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## Sex differences in the evolution of neutralizing antibodies to SARS-CoV-2

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**40-word-or less summary:**

We measured the levels of antibodies in 308 SARS-CoV-2-infected individuals, collected up to 6 months after symptom onset. Anti-Spike antibodies and NABs declined faster in males than in females, suggesting an association of sex with evolution of the humoral response.

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**Potential conflicts of interest:** SFK, AV, YM, CSM, NM, CP, DR, HM, LGI, YH, JDS, MG and AF have no competing interest to declare. LG, IS, TB, and OS are holder of a provisional patent on the S-Flow assay.

## **Abstract**

We measured Anti-Spike (S), Nucleoprotein (N) and neutralizing antibodies (NAbs) in sera from 308 RT-qPCR<sup>+</sup> healthcare workers with mild disease, collected at two time-points up to 6 months after symptom onset. At Month 1 (M1), anti-S and N antibody levels were higher in males > 50 years or with a body mass index (BMI) > 25. At M3-6, anti-S and anti-N antibodies were detected in 99% and 59% of individuals, respectively. Anti-S antibodies and NAbs declined faster in males than in females, independently of age and BMI, suggesting an association of sex with evolution of the humoral response.

**Keywords: SARS-CoV-2, antibodies, sex-related differences, COVID-19**

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## Introduction

The duration of humoral immune responses to SARS-CoV-2 is debated. Severe COVID-19 patients produce more antibodies than asymptomatic or mildly symptomatic individuals [1-3]. Some studies showed a rapid decrease in convalescents regardless of disease severity, others reported stable antibody titers within the first three months [1-7]. Anti-Spike (S) antibody amounts correlate with neutralization capacity, since S is the main, if not unique, target for neutralizing antibodies. Neutralizing antibody titers also vary depending on the time post-onset of symptoms (POS) and disease severity [1-3, 8]. Little is known about the influence of sex, age, body mass index (BMI) on the longevity of anti-SARS-CoV-2 antibodies, particularly in mildly symptomatic individuals, who represent the majority of COVID-19 cases.

## Methods

**Study design and participants.** We assessed the persistence of anti-SARS-CoV-2 antibodies in sera from mild COVID-19 healthcare workers in Strasbourg University Hospital. 308 donors with RT-qPCR-confirmed COVID-19 diagnostic were enrolled in the study, with samples longitudinally collected at Month 1 (M1) (median 31 days, range 11-58 days) and M3-6 (median 107 days, range 78-172 days) POS (Table S1).

SARS-CoV-2 RT-PCR testing on nasopharyngeal swabs was performed at least 10 days before inclusion. Participants completed a questionnaire covering sociodemographic characteristics, virological findings, and clinical data including myalgia, difficulty of breathing, fever, asthenia, rhinitis/pharyngitis, cough, headache, anosmia/dysgeusia, diarrhea.

**Ethics Committee Approval.** Results are part of an on-going prospective, interventional, monocentric, longitudinal, cohort study enrolling staff from the Strasbourg University Hospitals (ClinicalTrials.gov Identifier: NCT04441684). The protocol was approved by the institutional review board of Strasbourg University Hospitals.

## **Serological assays**

Commercial assays. The Biosynex® (COVID-19 BSS IgG/IgM) Lateral Flow Assay (LFA) detects IgM and IgG against the RBD with 99% specificity and 96% sensitivity after 22 days POS [9]. The EDI™ Novel coronavirus COVID-19 IgG ELISA assay detects IgG against N, with 96% specificity and 81% sensitivity after 28 days POS [9].

S-Flow assay. The S-Flow assay [10] was adapted by using 293T cells stably expressing the S protein (293T Spike cells) and 293T Empty cells as control. The positivity of a sample was defined as a specific binding above 40%, with 100% specificity (CI95%: 98.5-100) and 99.2% sensitivity (CI95%: 97.69%-99.78%) [10, 11]. Binding units (BU) were calculated to standardize the results. A standard curve with serial dilutions of a human anti-S monoclonal antibody (mAb 48) was acquired in each assay. The logarithm of the median of fluorescence of each sample was reported on the curve to obtain an equivalent value (in ng/mL) of mAb48 concentration in logarithm.

