Analysis of humoral immune responses in SARS-CoV-2 infected patients.

Lisa Henss[1], Tatjana Scholz[1], Christine von Rhein[1], Imke Wieters[3], Frauke Borgans[3], Fabian J. Eberhardt[3], Kai Zacharowski[4], Sandra Ciesek[5], Gernot Rohde[6], Maria Vehreschild[3], Christoph Stephan[3], Timo Wolf[3], Heike Hofmann-Winkler[7], Heinrich Scheiblauer[2], Barbara S. Schnierle[1*]

1 Paul-Ehrlich-Institut, Department of Virology, Paul-Ehrlich Strass 51-59, 63225 Langen, Germany

2 Paul-Ehrlich-Institut, IVD, Paul-Ehrlich Strass 51-59, 63225 Langen, Germany

3 Zentrum für innere Medizin, Infektiologie, Universitätsklinikum Frankfurt, Goethe Universität Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany

4 Klinik für Anästhesiologie, Intensivmedizin und Schmerztherapie, Universitätsklinikum Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany

5 Institut für Medizinische Virologie, Universitätsklinikum Frankfurt am Main, Goethe Universität Frankfurt, Paul-Ehrlich-Str. 40, 60596 Frankfurt am Main

6 Medizinische Klinik 1, Pneumologie/Allergologie, Universitätsklinikum Frankfurt – Goethe-Universität, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany

7 Deutsches Primatenzentrum GmbH, Leibniz-Institut für Primatenforschung, Kellnerweg 4, 37077 Göttingen, Germany

Brief summary

Humoral immune responses of 143 German COVID-19 patients were analyzed. Disease severity correlated with the amount of SARS-CoV-2-specific antibodies and their neutralization activity. Compared to patients with mild-moderate disease, patients with severe disease had only weakly neutralizing antibodies against coronavirus-NL63.

Footnote page

Conflict of interest statement:

The authors have non conflict of interest.

Funding:

This research was funded by the German Ministry of Health (BMG) by a grant to B.S.S.

Corresponding author contact information:

Barbara Schnierle

Paul-Ehrlich-Institut, Department of Virology, Paul-Ehrlich Strass 51-59, 63225 Langen, Germany

Telephone: 0049-6103-775504

FAX: 0049-6103-7775504

e-mail: barbara.schnierle@pei.de

Abstract

Background:

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection has caused a pandemic with tens of millions of cases and hundreds of thousands of deaths. The infection causes COVID-19, a disease of the respiratory system of divergent severity. Here, the humoral immune response of a cohort of 143 COVID-19 patients from the University Hospital Frankfurt/Main, Germany was characterized.

Methods:

SARS-CoV-2-specific antibodies were detected by enzyme-linked immunosorbent assay (ELISA). SARS-CoV-2 and hCoV NL63 neutralization activity was analyzed with pseudotyped lentiviral vectors.

Results:

COVID-19 severity increased with age and male patients encountered more serious symptoms than females. Disease severity correlated with the amount of SARS-CoV-2 specific IgG and IgA and the neutralization activity of the antibodies. The amount of SARS-CoV-2 specific IgG antibodies decreased with time after PCR conformation of the infection and antibodies directed against the nucleoprotein waned faster than spike directed antibodies. In contrast, for the common flu coronavirus NL63, COVID19 disease severity seemed to correlate with low NL63-neutralizing activities, suggesting the possibility of cross-reactive protection.

Conclusion:

The results describe the humoral immune responses against SARS-CoV-2 and might aid the identification of correlates of protection needed for vaccine development.

Keywords: Coronavirus, antibody, neutralization, SARS-CoV-2, COVID-19

Background

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) belongs to the *Coronaviridae* family and is the causative agent of pneumonia, defined as corona virus disease-2019 (COVID-19), which first emerged in the Hubei province in China [1]. The virus rapidly spread worldwide and the SARS-CoV-2 pandemic was declared by the World Health Organization (WHO), on March 11, 2020. Coronaviruses can cause different diseases in humans. Four endemic human coronaviruses, OC43, 229E, HKU1 and NL63 are the causative agents of common colds. Two other coronaviruses, the severe acute respiratory syndrome virus (SARS-CoV) and the Middle East respiratory syndrome virus (MERS-CoV), have a high pathogenic potential with 15-30% mortality in humans and have caused small epidemics of severe pneumonia [2].

Coronavirus serology has rapidly developed in the last few month and several commercial enzyme-linked immunosorbent assay (ELISA) kits are available. The coronavirus structural proteins, the surface glycoprotein termed spike (S) and the more abundant nucleocapsid (N) are the principle immunogens used for detection of anti-SARS-CoV-2 specific antibodies [3]. The spike protein consists of two subunits S1 and S2. S1 mediates the attachment of the virus to human cells via its receptor-binding domain (RBD) and S2 mediates the fusion of the viral and cellular membranes. Antibodies that bind to the spike protein, and in particular to the RBD domain, can neutralize coronaviruses. Recombinant RBD or S1 protein facilitates detection of coronavirus-specific antibodies by ELISA [4].

