

Anti-SARS-CoV-2 total immunoglobulin and neutralising antibody responses in healthy blood donors throughout the COVID-19 pandemic: a longitudinal observational study

Yukino Güttlin^{ab}, Diana Albertos Torres^{ab}, Alexander Gensch^b, Ann-Kathrin Schlotterbeck^b, Laurent Stöger^b, Stefanie Heller^b, Laura Infanti^c, Güliz Tuba Barut^{de}, Volker Thiel^{defg}, Karoline Leuzinger^h, Hans H. Hirsch^h, Andreas Buser^d, Adrian Egli^{abi}

^a Institute of Medical Microbiology, University of Zurich, Zurich, Switzerland

^b Department of Biomedicine, University of Basel, Basel, Switzerland

^c Regional Blood Transfusion Service Swiss Red Cross, Basel, Switzerland

^d Institute of Virology and Immunology, Bern and Mittelhäusern, Switzerland

^e Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern, Bern, Switzerland

^f Multidisciplinary Center for Infectious Diseases, University of Bern, Bern, Switzerland

^g European Virus Bioinformatics Center, Jena, Germany

^h Clinical Virology, University Hospital Basel, Basel, Switzerland

ⁱ Clinical Bacteriology and Mycology, University Hospital Basel, Basel, Switzerland

Summary

INTRODUCTION: Quantifying antibodies against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and neutralising antibodies may help to understand protection at the individual and population levels. Determination of neutralising antibodies using classical virus neutralisation tests (VNT) is considered the gold standard, but they are costly and time-intensive. Enzyme-linked immunosorbent assay (ELISA)-based surrogate VNTs (sVNT) or anti-SARS-CoV-2 spike protein receptor binding domain immunoglobulins (anti-S-RBD Ig) may be suitable alternatives to VNTs. We aimed to (a) explore the correlations between anti-S-RBD Ig, VNT, and sVNT measurements and (b) describe humoral immunity against SARS-CoV-2 after vaccination, natural infection, and vaccine breakthrough infection in healthy blood donors.

METHODS: We measured total anti-SARS-CoV-2 Ig in 5714 serum samples from 2748 healthy individuals visiting the Swiss Red Cross Blood Donation Centre in Basel from 03/2020 to 04/2022. We used the Elecsys[®] Anti-SARS-CoV-2 immunoassay (Roche) against the N- and S-receptor binding domain (RBD) proteins. In a subset of 548 samples from 123 donors, we conducted sVNTs against the Wuhan wild-type SARS-CoV-2 (SARS-CoV-2 Neutralizing Antibodies Detection Kit; Adipogen[™]). In 100 samples from 40 donors, we correlated sVNT and VNTs against the wild-type (D614G WU1) virus. Surveys were sent to the blood donors to collect data on their SARS-CoV-2 infection and vaccination status. Using this data, donors were categorised as “vaccination only”, “infection before vaccination”, “post-vaccine breakthrough infection”, and “natural infection only”.

RESULTS: Our longitudinal observation study cohort consisted of 50.7% males with a median age of 31 years

(range 18–75 y). Anti-SARS-CoV-2 N protein positivity rates per month indicate 57.1% (88/154) of the cohort was infected up to 04/2022. No differences in seropositivity were found between sexes, age groups, blood types (ABO or RhD), and cytomegalovirus serostatus. We observed a high correlation between anti-S-RBD Ig and inhibition percentage (Spearman's $\rho = 0.92$, Kendall's $\tau = 0.77$, $p < 0.0001$). We determined the sensitivity and specificity for the manufacturers' thresholds for detecting virus-neutralising effects and computed the “best” cut-off based on our real-world data. We categorised 722/1138 (63.5%) donors as vaccination only (82.3%), post-vaccine breakthrough infection (7.8%), infection before vaccination (5.8%), and natural infection only (4.2%). We observed a lower inhibition percentage in the natural infection-only group than in all other vaccinated groups. The infection before vaccination group had higher anti-S-RBD Ig titres after the first vaccine dose than the other vaccinated groups.

CONCLUSION: In total, 57.1% of healthy blood donors were infected with SARS-CoV-2, but natural infection without evidence of vaccination seems to result in substantially lower neutralising antibody levels. An estimate of antibody neutralisation may be helpful to assess reinfection risk. Total anti-S-RBD Ig correlates with surrogate virus neutralisation test results, a surrogate for neutralisation; therefore, we suggest that total anti-S-RBD Ig may estimate the level of neutralising antibodies. The threshold for protection from an unfavourable clinical outcome must be evaluated in prospective clinical cohorts.

Introduction

More than four years have passed since the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in December 2019. Enormous efforts have been

Yukino Güttlin
Institute of Medical Microbiology
University of Zurich
Gloriastrasse 30
CH-8006 Zurich
yguettlin[at]imm.uzh.ch

Adrian Egli
Institute of Medical Microbiology
University of Zurich
Gloriastrasse 30
CH-8006 Zurich
aegli[at]imm.uzh.ch

made to mitigate this new pandemic virus, including the rapid development of vaccines, global vaccination campaigns, public health countermeasures, and vigilance programs. In Switzerland, the first SARS-CoV-2 case was reported in February 2020, and various variants of concern have subsequently appeared. The severity of coronavirus disease 2019 (COVID-19) has decreased mainly due to vaccine-induced protection [1]. However, the Alpha (December 2020), Delta (May 2021), and Omicron (November 2021) variants of concern have nonetheless rapidly spread within the population. Understanding the specific immune response to SARS-CoV-2 at individual and population levels is crucial for understanding transmission between individuals, protecting individuals at high risk of severe disease, and helping to further improve epidemiological models.

One approach is monitoring humoral immunity using antibodies against SARS-CoV-2 and neutralising antibodies (nAb). Among the four structural proteins of SARS-CoV-2, the most immunogenic sites are the Nucleocapsid (N) protein and surface Spike glycoprotein (S) [2]. Previous work has shown that humoral immunity after infection mainly consists of anti-N and anti-S immunoglobulins (Ig). However, vaccines have mainly been designed against the S protein [3]. Therefore, anti-S and anti-S receptor binding domain (RBD) Ig seropositivity alone serves as a vaccination indicator. In contrast, anti-N Ig seropositivity can be used as a proxy for infection. Among the range of antibodies against the S protein, nAbs neutralise the virus' ability to enter and infect new host cells by blocking the viral RBD [4]. Therefore, neutralising antibodies are considered to correlate with protection from SARS-CoV-2 infections [5, 6].

The gold standard method to determine neutralising antibody levels is the classical live virus neutralisation test (VNT) [7]. However, VNTs require a biosafety level 3 (BSL3) laboratory and are costly and time-intensive. So-called surrogate virus neutralisation tests (sVNT) based on an enzyme-linked immunosorbent assay (ELISA) were developed to overcome the limitations of VNTs. In these as-

says, neutralising antibodies present in the sera and angiotensin-converting enzyme 2 (ACE2) compete for binding to the SARS-CoV-2 S-RBD.

In this study, we prospectively collected more than 5000 longitudinal serum samples from healthy blood donors at the Regional Swiss Red Cross Blood Transfusion Center in Basel, Switzerland. We monitored the humoral immunity against SARS-CoV-2 from March 2020 to April 2022. Using our real-world dataset, we aimed to (a) explore the correlations between anti-S-RBD Ig, virus neutralisation tests, and surrogate virus neutralisation tests and (b) describe humoral immunity against SARS-CoV-2 after vaccination, natural infection, and post-vaccine breakthrough infections.