**Cells.** 293T cells (ATCC® CRL- 3216™) were transduced to express SARS-CoV-2 S (GenBank: QHD43416.1) or with an empty lentivector and selected with puromycin [10] [12]. 293T cells stably expressing ACE2 and inducible TMPRSS2 (293T-ACE2-iTMPRSS2) were produced by lentiviral transduction and selection with puromycin and blasticidin. Cells were regularly tested for absence of mycoplasma.

**Neutralization of pseudotyped lentiviral particles.** The assay was performed as described [10].  $2 \times 10^4$  293T-ACE2-TMPRSS2 were plated in 96-well plates. Sera were diluted at 1:100 and incubated with Spike-pseudotyped lentiviral particles (provided by Theravectys-Pasteur laboratory) for 15 minutes at RT before addition to cells. After 48h, the luciferase signal was measured with EnSpire® Plate Reader (PerkinElmer). The percentage of neutralization was calculated as:  $100 \times \left(1 - \frac{\text{mean}(\text{luciferase signal in sample duplicate})}{\text{mean}(\text{luciferase signal in virus alone})}\right)$ . A titration of a mAb 48 was performed on each plate as control.

**Statistical analysis.** Baseline characteristics between men and women were compared using a Chi-square test for categorical variables and Student's t-test for continuous variables. Correlations between antibody measures at M1 and characteristics of participants were estimated using linear regression models for factors associated with BU and neutralization levels, and logarithmic regression models for factors associated with IgM and IgG positivity. The difference in BU and neutralization levels between M1 and M3-6 was then estimated and standardized by the time interval between the two timepoints. Factors associated with these standardized differences were investigated using linear regression models. Factors that were associated with the outcome with a p-value <0.15 in univariate analysis were introduced in the multivariate model. A p-value <0.05 was considered statistically significant. Subjects were divided into "sustainer" or "decayer" categories, for anti-S, anti-N IgG and neutralizing antibodies, as reported [7]. We calculated the fraction of antibody value at M3-6 divided by value at M1. "Sustainers" were defined by a fraction  $\geq 1$ , and "decayers" by a fraction <1. The half-life of decayers, extrapolated from the equation of the segment formed by the two timepoints, corresponds to the week for which antibodies reach half of M1 level.

Analyses were performed using Stata (Stat Corp., College Station, TX, USA), Excel 365 (Microsoft), RStudio Desktop 1.3.1093 (R Studio, PBC) or Prism 8 (Graphpad Software).

## Results

We analyzed the longitudinal antibody response in a monocentric cohort of 308 RT-qPCR confirmed staff from Strasbourg University Hospitals (Figure S1). The cohort included 75% females, with median age of 39 years (Table S1). The participants were nurses, doctors, caregivers and administrative staff. Contact with a COVID-19 patient, within or outside of the hospital, was reported by 37% of individuals and 94% had mild symptoms consistent with COVID-19 (Table S1). Sixteen participants were hospitalized for moderate disease. None progressed to severe illness. The median time from onset of symptoms to

RT-qPCR testing was 3 days. All individuals were sampled twice, firstly at M1 with a median of 31 days POS (range: 11-58) and secondly at M3-6 at a median of 107 days POS (range: 78-172).