In the current pandemic, the urgent need for effective therapeutic measures requires a profound understanding of the pathogenesis of SARS-CoV-2. Here, we analyzed the humoral immune response of German COVID-19 patients in order to characterize the disease and support the identification of correlates of protection needed for the development of vaccines and therapeutic antibodies.

Methods

Cell culture

HEK293T-hACE2 [5] and HEK293T (ATCC CRL-3216) cells were cultured at 37°C under 5% CO_2 and grown in Dulbecco's modified Eagle medium (DMEM; Sigma, Taufkirchen, Germany) supplemented with 10% fetal bovine serum (Sigma, Taufkirchen, Germany) and 5% L-glutamine (200 mM; Lonza, Verviers, Belgium) and 1% penicillin/streptavidin (Fisher Scientific, Schwerte, Germany).

Patient serum samples

Human naïve serum was obtained from the German Red Cross from volunteer blood donors and was collected prior to the introduction of SARS-CoV-2 into Germany. Human serum of SARS-CoV-2 PCR positive patients was obtained from the University Hospital, Frankfurt am Main under the "COVID Capnetz" ethical approval (#11/17). The study was reviewed and approved by the Frankfurt University's ethics committee. The clinical symptoms of the patients were documented by the medical staff of the University Hospital Frankfurt. The score for classification of the severity of symptoms was determined as recommended by the "Clinical Characterisation and Management Working Group of the WHO Research and Development Blueprint Programme" with some modification which resulted the following scoring system [6]. Score 1: Outpatient, SARS-CoV-2 positive, no impairments in mobility; Score 2: Outpatient, SARS-CoV-2 positive, impairments in mobility; Score 3: Hospitalization, SARS-CoV-2 positive, no oxygen therapy; Score 4: Hospitalization, SARS-CoV-2 positive, oxygen (mask/ nasal cannula); Score 5: Hospitalization, SARS-CoV-2 positive, NIV or high flow oxygen; Score 6: Hospitalization, SARS-CoV-2 positive, intubation and mechanical ventilation; Score 7: Hospitalization, SARS-CoV-2 positive, ventilation plus additional support (pressors, RRTT, ECMO).

The samples were single donations with three exceptions, the two patients with score 7 and one patient with initial score 5. The score 7 patients donated blood twice and at the second donation they improved their clinical score to score 6. The score 5 patient showed more clinical signs at the second blood donation and had a score 6. Blood samples were collected between 7 to 130 days after PCR confirmation of the infection.

Pseudotype-based neutralization assay

Lentiviral vectors were prepared in HEK293T cells by co-transfection using Lipofectamine® 2000 (Thermo Fisher, Darmstadt, Germany) as described previously [7]. Plasmids encoding HIV-1 gag/pol, rev, the luciferase-encoding lentiviral vector genome and the SARS-CoV-2 delta 19 spike (#MN908947) or the NL63 delta 19 spike gene (#AFV53148.1) were transfected. The coronavirus genes were synthesized (Eurofins, Ebersberg, Germany) and cloned into the vector pIRES-GFP as described before [8]. Vectors were concentrated by ultracentrifugation and stored at –80°C. Pseudotyped vectors and serially diluted human serum (1:60 to 1:14,580) were incubated in triplicates for 30 min. at 37°C and used to transduce HEK293T-hACE2 cells. After 48 hours, luciferase substrate was added to measure luciferase activity. The reciprocal area under the curve (AUC) value calculated for each sample corresponds to the neutralization activity.

ELISAs

The following CE marked ELISA kits used were: Liaison SARS-CoV-2 S1/S2 IgG (Diasorin SpA, Saluggia, Italy) and Architect SARS-CoV-2 IgG (Abbott GmbH, Wiesbaden, Germany). The tests were carried out and interpreted according to the manufacturers' instructions, as stated in the package inserts.

An ELISA utilizing the SARS-CoV-2 RBD as antigen was established in-house, following a protocol from Stadelbauer *et al.* 2020 [9]. The RBD was transiently expressed from HEK293T cells and purified

by Ni-affinity chromatography. The expression plasmid was generously provided by Florian Krammer, Icahn School of Medicine at Mount Sinai, New York, USA and is described in [9]. RDB was used to coat 96 well microtiter ELISA plates at a concentration of 2 µg/ml and antibodies directed against either human IgG (Merck, Darmstadt, Germany, Cat# 6029) or IgA (ThermoFischer, Dreieich, Germany, Cat# A18781) –horseradish-peroxidase coupled were used to detect binding antibodies. The values obtained with samples from 5 naïve individuals and two standard derivations of these values were subtracted as cut-off.