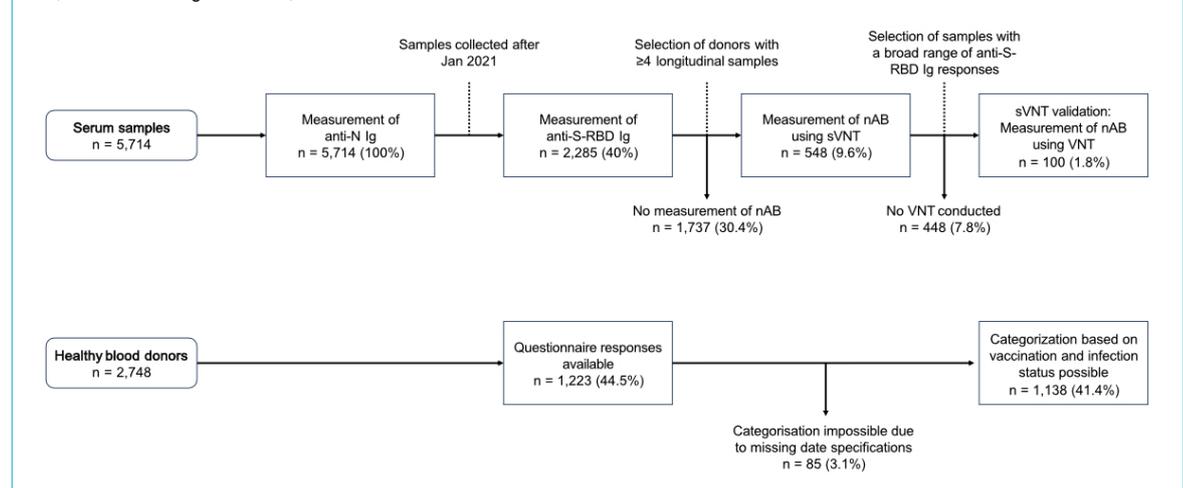
Materials and methods

Collection of serum samples, metadata, and data curation

We collected serum samples from healthy blood donors residing in the canton of Basel Stadt (BS), Switzerland, at the blood donation centre from March 2020 to the end of April 2022. The serum samples were stored at -80°C until batchwise determination of humoral immunity. The numbers of samples or individuals included in the various analyses described below are summarised in figure 1.

Data collected on donors included sex, birth year, blood type (AB0 and RhD), and cytomegalovirus (CMV) serostatus. Specific data on past SARS-CoV-2 infections and vaccinations and the respective dates were collected retrospectively through a questionnaire sent to the donors in October 2022. We received 1223 responses from 2748 requests (44.5%). In case of ambiguous vaccination or infection date entries, the dates were manually determined (figure S1 in the appendix) while minimising date errors; this manual date determination was applied for 121/1223 (9.9%) donors. Lastly, if donors were vaccinated with the Janssen vaccine (Ad26.COVID-S) for their first dose, we defined them as fully vaccinated after only one dose ($n = 7$, 0.6%). Standard time intervals between the vaccine doses

Figure 1: Overview of the subsampling criteria and the numbers of samples or healthy blood donors included in the different analyses. The upper part shows the subsampling criteria and the number of samples in the different subsets for the various analyses. The lower part summarises the questionnaire responses and the vaccination and infection status categorisation results. anti-N: anti-SARS-CoV-2 nucleocapsid protein; anti-S-RBD: anti-SARS-CoV-2 spike glycoprotein receptor binding domain; Ig: immunoglobulin; sVNT: surrogate virus neutralisation test; nAb: neutralising antibodies; VNT: classical virus neutralisation test.



and vaccination schemes were considered for each vaccine for our analyses, as readily summarised by Ghasemiyeh et al. [8]. The study was conducted according to the Declaration of Helsinki and approved by the Ethikkommission Nordwest- und Zentralschweiz (EKNZ 2020-00769), Switzerland. Informed consent was obtained from all subjects involved in this study.

Detection of anti-SARS-CoV-2 N and S protein antibodies

We tested for SARS-CoV-2 anti-N and anti-S-RBD antibodies using the Elecsys® Anti-SARS-CoV-2 and Elecsys® Anti-SARS-CoV-2 S immunoassays (Roche Diagnostics, Switzerland) following the manufacturer's instructions. Their results are reported as semi-quantitatively determined Ig levels. The limit of quantification for the anti-S-RBD measurements was 0.4–2500 U/ml. We classified samples with an output value of ≥ 1.0 cut-off index (COI) and ≥ 0.8 U/ml as reactive (i.e. positive) for anti-N and anti-S-RBD Ig, respectively. Furthermore, Roche determined a cut-off for the presence of neutralising antibodies as ≥ 15 U/ml. Those samples showed 50% neutralisation at a sample dilution of over 1:20 in a plaque-reducing neutralisation (PRNT) assay and, therefore, could functionally neutralise the live virus in vitro [9]. We tested all samples for anti-N Ig; however, serial testing of anti-S-RBD Ig started in January 2021 with vaccine availability in Switzerland.

Detection of neutralising antibodies by surrogate virus neutralisation tests

We used an ELISA-based surrogate virus neutralisation test to measure the neutralising activity. We used the SARS-CoV-2 Neutralising Antibodies Detection Kit (AG-48B-0002-KI01, Adipogen™, Switzerland) according to the manufacturer's instructions. Briefly, neutralising antibodies in the serum compete against the human recombinant angiotensin-converting enzyme 2 conjugated to horseradish peroxidase for binding to the SARS-CoV-2 S-RBD (Wuhan wild-type). After peroxidase activity was quantified using 3,3',5,5'-tetramethylbenzidine, the percentage inhibition (inh%) was calculated as follows:

$$\text{inh \%} = \left(1 - \frac{OD_{\text{sample}}}{OD_{\text{negative control}}}\right) \times 100$$

The measured reduction in the optical density (OD) indicates the inhibition of the interaction between the RBD and angiotensin-converting enzyme 2, indicating the presence of neutralising antibodies. The cut-off value for positivity was set at 20% inhibition, according to the manufacturer. We conduct the ELISA assay on samples from donors with at least four longitudinal samples. We also included some donors with fewer than four longitudinal samples, especially those in the "natural infection only" category (see the classification of donors section below).

Validation of surrogate virus neutralisation tests by live virus serum neutralisation test

We validated the surrogate virus neutralisation tests by comparing a subset of 100 serum samples from 40 donors to neutralisation titres observed in a classical VNT using live SARS-CoV-2 viruses (D614G WU1, BetaCoV/Germany/BavPat1/2020, Acc. No. EPI_ISL_406862) and

transmembrane serine protease 2 (TMPRSS2)-expressing Vero E6 cells (VeroE6/TMPRSS2; NIBSC Research Reagent Depository, UK). The samples were selected to include a wide range of anti-S-RBD Ig levels, including negative samples (≤ 0.4 U/ml). First, the serum was serially diluted twofold from 1:10 to 1:1280 and mixed with 100 plaque-forming units of virus per well. After 1 h of incubation, the mixture was added to confluent VeroE6/TMPRSS2 cells. Two positive controls, a vaccinee serum from an individual immunised with monovalent mRNA vaccine (from an ongoing study at the Institute of Virology and Immunology [IVI], Bern and Mithelhäusern, Switzerland) and guinea pig serum immunised with pseudotype VSV-SARS-CoV-2 (IVI, in house), as well as a negative control, unimmunised guinea pig serum (IVI, in house), were also tested. The neutralisation titre was defined as the highest dilution at which the serum was still protective against the virus, determined by the cytopathic effect (i.e. the titre at which the serum was still protective against the virus). Samples that did not neutralise the virus at the lowest dilution of 1:10 are reported as " $<1:10$ ". Therefore, all samples with a dilution of $\geq 1:10$ were classified as positive and $<1:10$ as negative for neutralising activity (see appendix).