Seropositivity rates were estimated with four assays. The dynamics of the immune response was assessed by comparing antibody levels at different times POS (Fig. 1). All participants had anti-S IgG by S-Flow at M1 and 3 participants (1%) became negative at M3-6. Quantitative measurement with standardized Binding Units (BU) demonstrated a slight but significant decrease of anti-S IgG amounts between M1 and M3-6 (Fig. 1B). The LFA (Biosynex™) detecting anti-S IgG and IgM, was less sensitive than S-Flow, with 85% individuals IgG seropositive at the two time-points. IgM were detected in 93% of participants at M1 and only 79% at M3-6, likely reflecting the contraction of the IgM response. Measurement of anti-N IgG with an ELISA (EDI™) gave similar results than LFA at M1, but only 59% of individuals remained positive at M3-6 (Fig. 1A). The neutralization activity, measured with pseudotyped lentiviral particles, also declined overtime. With a positive neutralization threshold set at 20%, 95% and 84% of the sera were positive at M1 and M3-6, respectively (Fig. 1A). Applying more stringent thresholds (50% or 80%) confirmed this decline (Fig. S2). We observed a correlation between neutralization activity and anti-S or anti-N IgG in the sera (not shown). Plotting the median values of anti-S, neutralizing and anti-N antibodies at different time intervals confirmed a slow decline over time, with large inter-individual variations (Fig. S3A).

We then determined whether these variations may be attributed to biological or clinical characteristics of the participants. We analyzed the associations between antibody levels (anti-S IgG, neutralizing activity or anti-N IgG) and sex, age, BMI and type of symptoms, at M1 and M3-6. We calculated the slope of the curves between the two time-points, to assess the impact of the participants' characteristics on the evolution of the response. Levels of anti-S and neutralizing antibodies were higher in males than in females at M1 but not at M3-6 (Fig. 2A). Accordingly, the slope of antibody decline was significantly steeper in males (Fig.



2B). A multivariate analysis showed that anti-S and neutralizing antibodies were higher at the first time-point and declined faster in males, independently of other factors (Table S3). There was no significant difference between males and females regarding the decline of anti-N IgG.

The majority of individuals showed a decline whereas others displayed stable antibody amounts. We categorized the subjects, based on the stability of the humoral response, into “sustainers” and “decayers” [7] (Figure S3B). The proportion of decayers varied between 71 and 83% for the three assays. Among decayers, the median half-life of antibody levels was 41.0 weeks (IQR: 24.3-71.8) for anti-S IgG, 19.9 weeks (IQR: 14.4-36.0) for neutralizing antibodies, and 18.4 weeks (IQR: 15.2-25.7) for anti-N IgG (Figure S3C). We also noted that female subjects were in higher proportion sustainers than decayers compared to males (Fig. 2C), in line with our observation that antibodies persist for longer periods of time in women (Fig. 2B).

Categorization of the participants by age ( $\leq 30$ , 30-50 and  $> 50$  years old) and BMI (17-25,  $\geq 25$ ) further showed that older participants and those with a high BMI had higher antibody titers at M1, as seen with anti-S, neutralization and anti-N IgG (Figure S4). However, the decline of antibody levels occurred at the same rate, regardless of age or BMI (Table S3 and Figure S4).

There was no association of reported clinical signs, except anosmia/ageusia or cough, with the amount of antibodies at M1 nor with their evolution (Tables S2 and S3). This likely reflects the homogeneity of symptoms, as all participants suffered from a mild-to-moderate disease. As reported [10], the antibody levels at M1 were higher in hospitalized individuals but decreased at the same rate than non-hospitalized patients (Table S1 and Figure S5). Multivariate analyses indicated that high antibody levels at M1 were associated with a more rapid decline, independently of any other parameters (Table S3).

## Discussion

Assessing the long-term humoral response is critical to evaluate immune protection at the population level. Commercially available assays have been validated with sera collected from acutely or recently infected individuals. Differences in the sensitivity of ELISA tests, including those detecting anti-N antibodies, may be dependent on the days POS [13, 19]. We performed here a longitudinal analysis of the humoral response in a cohort of 308 RT-qPCR confirmed SARS-CoV-2 infected patients with mild disease. Antibodies declined over the 172 days of analysis. We observed a sharp decrease of anti-N seroprevalence between M1 and M3-6 that may reflect a lower abundance of anti-N antibodies in mild disease, a different kinetic of the anti-N response, or a lower sensitivity of the test. The poor performance of some serological assays for long-term analyses may explain discrepant results regarding the stability or waning of antibody titers in convalescent patients.

