or fifth vaccination to subsequent time points. Two-tailed *p*-values <0.05 were considered statistically significant. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

against BA.2 continued to decline at later time points, with decline being 26.9× for D614G and 14.5× for BA.2 between 3D3wk vs. 3D24mo time point samples. The fold differences were statistically significant when comparing three-dose time points, but less or non-significant when comparing four-dose time points.

In contrast to the slower decay of the neutralizing antibodies against D614G and Omicron BA.2 post-fourth dose, the decay rate of Omicron XBB.1.5 and JN.1 neutralization capacity was equivalent after the third and fourth doses (Fig. 8 C—D). In addition, the decay of the neutralizing antibodies against XBB.1.5 and JN.1 was substantially lower compared to the fold differences in D614G and BA.2, likely due to antibodies declining to undetectable levels. Post the fifth vaccine dose the decay rate was highest against BA.2 (1.9× fold difference) and lowest against JN.1 (1.2× fold difference).

4. Discussion

SARS-CoV-2 vaccinations are effective in preventing severe COVID-19, and yearly booster doses are administered especially to the elderly and people with predisposing conditions. Here, we continued our follow-up of COVID-19 vaccinated HCWs [2,7,11,13] to study the longevity and neutralization capacity of the antibodies against recent

SARS-CoV-2 subvariants induced by up to the fifth vaccine booster dose and breakthrough infections. We and others [2,7] have shown that three or four COVID-19 vaccine doses do not prevent infection by SARS-CoV-2 Omicron subvariants BA.2, BA.5 and XBB.1.5. The emergence of new Omicron subvariants with increasing number of mutations facilitates the escape of vaccine-induced antibodies, and hence the subvariant-specific booster vaccines are implemented. In our cohort, the fourth and fifth vaccine doses included Omicron subvariant-specific mRNA vaccines. The vaccinees were followed up until 24 months post the third, 12 months post the fourth, and 3 months post the fifth vaccine dose, the time period when Omicron subvariant XBB.1.5 circulated and a new subvariant, JN.1 emerged in Finland. The ability of the new Omicron subvariants to escape vaccine-induced immunity was clearly observed in our vaccinee cohort as an increasing number of vaccinees suffered from breakthrough infections.

Booster vaccinations are intended to boost up the waning immunity [14] and increase or direct the specificity of the antibodies against new virus variants. In our cohort in uninfected vaccinees, the booster doses increased the S1-specific antibodies to the same level, indicating that the antibody levels obtained by different booster vaccines will not induce any higher antibody titers. However, the decay rate of the antibodies was somewhat slower after the fourth and fifth booster vaccine doses, indicating that the booster vaccinations could provide a longer lasting antibody response. Importantly, all uninfected vaccinees had circulating antibodies in all time points post any booster doses, confirming long-lasting humoral immune response, which is well in line with observations by other studies [15]. As expected, breakthrough infections induced high S1-specific antibody levels, reaching even higher antibody levels than that seen in individuals who had only been vaccinated without contracting an infection. However, in some individuals the N-specific antibody levels remained low or undetectable despite elevated S-specific antibody levels indicating infection. Lower N-specific antibody levels and faster half-life of N-proteins combined with long periods between samplings and a relatively aged cohort might explain the low levels even after breakthrough infections. Interestingly, multiple breakthrough infections did not continue to further raise S1-specific antibodies, as was seen for N-specific antibodies, indicating that the S1-specific antibody response reaches the maximum titers either with repeated vaccinations or with a combination of vaccinations and infections. Individuals who had multiple breakthrough infections likely had been infected with multiple subvariants, as breakthrough infections induce high neutralizing antibody responses that should confer protection against infections from the same subvariant. Later subvariants also had enhanced capability to escape the immune response, which would reduce the efficiency of cross-protective antibodies induced by the previous breakthrough infection. Thus, our results are in accordance with other studies [15] showing that additional boosters by vaccinations or breakthrough infections do not elevate the antibody levels higher than the previous boosters. It is, however, noteworthy that additional booster vaccinations result in a slower decay of circulating antibodies and may thus provide somewhat longer protection against an infection/reinfection. This is probably due to further activation of memory B cells leading to production of broadly cross-reactive antibodies [16].