A total of 82 and 18 samples were classified as positive (inhibition percentage $\geq 20\%$) and negative (inhibition percentage $<20\%$), respectively, for neutralising antibodies in the surrogate virus neutralisation tests. In the classical VNT, 75 samples had a serum dilution $\geq 1:10$ and 25 samples had a dilution below 1:10 and, therefore, were classified as positive and negative, respectively. Next, the sVNT results were plotted against the classical VNT results, and the Kendall correlation coefficient (τ) was computed (figure S2 in the appendix). The sVNT results were significantly correlated with the classical VNT results (Kendall's $\tau = 0.73$, $p < 1e^{-4}$). Assuming the classical VNT results are the ground truth, the wild-type sVNT had a sensitivity of 96%, specificity of 60%, positive predictive value of 87.8%, and negative predictive value of 83.3%.

Classification of donors into infection and vaccination status categories based on questionnaire data

To compare serological responses and describe humoral immunity against SARS-CoV-2 after vaccination, natural infection, and breakthrough infections, and based on the information collected with the questionnaire, we classified the donors into four categories: (a) "vaccination only" (vac), if donors specified to have been vaccinated but not infected; (b) "natural infection only" (inf), if donors specified to have been infected but not vaccinated; (c) "infection before vaccination" (infvac), if donors specified to have been vaccinated and infected, and the date of the first infection was before the date of the first vaccine dose; (d) "post-vaccine breakthrough infection" (bt), if donors specified to have been vaccinated and infected, and if the date of the first infection was after complete vaccination. We only considered infections confirmed by a PCR or rapid antigen test. Further, we considered donors completely vaccinated after receiving a second dose of the mRNA or AstraZeneca vaccines or the first dose of the Janssen vaccine [8]. Please note that only infection and vaccination dates before a donor's last collected serum sample date were considered for classification, allowing the cor-

rect classification of their infection and vaccination status within the study's timeframe and set of samples.

Statistical data analysis

The statistical data analyses were conducted using R Studio (version 2022.07.2) with the R (version 4.2.1; 2022-06-23) [10] packages Tidyverse (1.3.2) [11] and plotROC (2.3.0) [12]. Correlations were evaluated by computing Spearman's rank correlation coefficient (ρ) and Kendall's rank correlation coefficient (τ). Seroprevalence was compared between different cohort characteristics (sex, age, blood types, and cytomegalovirus seropositivity) by computing a Fisher's exact test between each month, and the p-values were adjusted using the Benjamini and Hochberg [13] correction method. The timings of various variants of concern appearing in Switzerland were determined as the first month where the proportion of the specific variant of concern exceeded 2% of all sequenced samples in the Global Initiative on Sharing All Influenza Data [14] from the Swiss Pathogen Surveillance Platform (www.spsp.ch) provided on the CoV-Spectrum website (<https://cov-spectrum.org>). The area under the receiver operating characteristic curve (AUROC) was computed to define an optimal anti-S-RBD Ig cut-off for neutralising antibody prediction. Confusion matrices were constructed for each cut-off to calculate the specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV).

Results

Cohort of healthy blood donors

In total, we analysed 5714 serum samples from 2748 healthy blood donors. The median number of samples col-

lected per donor was 1 (interquartile range [IQR] = 1–3; range = 1–19). The cohort comprised 50.7% males (n = 1392) and 49.3% females (n = 1356) with a median age of 31 years (IQR = 26–44; range = 18–75). The most common blood types were 0 (45.1%, n = 1169) and A (40.4%, n = 1048), with much fewer donors having blood types B (10.1%, n = 261) and AB (4.4%, n = 113). Most donors were RhD positive (80.1%, n = 2075) and seronegative for cytomegalovirus (60.3%, n = 289). Table 1 summarises the cohort characteristics overall and separately for each year (2020–2022).

Serological responses against SARS-CoV-2 N- and S-proteins

Overall serology results

We tested 5714 and 3319 serum samples for anti-N and anti-S-RBD Ig, respectively. The anti-S-RBD Ig measurements are shown from January 2021 onwards once vaccines became available in Switzerland. Figure 2 shows the percentage of positive tests for each month (anti-N Ig levels ≥ 1.0 COI, anti-S-RBD Ig levels ≥ 0.8 U/ml). We noted a slight increase in anti-N Ig responses after the Alpha (December 2020) and Delta (May 2021) variants appeared in Switzerland. However, the positivity rate plateaued around 10% from December 2020 to November 2021. With the start of the Omicron wave in November/December 2021, we noted an increase in positive tests to 57.1% in April 2022. We observed an increase in the anti-S-RBD Ig seroreactivity during six months, from 8.5% in January to 87.1% in July 2021. In the following months, the positivity rate plateaued and finally reached 98.7% in April 2022.

Table 1:

Descriptive characteristics of the study cohort overall and by year of sample collection. The characteristics were determined at the time of study inclusion, and donors with unknown characteristics were not included in the percentage calculations.

Characteristic		Overall n = 2748*	2020 n = 1705*	2021 n = 862*	2022 n = 181*
Sex	Female	1356 (49.3%)	830 (48.7%)	432 (50.1%)	94 (51.9%)
	Male	1392 (50.7%)	875 (51.3%)	430 (49.9%)	87 (48.1%)
Age	Mean (SD)	36 (13)	37 (14)	33 (12)	34 (12)
	Median (IQR)	31 (26, 44)	32 (26, 48)	29 (25, 38)	30 (25, 40)
	Range	18, 75	18, 74	18, 75	18, 75
Age group	18–29 years	1195 (43.5%)	678 (39.8%)	435 (50.5%)	82 (45.3%)
	30–39 years	720 (26.2%)	434 (25.5%)	233 (27.0%)	53 (29.3%)
	40–49 years	290 (10.6%)	196 (11.5%)	74 (8.6%)	20 (11.0%)
	50–59 years	306 (11.1%)	220 (12.9%)	67 (7.8%)	19 (10.5%)
	≥ 60 years	237 (8.6%)	177 (10.4%)	53 (6.1%)	7 (3.9%)
Blood type	A	1048 (40.4%)	639 (38.9%)	344 (44.2%)	65 (38.2%)
	B	261 (10.1%)	168 (10.2%)	72 (9.2%)	21 (12.4%)
	AB	113 (4.4%)	70 (4.3%)	37 (4.7%)	6 (3.5%)
	0	1169 (45.1%)	765 (46.6%)	326 (41.8%)	78 (45.9%)
	Unknown	157	63	83	11
Rh factor	Positive	2075 (80.1%)	1300 (79.2%)	633 (81.3%)	142 (83.5%)
	Negative	516 (19.9%)	342 (20.8%)	146 (18.7%)	28 (16.5%)
	Unknown	157	63	83	11
Cytomegalovirus serology	Positive	190 (39.7%)	144 (39.7%)	39 (41.9%)	7 (30.4%)
	Negative	289 (60.3%)	219 (60.3%)	54 (58.1%)	16 (69.6%)
	Unknown	2269	1342	769	158

SD: standard deviation; IQR: interquartile range.

Serology results by donor characteristics

The percentages of positive tests (anti-N Ig levels ≥ 1.0 COI, anti-S-RBD Ig levels ≥ 0.8 U/ml) per sample collection month were calculated for each group within the donor characteristics. We found no statistically significant differ-

ences in seroreactivity between sexes, blood types (ABO and RhD), and cytomegalovirus positivity. However, we observed statistically significant differences in positivity rates among age groups for the anti-S-RBD Ig measurements in May 2021 ($p_{\text{adjusted}} < 0.05$) (figure 3).

Figure 2: Cumulative monthly seropositivity rates for anti-N and anti-S-RBD Ig in sera from healthy blood donors. The percentage of positive tests for anti-N (blue) and anti-S-RBD Ig (green) was calculated for each month. Samples were classified as positive if the anti-N Ig level was ≥ 1.0 COI and the anti-S-RBD Ig was ≥ 0.8 U/ml. The blue arrows show the appearance of a new SARS-CoV-2 variant of concern in Switzerland. The green arrows show time points when the vaccine and booster doses were made available in Switzerland. COI: cut-off index; anti-S-RBD: anti-SARS-CoV-2 spike glycoprotein receptor binding domain.