Neutralizing antibody levels were assessed using pseudotyped lentiviral particles. One limitation of our study is the use of a single dilution of the sera (1:100). However, we and others previously reported a correlation between the percentage of neutralization at this non-saturating dilution and titers obtained with pseudovirus or infectious virus and serial dilutions of the sera [10, 13]. Neutralizing antibody levels decreased twice as fast as anti-S IgG, with half-lives of 19.9 and 41 weeks, respectively. We further report sex differences in the longevity of the immune response. Males displayed higher antibody levels shortly after infection, but a steeper decrease, so that the difference was no longer visible at M3-6. In line with our results, previous reports showed a stronger induction of the immune response against SARS-CoV-2 in male patients [14]. Multiple studies have demonstrated that women develop more robust responses to infections and vaccination and are more sensitive to auto-immune diseases than men [15]. This may be linked to sex hormones, X chromosomal and environmental factors. SARS-CoV-2-infected women mount more robust T cell activation than male patients [14], which will impact the duration of the response. We limited our analyses to the first six months of convalescence after infection. Future work will help

determining whether the sex differences reported here are amplified over time and may be linked to differences in antigen persistence [8]. It will also be of interest extending our analysis on antibody longevity to other categories of persons, including asymptomatic individuals who represent most of SARS-CoV-2 cases, patients who recovered from severe forms of COVID-19, and vaccine recipients. Whether vaccines provide a longer protection in women than in men, and whether this different evolution will impact the sensitivity to viral variants remain outstanding questions.

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**Authors contribution.**

Conceptualization and Methodology: AF, OS, SFK

Cohort management and sample collection: AV, FG, CSM, MJW, CSM, DR, NM, YH, JDS, AF, MG, SFK

LFA and EDI: AV, FG, MJW, LGI

S-Flow and seroneutralization: TB, LG, IS, OS

Data assembly and manuscript writing: LG, AV, FG, TB, YM, AF, SFK, OS

Funding acquisition: AF, OS, SFK

Supervision: OS, SFK

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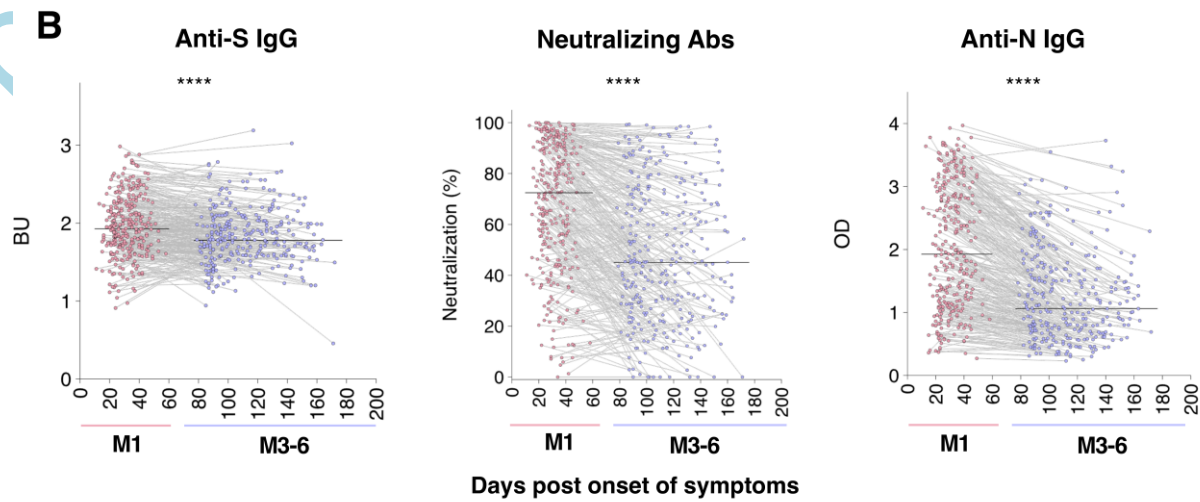
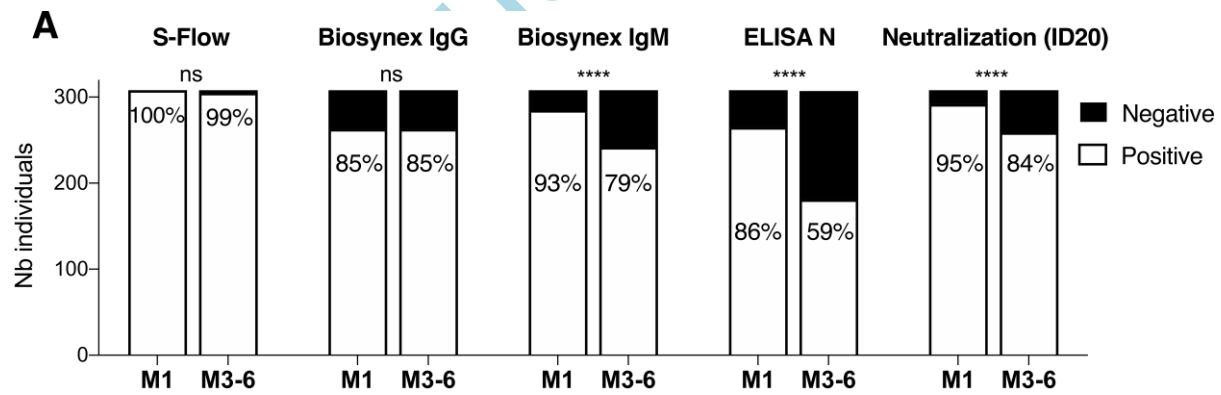
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## Figure legends