Subvariant-specific booster vaccines are being administered in the hope of inducing more specific response towards emerging new SARS-CoV-2 subvariants. In our cohort, the fourth vaccine doses contained mRNA for XBB.1.5 spike protein or for original SARS-CoV-2 and Omicron BA.1 or Omicron BA.4/5. Based on our results on neutralizing antibodies in uninfected vaccinees, the fourth vaccine dose restored the nearly vanished neutralizing antibodies against the original SARS-CoV-2 D614G variant and Omicron BA.2 subvariant. As expected, the fourth vaccine dose also induced neutralizing antibodies against the XBB.1.5 subvariant, but interestingly, the neutralizing antibodies were at the same level as against the later JN.1 subvariant. The fifth vaccine doses in our cohort were mRNA for XBB.1.5 spike protein or for original SARS-CoV-2 and Omicron BA.4/5. The fifth vaccine dose was able to induce neutralizing antibodies against the original and BA.2 subvariants as well as it induced high neutralizing titers against XBB.1.5 and JN.1 subvariants. It has been shown that the neutralizing antibodies induced by bivalent vaccines decay faster than those induced by a monovalent vaccine [17]. Due to small sample sizes, we were unable to address this observation further by separating vaccinee cohorts to vaccine-specific subsets. However, our results clearly show that the fourth and fifth vaccine doses increase neutralizing antibodies that readily cross-react with past and forthcoming subvariants, indicating immune imprinting elicited by the first vaccinations [18–20]. This cross-protection is evident also in the effectiveness data showing a beneficial effect of bivalent boosters against severe COVID-19 in Finland during the XBB era [21].These subvariant-specific vaccines have been shown to be effective against the forthcoming subvariants as well, such as XBB.1.5-adapted vaccine inducing neutralizing antibodies against JN.1 [22,23].

In the time period after the fourth vaccine, the subvariant XBB.1.5 was circulating in Finland, and the subvariant JN.1 emerged before the fifth vaccine doses were given. The combined effect of five vaccinations and Omicron breakthrough infections is evident in our cohort, showing equally high neutralizing antibody levels against all subvariants, including the JN.1. In line with the results from others [17,19], vaccinations together with breakthrough infections with any Omicron subvariant induced neutralizing antibodies against all subvariants, most likely due to repeated Omicron exposure redirecting B cell response from original vaccine imprinting towards more specific responses against Omicron subvariants [24].

5. Conclusions

Our results indicate that the fifth vaccine dose boosts neutralization capacity of serum antibodies against recent Omicron JN.1, while still inducing the same boosting effect against previous subvariants D614G, BA.2 and XBB.1.5. No exhaustion of vaccine-induced immune responses was seen even after the fourth and fifth vaccine doses. Furthermore, the vaccinees who contracted a breakthrough infection in addition to receiving multiple vaccinations exhibited even stronger neutralization of all studied subvariants. S1-specific antibody levels decayed somewhat slower after the fourth vaccine dose when compared to the post-third dose. Even though N-specific antibody levels were induced by breakthrough infections, many vaccinees lacked an immune response against the N protein suggesting that an anti-N IgG response is not a fully reliable marker for a natural SARS-CoV-2 infection among vaccinees. S1-specific antibody levels were high and at similar levels regardless of the number of breakthrough infections.

In conclusion, our data agrees with the existing literature in that booster vaccinations induce high neutralizing antibody levels against JN.1, and that these antibodies cross-react with previous SARS-CoV-2 subvariants. Additionally, booster vaccinations in combination with breakthrough infections induce very high neutralizing antibody levels.

6. Limitations of the study

Our study was limited by the relatively high average age of the participants, especially in the booster dose groups. This may have skewed the antibody responses to a lower level than the general population, as immune responses generally weaken in aged individuals. The long periods between samplings and the lack of knowledge on the infections status of individuals during these periods may have resulted in elevated anti-S1 and neutralizing antibodies without the accompanying elevated anti-N antibodies may have increased average antibody levels and half-lives in our analysis. Our cohort was largely female, which may make our results less applicable to male population.

CRediT authorship contribution statement

Arttu Reinholm: Writing – review & editing, Writing – original

draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Hira Khan:** Writing – review & editing, Investigation. **Terhi Laakso:** Writing – review & editing, Investigation. **Sari Maljanen:** Writing – review & editing, Resources, Data curation. **Pinja Jalkanen:** Writing – review & editing, Methodology, Investigation. **Marianne Gunell:** Writing – review & editing, Resources, Methodology, Formal analysis. **Teemu Kallonen:** Writing – review & editing, Resources, Methodology, Formal analysis. **Pamela Österlund:** Writing – review & editing, Supervision, Resources, Conceptualization. **Olli Ritvos:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Arttu Nousiainen:** Writing – review & editing, Resources, Data curation. **Hanni K. Häkkinen:** Writing – review & editing, Resources, Data curation. **Hanna Välimaa:** Writing – review & editing, Resources, Data curation. **Anu Kantele:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization. **Johanna Lempainen:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization. **Ilkka Julkunen:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Pekka Kolehmainen:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization. **Laura Kakkola:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Extended Fig. 1.

Average (red line) and individual (black lines) half-life times for S1-specific antibodies after three and four vaccination doses. Curves were calculated with nonlinear, one phase decay model with 95 % confidence interval (red dotted lines) from sample sets with three or more samples.

Extended Fig. 2.

Average (red line) and individual (black lines) half-life times for neutralization capacity against SARS-CoV-2 variant D614G and Omicron subvariants BA.2, XBB.1.5, and JN.1 after three and four vaccination doses. Curves were calculated with nonlinear, one phase decay model with 95 % confidence interval (red dotted lines) from sample sets with three or more samples.

Data availability

Data will be made available on request.

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