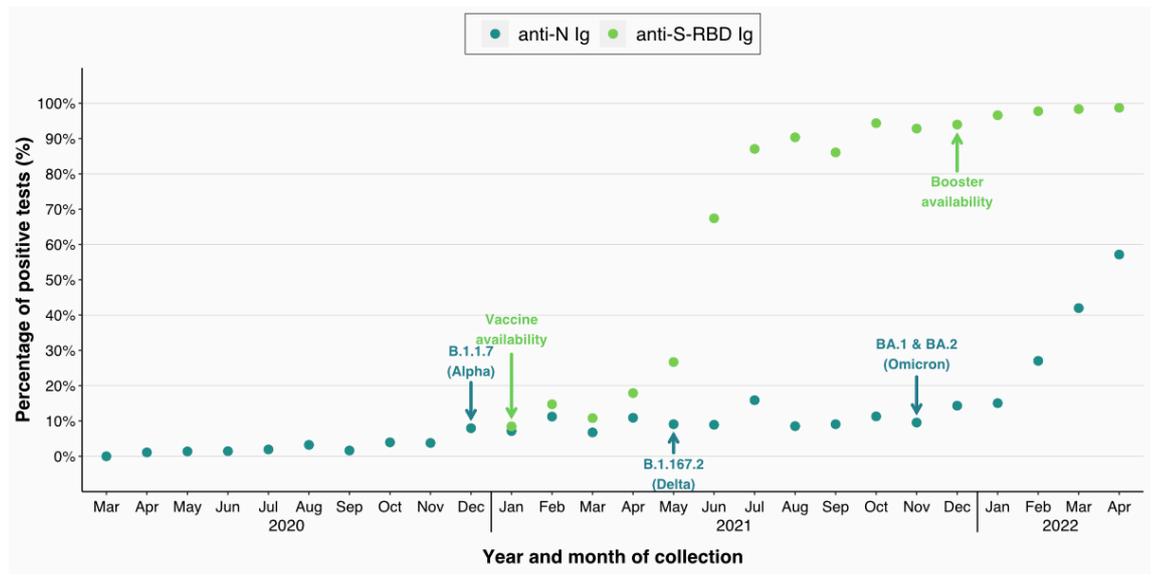
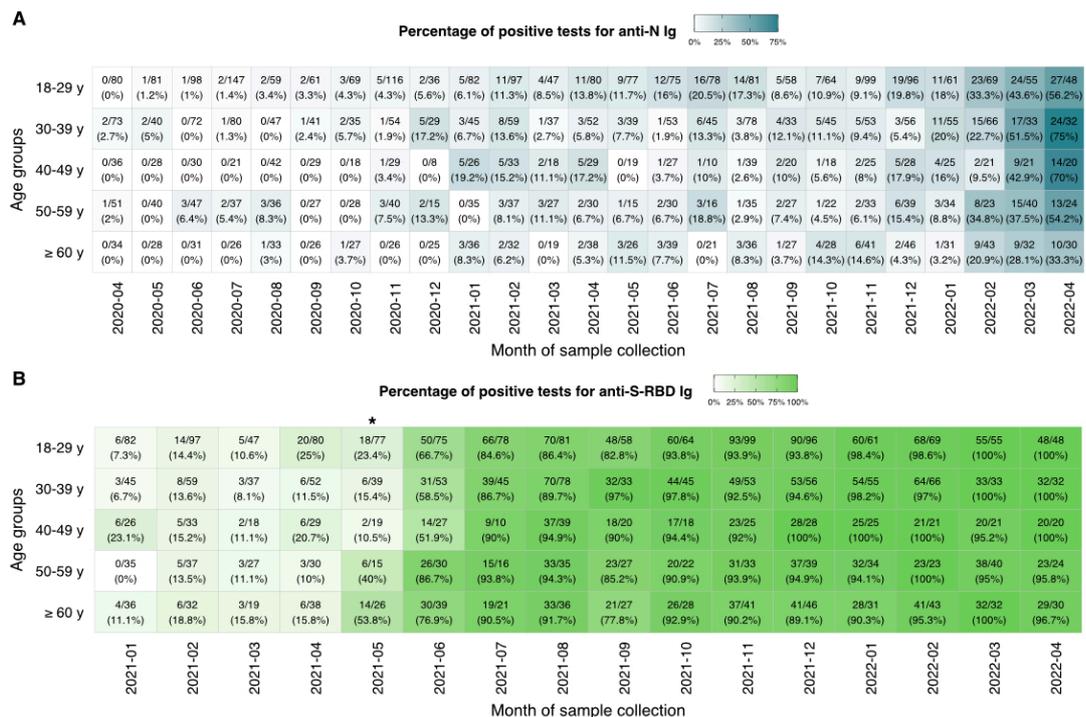


Figure 3: Serological responses by age group over time. The proportions (positive tests / total tests) and percentages of positive tests for anti-N Ig (A) and anti-S-RBD Ig (B) were calculated for each month and age group and are shown on a colour scale where light or dark hues correspond to lower or higher percentages, respectively. Samples were classified as positive if the anti-N Ig level was ≥ 1.0 COI and the anti-S-RBD Ig was ≥ 0.8 U/ml. The asterisk denotes the month where a significant difference was found between age groups (Fisher's exact test with a Benjamini and Hochberg correction for multiple comparisons; $p_{\text{adjusted}} < 0.0451$). COI: cut-off index; anti-S-RBD: anti-SARS-CoV-2 spike glycoprotein receptor binding domain.



Measurement of neutralising antibodies using the surrogate virus neutralisation tests