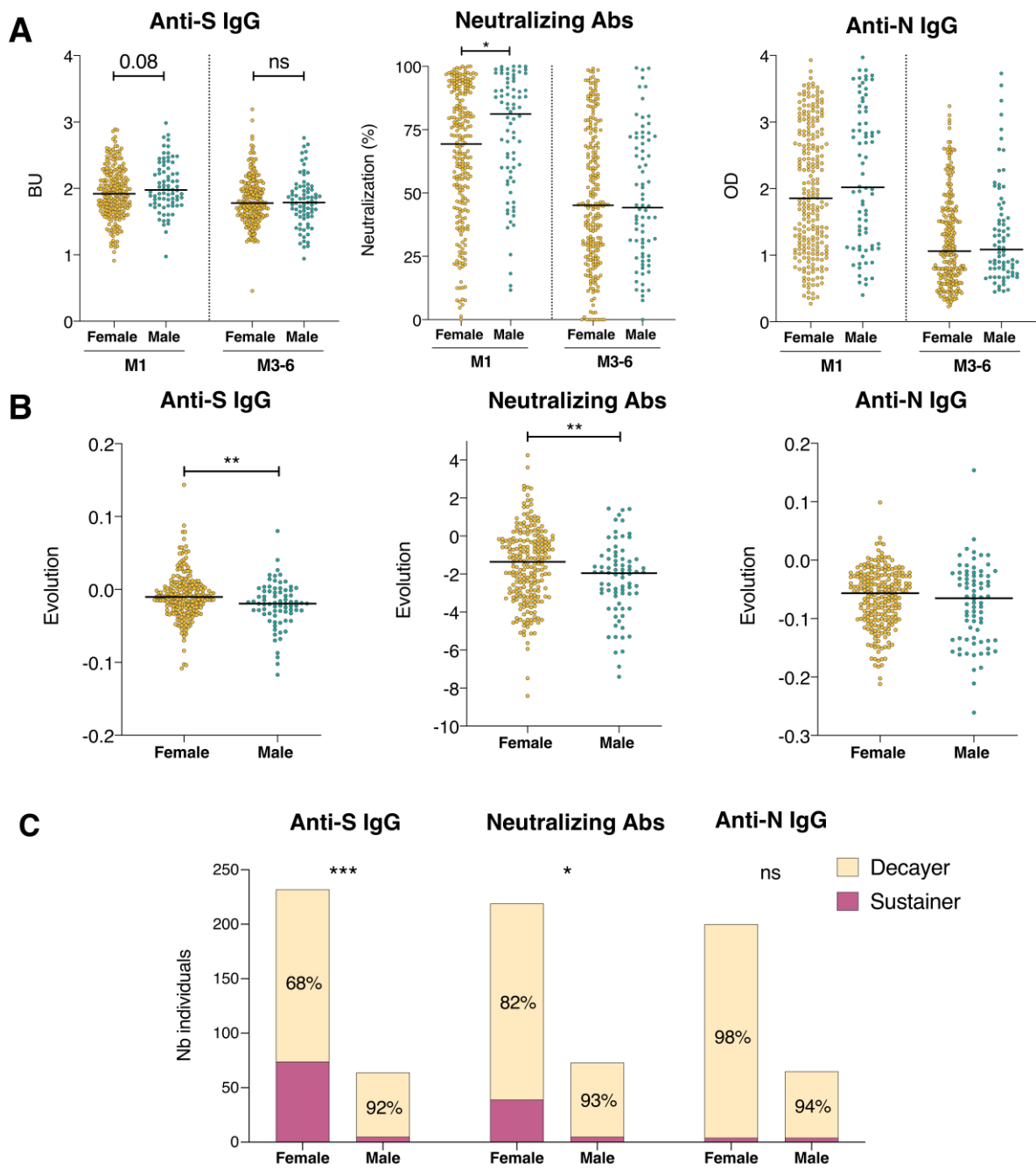
**Fig. 1 Temporal evolution of anti-SARS-CoV-2 antibodies. A.** The number of individuals displaying anti-S (S-Flow or Biosynex™ tests), anti-N (ELISA N EDI™) or neutralizing antibodies were plotted at Month 1 (M1) and M3-6 POS. The percentages of positive cases are indicated in the bars. Neutralization positivity was defined as a neutralizing activity against lentiviral pseudotypes higher than 20%, at a 1:100 serum dilution (defined as Inhibitory dose (ID20)). Differences between time-points were analyzed with a Chi-square test, \*\*\*\*  $p < 0.0001$ . **B.** Levels of antibodies defined as Binding Units for anti-S IgG (S-Flow assay), percentage of neutralization, and Optical Density (OD) for anti-N IgG (ELISA) were plotted against the days POS. Pink and purple points stand for M1 and M3-6 time-points, respectively. Each grey line connects the time-points from a same donor. The black line represents the median of all samples for each time-point. Paired Wilcoxon test was performed between M0 and M3-6, \*\*\*\*  $p < 0.001$ .

**Fig. 2 Sex differences in anti-SARS-CoV-2 antibody levels at the two samplings and their temporal evolution. A.** Anti-S IgG (in BU), percentages of neutralization and anti-N IgG (in OD) were compared between males (green dots) and females (orange dots) at M1 or M3-6. The black line represents the median of all samples for each time-point. Samples from females and males were compared with a Mann-Whitney test, \*  $p < 0.05$ , ns: not significant. p-value or non-significance (ns) are indicated on the segments. **B.** Weekly evolution of antibody levels between M1 and M3-6 was calculated as  $(\text{level at M3-6} - \text{levels at M1}) / (\# \text{ weeks POS M3-6} - \# \text{ weeks POS M1})$ . Color coding and graphical parameters are as in A. The dotted line represents a stable antibody level (evolution of 0). Statistical analysis Mann-Whitney test, \*\* $p < 0.01$ . **C.** Each subject was defined as “sustainer” (purple) if the fraction antibody at M3-6 / antibody at M1 was  $\geq 1$  or “decayer” (yellow) if the fraction was  $< 1$ . The proportion of sustainers and decayers is compared between females and males. Differences were assessed with a Chi-square test. \*:  $p\text{-value} < 0.05$ , \*\*\*:  $p\text{-value} = 0.0001$ .



**Fig. 1** Temporal evolution of anti-SARS-CoV-2 antibodies





**Fig. 2 Sex differences in anti-SARS-CoV-2 antibody levels at the two samplings and their temporal evolution.**