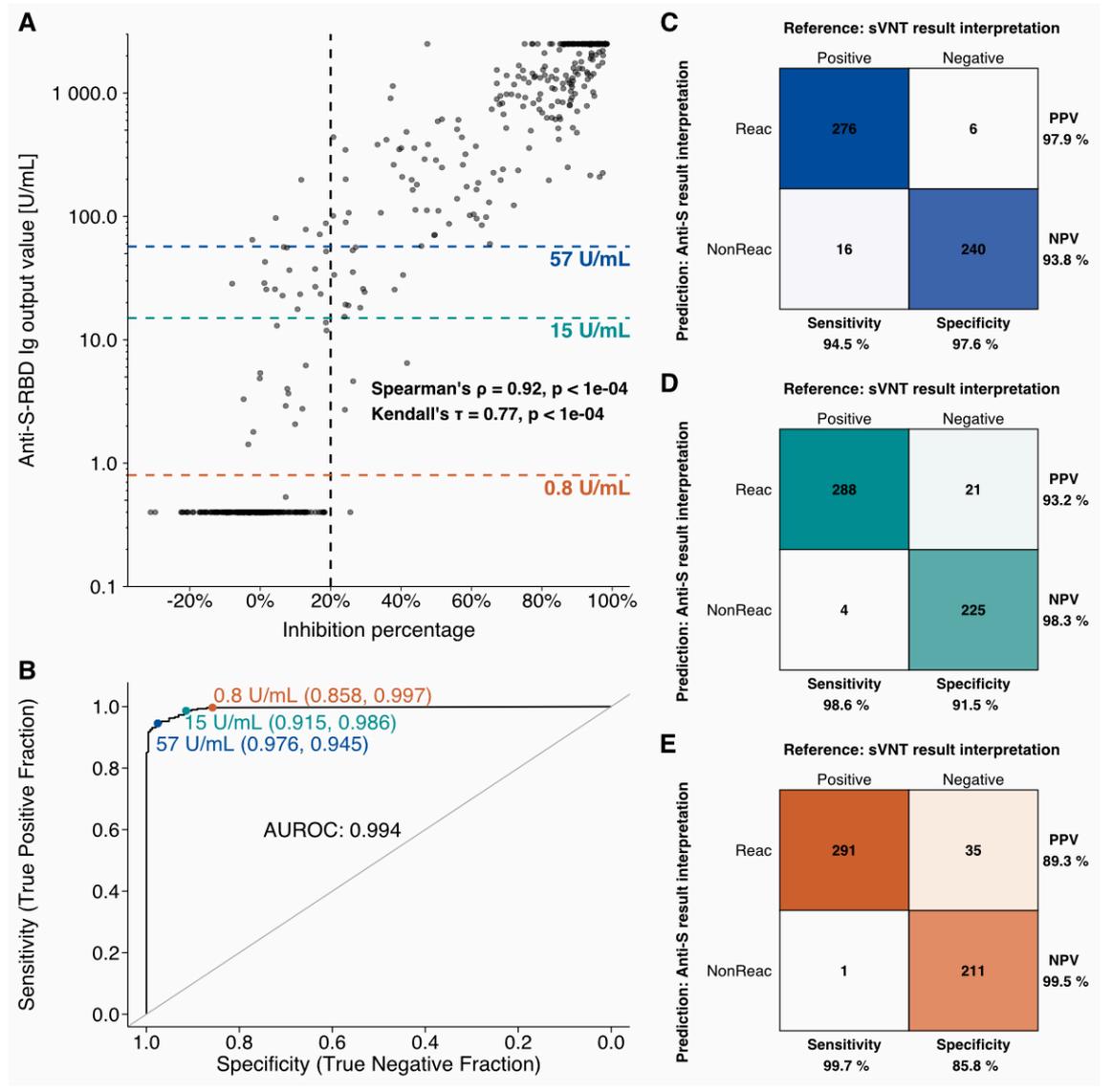
We conducted a correlation analysis to evaluate whether the anti-S-RBD Ig measurements could predict the presence of neutralising antibodies (surrogate virus neutralisation tests) in serum. We excluded 10 samples from the 548 samples due to a lack of sample material to measure anti-S-RBD Ig, resulting in 538 samples for this analysis. The strength of the correlation was assessed using Spearman's ρ (0.92, $p < 0.0001$) and Kendall's τ (0.77, $p < 0.0001$) figure 4A).

The AUROC curve revealed good predictive performance of anti-S-RBD Ig levels for neutralising antibody presence

(figure 4B, AUROC = 0.994). Based on our dataset, the appropriate cut-off for anti-S-RBD Ig levels predicting nAb presence was 57 U/ml, representing the anti-S-RBD Ig titre at the maximum Youden's index (sensitivity + specificity - 1). Based on this cut-off, the presence of nAb in the sera could be predicted with a sensitivity of 94.5% and a specificity of 97.6% (positive predictive value = 97.9%, negative predictive value = 93.8%).

Figures 4C-E show confusion matrices for three different anti-S-RBD Ig titre cut-offs to predict nAb presence: 57 (figure 4C), 15 (figure 4D), and 0.8 (figure 4E) U/ml. The latter two cut-offs (15 and 0.8 U/ml) are those specified in

Figure 4: Correlation analysis of Elecsys® Anti-SARS-CoV-2 S results vs wild-type SARS-CoV-2 surrogate virus neutralisation test (sVNT) results. **A** The anti-S-RBD Ig output values plotted against the inhibition percentages (i.e. the sVNT results), where each dot represents a serum sample. The black vertical dashed line denotes the cut-off for the presence of neutralising antibodies (nAb) at 20% inhibition, as measured in the sVNT. The orange (0.8 U/ml) and teal (15 U/ml) dashed lines denote the cut-off for anti-S-RBD Ig reactivity and nAb presence, respectively. The blue (57 U/ml) dashed line denotes the anti-S-RBD Ig output value at the highest Youden's index. Spearman's ρ and Kendall's τ were calculated. **B** The receiver operating characteristic curve and calculated AUROC. The orange, teal, and blue points represent the above-mentioned cut-offs and their respective specificity and sensitivity values are shown in brackets. Confusion matrices were plotted for the cut-offs **(C)** 57 U/ml, **(D)** 15 U/ml, and **(E)** 0.8 U/ml. anti-S-RBD Ig: anti SARS-CoV-2 spike receptor binding domain immunoglobulins; AUROC: area under the receiver operating characteristic curve; ROC: receiver operating characteristic; Reac: reactive for anti-S-RBD Ig; NonReac: non-reactive for anti-S-RBD Ig; PPV: positive predictive value; NPV: negative predictive value; anti-S-RBD: anti-SARS-CoV-2 spike glycoprotein receptor binding domain.



the Elecsys[®] package insert for detecting the presence of nAb and anti-S-RBD Ig seropositivity, respectively.

Humoral immunity after vaccination, natural infection, and breakthrough infection

In total, 1223/2748 questionnaires (44.5%) were answered. The responding donors were classified into their respective infection and vaccination status groups. Due to missing data entries in the questionnaire, 85 donors (7%) could not be classified. To summarise, 594/1138 donors (52.2%) were classified as vac (“vaccination only”), 56/1138 donors (4.9%) as bt (“post-vaccine breakthrough infection”), 42/1138 donors (3.7%) as infvac (“infection before vaccination”), and 30/1138 donors (2.6%) as inf (“natural infection only”). There were 416/1338 donors (36.6%) who were not vaccinated or infected between the collection of their first and last serum samples.

The times of the collected samples were aligned to the first vaccination date to assess whether humoral immunity levels varied between the donors’ infection and vaccination backgrounds. For each donor, the time difference (in days) between the collection date of each sample and the first vaccination date was calculated. To assess whether vaccination would always result in an increase in the anti-S-RBD Ig titre, we selected one sample collected immediately before and after the first vaccination date for each donor (figure 5A). With only a few exceptions, the anti-S-RBD Ig titre increased after the first vaccination dose in all vaccination categories (vaccination only, infection before vaccination, and post-vaccine breakthrough infection). In the vaccination-only and post-vaccine breakthrough infection groups, almost all samples collected before vaccination were negative for anti-S-RBD Ig. However, afterwards, the output values were widely distributed. In contrast, most samples in the infection before vaccination group were reactive to anti-S-RBD Ig before vaccination, with the maximum output values reached after the first vaccine dose.

Next, we binned all measurements by donor and month after their respective first vaccination date (figure 5B). As noted above, the vaccination-only and post-vaccine breakthrough infection groups showed similar anti-S-RBD Ig courses over time. The median anti-S-RBD Ig output values were very low within a month of the first vaccination dose (≈ 30 U/ml) and then steeply increased within two to three months. After a decrease in output values by four months, especially in the vaccination-only group, a second steep increase was observed from eight months onwards. In contrast, in the infection before vaccination group, the anti-S-RBD Ig output values were already elevated within one month of the first vaccination dose, and the median did not decrease over time. However, this group had a small sample size ($n_{\max} = 8$).

Finally, we compared the inhibition percentage determined in the surrogate virus neutralisation test between the infection and vaccination categories (figure S3). The post-vaccine breakthrough infection group had the highest median inhibition percentage (91.5%), followed by the vaccination-only (87.4%) and infection before vaccination (83.9%) groups. The lowest median inhibition percentage was observed in the natural infection-only group (32.5%). However, the natural infection group had a small sample

size ($n = 6$); therefore, its results should be interpreted cautiously.

Discussion

Three key findings emerge from our data. First, in April 2022, four months after the appearance of the Omicron variants in Switzerland, almost 60% of the healthy blood donors in Basel were reactive to anti-N Ig and 99% to anti-S-RBD Ig, which can be cautiously interpreted as the infection and vaccination rates, respectively. Second, we also observed a high correlation between anti-S-RBD Ig and surrogate virus neutralisation tests ($p < 0.0001$). Third, infection followed by vaccination resulted in a higher and more prolonged anti-S-RBD Ig level than vaccination alone.

Data available on the percentage of SARS-CoV-2 positive tests (PCR and rapid antigen) and the number of administered vaccinations for the canton of Basel Stadt match our findings [15, 16] (figure S4). However, the infection and vaccine rates over time are slightly lower than in our data (figure 2), potentially because many individuals in the cohort donated blood repeatedly, resulting in an essentially cumulative positivity rate. Furthermore, our data might have a sampling bias since blood donors potentially follow vaccine recommendations and public health precautions more strictly.

Surprisingly, we did not observe a sex difference in humoral immunity in our cohort, and the percentage of positive tests (anti-N Ig levels ≥ 1.0 COI, anti-S-RBD Ig levels ≥ 0.8 U/ml) only differed significantly between age groups for only one month. A systematic review by Notarte et al. [17] showed that older males had lower humoral responses amongst various criteria. They suggested this could be due to a functional decline in the immune system with age and the immunomodulating properties of hormones, leading to higher antibody production in females [18, 19]. We performed a similar analysis of the anti-S-RBD Ig titres by calculating their median per month after the first vaccination dose using data obtained through the questionnaire. However, we did not observe any differences in the antibody levels, even in the various subgroups.

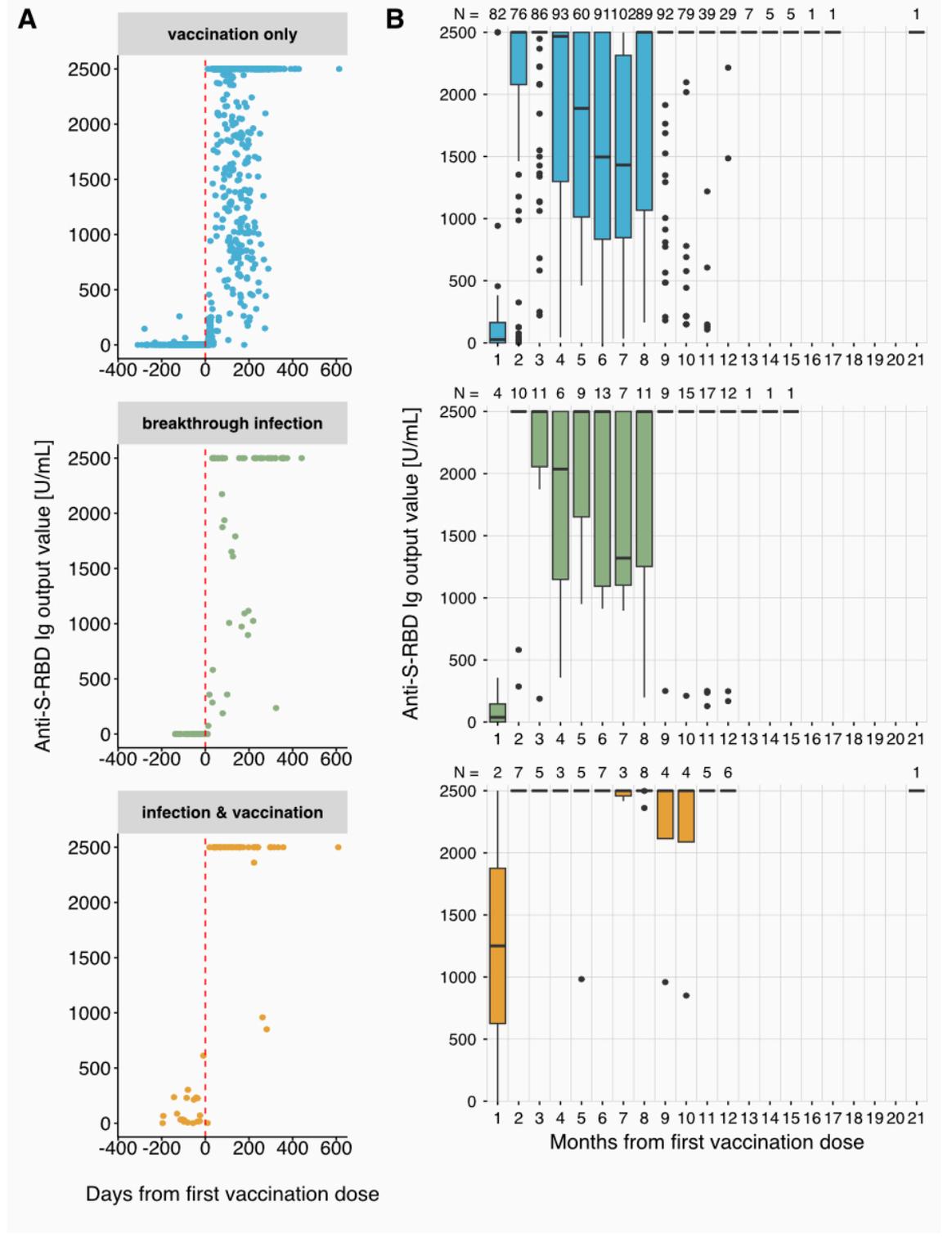
We evaluated the functional anti-S antibodies, specifically the neutralising antibody levels, which have been proposed to correlate with protective immunity [5, 6]. We showed that anti-S-RBD Ig measurements correlated well with the presence of nAb. Many studies have thoroughly investigated this relationship previously. For example, Kitagawa et al. [20] observed strong correlations between the inhibition percentages measured in the surrogate virus neutralisation tests and the anti-S IgM and IgG measurements (Spearman’s $\rho = 0.95$ and 0.96 , respectively; $p < 0.001$). Furthermore, Roche states on their website and in the package insert for the Elecsys[®] Anti-SARS-CoV-2 S immunoassay [9] that an anti-S-RBD Ig titre of over 15 U/ml indicates the presence of nAb. In contrast, we determined a cut-off of 57 U/ml in our dataset. The difference in cut-off may be due to the different methods used to determine nAb presence. We conducted a surrogate virus neutralisation test, whereas Roche compared their measurements with the results of an in-vitro plaque-reducing neutralisation assay, considering samples achieving 50% neutralisation at a sample dilution of $>1:20$ as positive for neu-

tralis activity. Different cut-offs have been described as associated with neutralisation, possibly due to differences in sample sets/cohorts and statistical approaches. We computed the Youden's index to determine a suitable cut-off.

Choosing a cut-off may be optimised for better sensitivity or specificity.

We also observed that, in almost all cases, a single vaccine dose led to an increase in anti-S-RBD Ig titres within one

Figure 5: Anti-S-RBD Ig titres by infection and vaccination status. **A** Samples collected right before and after the specified first vaccination date were selected for each donor and normalised to the first vaccination date. The anti-S-RBD Ig titres of the abovementioned samples were grouped by infection and vaccination status. The red dashed line denotes the first vaccination date (day 0). **B** The median anti-S-RBD output values summarised by month from the donors' respective first vaccination dose. Colours represent the donors who were only vaccinated (vac, blue), who have experienced a post-vaccine breakthrough infection (bt, green), and who have been naturally infected before being vaccinated (infvac, orange). In **B**, the respective number of samples included in the calculations per month is shown above each bar. Anti-S-RBD Ig: anti-SARS-CoV-2 spike receptor binding domain Immunoglobulins.



month. Similarly, we showed that the first vaccine dose given to previously naturally infected donors acted as a “booster dose”. The median anti-S-RBD Ig titres were substantially higher immediately after the first vaccination and persisted longer in the infection before vaccination group compared to the vaccination-only and post-vaccine breakthrough infection groups. This “hybrid immunity” has already been previously associated with an increased humoral response to SARS-CoV-2 [21–24]. Furthermore, we observed a lower inhibition percentage, as measured in the surrogate virus neutralisation test, in the “natural infection only” group than in all other vaccinated groups, suggesting that less neutralising antibodies may be elicited after only a natural infection. Assis et al. investigated the difference in humoral immunity responses in naturally infected individuals and mRNA vaccinees. They observed that the vaccines elicited higher and broader antibody levels than natural infections alone. Notably, they observed that sera of vaccinees had a higher antibody titre specifically against receptor binding domain segments [25]. Our findings may support this observation since we observed a significant correlation between anti-S-RBD Ig measurements and neutralising antibodies.

Interestingly, 24 donors had a sample that was nonreactive for anti-S-RBD Ig after vaccination. However, almost all samples were collected within 21 days of vaccination, and the antibodies may have been undetectable due to the short interval between sample collection and the first vaccine dose. Leuzinger et al. found that SARS-CoV-2 antibody assays had lower sensitivity in the first one to two weeks after a positive SARS-CoV-2 diagnosis [26]. Furthermore, as the second dose had not yet been administered, it could be assumed that a single vaccine dose might be insufficient to induce antibodies in those donors. Two samples were still negative for anti-S-RBD Ig 36 and 178 days after the first vaccine dose, respectively. Examining the questionnaire of the first donor, we could see that no second vaccination had been administered at the time of the sample collection, although 36 days had passed since the first dose. The donor also specified having no infection in the meantime. Again, the first dose might have been insufficient to elicit measurable antibodies for this donor.

One limitation of our study was the self-reported questionnaire, which may contain errors. Second, our study was not a prospective vaccine trial, and our data consisted of real-world humoral surveillance monitoring. Third, the vaccination-only group contained six samples that were collected before the first vaccination dose but tested positive for anti-S-RBD Ig (≥ 0.8 U/ml) (figure 5A). A closer inspection of the serological results revealed that five of these samples were also positive for anti-N Ig (≥ 1.0 COI). Furthermore, one was positive for anti-S-RBD Ig but not anti-N Ig before the first vaccination date. Therefore, we could conclude that either the anti-S-RBD Ig measurements were falsely positive, vaccination dates were incorrectly specified, or donors had a subclinical or asymptomatic infection before vaccination or simply forgot about the infection. Fourth, we lack data on the vaccination and infection status of 58.6% (1610/2748) of the donors since there was either no response to the questionnaire or missing data entries, making it impossible for them to be classified into the respective status, which may have introduced an un-

known bias in some of our analyses. Fifth, the surrogate virus neutralisation test used only wild-type RBD. The receptor binding domains of the different variants of concern are structurally divergent from the wild-type and each other due to mutations, and it has been shown that those mutations can lead to immune evasion [27, 28]. Therefore, if an individual tests positive for neutralising antibodies according to our threshold for the anti-S-RBD Ig titre, those nAbs might be unable to effectively neutralise the other variants of concern and newly emerging variants to the same extent. Finally, we do not have complete data for all donor characteristics (e.g. the blood type [ABO and rhesus] and cytomegalovirus serology status), which may have prevented us from observing a possible connection between those characteristics and the serological responses over time.

Our dataset included samples collected over more than two years from the very start of the COVID-19 pandemic, covering several infection waves caused by various novel variants of concern. The collection of comprehensive metadata also allowed the influence of different donor characteristics on humoral immunity to be examined. Understanding the dynamics of the humoral immunity against SARS-CoV-2 may provide insights into the level of protection against infection and may assist policy-making for future pandemics.

Conclusions

This study aimed to (a) explore the correlation between anti-S-RBD Ig and surrogate virus neutralisation test measurements and (b) describe humoral immunity against SARS-CoV-2 after vaccination, natural infection, and breakthrough infection. We observed that 57.1% of the investigated cohort was infected with SARS-CoV-2. Furthermore, one vaccine dose already leads to a substantial increase in the anti-S-RBD Ig titre, while a “hybrid immunisation” (i.e. vaccination after a previous natural infection) seems to result in a higher and longer-lasting anti-S-RBD Ig titre after the first vaccine dose. We also observed that vaccinations elicited higher neutralising antibody levels than natural infections alone. An estimate of neutralisation may be helpful to assess the risk of re-infection. Since anti-S-RBD Ig levels correlated with surrogate virus neutralisation test results, we suggest that anti-S-RBD Ig may be used to estimate the level of neutralising antibodies. The threshold for protection from an unfavourable clinical outcome must be evaluated in prospective clinical cohorts. Our study provides insights into the dynamics of humoral immunity against SARS-CoV-2 at a single-city resolution. The analysis of our longitudinal real-world dataset, collected over two years from the start of the COVID-19 pandemic, may contribute to understanding the course of a pandemic and, thus, help better prepare for and manage future pandemics.

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Potential competing interests

AE is medical advisory for Sefunda and PhAST. All other authors have completed and submitted the International Committee of Medical Journal Editors form for disclosure of potential conflicts of interest. No potential conflict of interest related to the content of this manuscript was disclosed.

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Appendix

Discussion about a protective titre of neutralising antibodies against SARS-CoV-2 reinfection

The protective titre of neutralising antibodies against a SARS-CoV-2 infection has been discussed extensively, mainly in connection with the assessment of vaccine efficacy, the therapeutic use of convalescent plasma or sera for patients suffering from severe COVID-19, and pandemic surveillance and public health policy-making [29]. The gold standard for quantifying neutralising antibodies against SARS-CoV-2 in serum samples is the live virus plaque reduction neutralisation test (PRNT). Briefly, diluted serum samples are mixed with live SARS-CoV-2 viruses and added to confluent *TMPRSS2*-expressing Vero-E6 cells. After incubation, the cells are fixed and stained with crystal violet, and the plaques are counted in each well and compared to the number of plaques in the control wells. Finally, the PRNT50 or PRNT90 value, the dilution at which the serum showed 50% or 90% reduction of plaques compared to the controls, respectively, is computed [30].

Khoury et al. modelled the relationship between neutralising antibody titres and protection from a detectable or severe SARS-CoV-2 infection [5]. They found that the

threshold for neutralising antibody titres at which the serum was 50% protective against a symptomatic or severe infection was 20.2% or 3.0% of the mean convalescent neutralising antibody titre, respectively. These values translate into a plaque-reducing neutralisation serum dilution of between 1:10 and 1:30 but also up to 1:200 in one of their evaluated datasets. Using this mathematical model, Lau et al. determined the threshold for 50% protection from a symptomatic infection at a PRNT50 and PRNT90 titre of 1:25.9 (95% CI = 1:24.7–1:27.6) and 1:8.9 (95% CI = 1:8.6–1:9.4), respectively, in their cohort [31].

In our study, the threshold for the presence of neutralising antibodies was set to a serum dilution of 1:10. If the serum did not show a plaque reduction of 90% at a serum dilution of 1:10, it was considered negative for neutralising antibodies. Previous studies have also followed this method [30, 32]. Kohmer et al. defined the serum dilution of 1:10 as a “borderline” result and considered serum dilutions $\geq 1:20$ as positive for neutralising antibodies [33]. However, we did not define a threshold for the neutralising antibody titre showing a protective effect against SARS-CoV-2 infection.

Supplementary figures

Figure S1: Schematic of the manual determination of infection and vaccination dates. The upper light blue boxes describe the conditions that must be met for manual date determination. The bottom dark blue boxes represent the method for manually determining the date for each condition. The values next to the arrows represent the frequency at which the specific type of manual date determination was conducted. Median dates were selected for cases with ambiguous date specifications (leftmost column) to minimise errors. Specific date intervals were chosen based on the standard vaccination scheme of the different vaccines (three columns on the right).

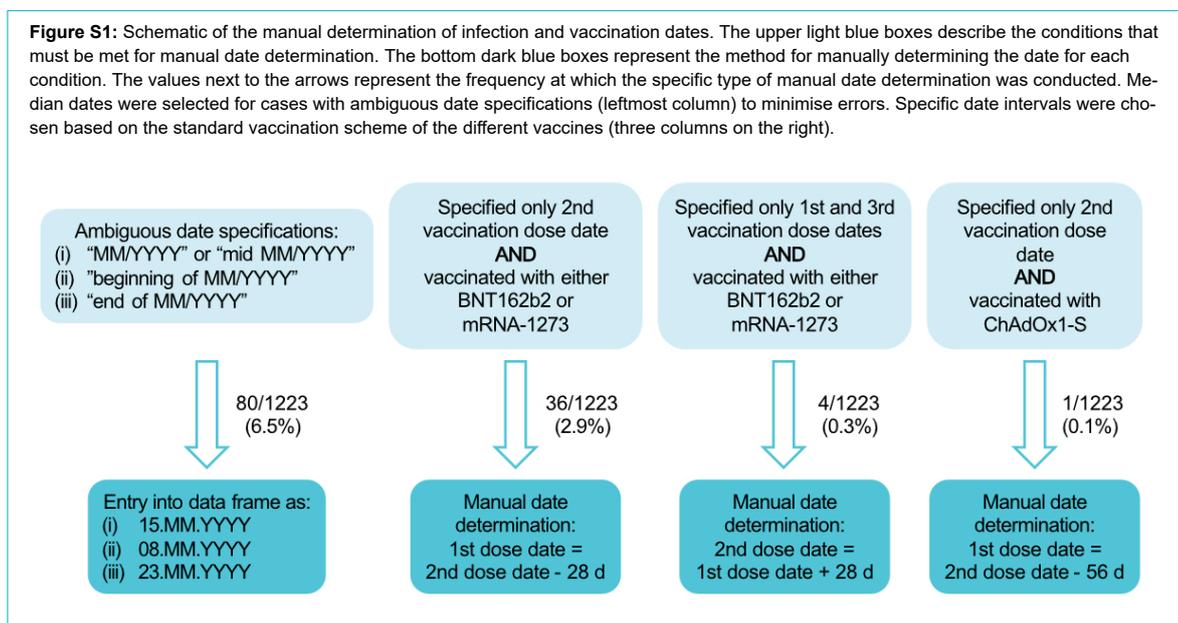


Figure S2: Correlation analysis: classical virus neutralisation test (VNT) vs surrogate virus neutralisation test (sVNT). Tested with Wuhan wild-type SARS-CoV-2 in TMPRSS2-expressing Vero E6 cells and SARS-CoV-2 Neutralizing Antibodies Detection Kit (Adipogen™). **A** The x-axis shows the classical VNT results (i.e. the highest serum dilution at which the serum still protected against the virus). All samples that did not show live virus neutralisation at the lowest dilution tested were assigned the value 1. The y-axis shows the sVNT results (i.e. the calculated inhibition percentages). The points represent each serum sample. Kendall's tau (τ) and the p-value were computed. The red dotted lines denote the respective methods' cut-off for neutralising activity. **B** The confusion matrix. Samples were classified as positive for neutralising antibodies at an inhibition percentage $\geq 20\%$ and a serum dilution of $\geq 1:10$ for the sVNT and VNT, respectively. PPV: positive predictive value; NPV: negative predictive value.

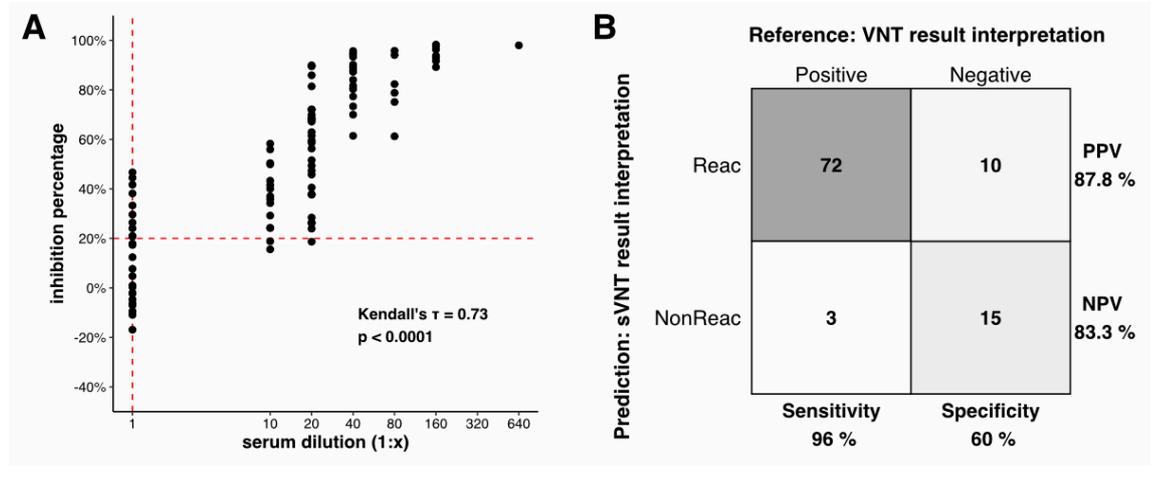


Figure S3: Inhibition percentages grouped by infection and vaccination status. Samples (dots) with an inhibition percentage measured by the surrogate virus neutralisation test (sVNT) and the infection and vaccination status determined by the questionnaire responses were included. The areas around the dots represent the distribution density, the yellow line denotes the median, and the red dashed line denotes the cut-off of the sVNT at 20% inhibition. The values above the violin plots represent the number of samples included in the calculations for each category. bt: post-vaccine breakthrough infection; vac: vaccination only; infvac: infection before vaccination; inf: natural infection only.

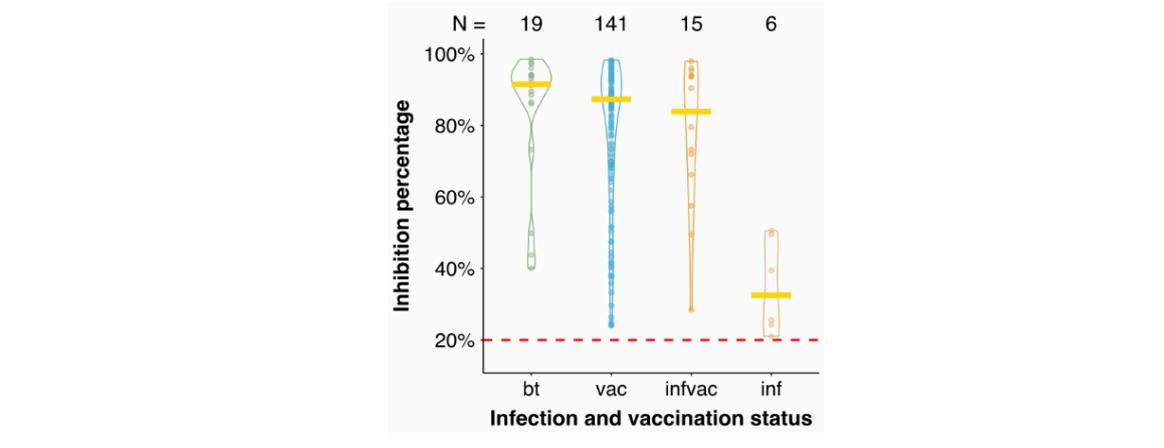


Figure S4: The percentages of positive SARS-CoV-2 PCR and rapid antigen tests and vaccinated individuals in the canton of Basel Stadt, Switzerland, from March 2020 to April 2022. The percentages were calculated for each month. The blue squares represent the percentage of positive tests per month, and the green squares represent the percentage of vaccinated individuals (who received at least one dose of a vaccine against SARS-CoV-2).