Statistical analysis of the neutralization experiments and software

AUC values were determined using the GraphPad Prism 7.04 software (La Jolla, CA, USA). Mean values and standard deviations were calculated in Excel.

Results

Characterization of COVID-19 patients

Serum samples from 143 patients with PCR-confirmed SARS-CoV-2 infections with various clinical symptoms were collected between March 9th and June 25th 2020 in Frankfurt/Main, Germany. The patients' age ranged from 18 to 81 years and samples were collected at different times after confirmation of infection by PCR (day 7-130). Patients were scored for clinical symptoms, with a score of 1-2 for mild symptoms without the need for hospitalization. A score of 3-4 indicates moderate disease with patients being hospitalized and score 4 patients needing oxygen. A score of 5-7 characterizes severe disease with patients needing oxygen, artificial respiration and intensive care. Most patients (77.39 %) had mild disease and 14.38 % had moderate disease and only 8.21 % of the patients had severe disease. The mean age increased with disease severity in females from 40.5 to 62.2 years as well as in male patients from 45.7 to 53.3 (Figure 1). There was a significant difference in age when patients with mild to moderate disease were compared to patients with severe disease (Figure 1). Of the patients with clinical score 1-3, females were significantly younger with a mean age of 40.5 years than male patients, with a mean age of 45.7 years (Figure 1). This gender difference was not significant for patients with severe disease. However, only 8% of the patients had severe disease, which might not be representative. A total of 71 male and 75 female patients were analyzed and with one exception, only male patients had severe disease (score 5-7) (Figure 1).

SARS-CoV-2 neutralization activity of patient serum samples

First, the humoral immune responses against SARS-CoV-2 were studied by the ability of serum to neutralize SARS-CoV-2. Neutralization activity was determined using lentiviral vectors pseudotyped with the SARS-CoV-2 spike protein. These vectors acquire the host spectrum of SARS-CoV-2 and a

rapid readout of the results is made possible by the transfer of the luciferase gene, serving as a surrogate measure of infection. Addition of neutralizing antibodies directed against SARS-CoV-2 reduces the luciferase activity. Neutralization activity of serum was determined using the area under the curve (AUC) generated by serum dilutions. Neutralization activity depicted as the reciprocal AUC increased with the severity of disease and was highest in patients with a disease score of 7 (Fig. 2A). Overall, the neutralization activity of patient-derived antibodies was quite diverse, with occasional high level of neutralization activity of serum samples of patients with mild disease. However, when patients with a clinical score of 2 were analyzed for gender distribution of neutralizing antibodies, male patients had more neutralizing antibodies than females, which corresponds to the higher number of male patients with severe disease(Fig. 1)B).

SARS-CoV-2 spike binding antibodies

The presence of spike binding antibodies in patient serum was analyzed by an in-house ELISA coated with the SARS-CoV-2 S1 RBD according to a previously developed protocol [9],[4]. The amount of RBD-binding IgG (Fig. 3A) and IgA (Fig. 3B) increased with the severity of disease and showed a significant increase when comparing patients with a score of 1 to those with a score of 4 -7. There were only two patients with clinical score 7 and the data might not be representative. The clinical score also correlated with the amount of S1/S2-binding IgG determined by the quantitative ELISA from Liaison (Diasorin SpA, Saluggia, Italy) (Fig. 3C). Compared to mildly affected patients there was a significant increase in S1/S2-binding IgG detected in the serum of patients with a score of 4 and higher. This increase in IgG binding was less obvious when the Architect nucleocapsid protein (N) ELISA (Abbott GmbH, Wiesbaden, Germany) was used (Fig. 3D), indicated by the lower significance of the difference between patients with a score of 6 and 1.

Analysis of patients with a clinical score of 2

Most patients had a clinical score of 2 (N=96); however, these single patient serum samples were collected at different time points after PCR-based diagnosis. A decrease in antibody titers, especially antibodies directed against N has been described before [10]. Therefore, the patients with this clinical score were investigated for the presence of antibodies directed against the viral N protein (Fig 4A) and a significant decrease in antibody binding to N was observed in samples from 81-130 days after diagnosis. A similar decrease was observed for IgG directed against RBD (Fig. 4B) and is suggestive of short lasting antibody responses. However, for IgA responses there was no significant decrease detectable (Fig. 4C), although the mean OD values decrease from 0.34 for samples at days 32-45 to 0.20, 0.18 and 0.099 for samples collected at the later time points. Overall, the IgA detection was not very sensitive and might underrate the decrease in SARS-CoV-2-specific IgA.

HCoV-NL63 neutralizing activity

Preexisting immune responses against the common cold causing human CoVs have been described before [11], [12] and consequently neutralization of one human CoV, NL63, by the patient material was studied. Neutralization activity was determined with lentiviral vectors pseudotyped with the NL63 spike protein and, as before, this is depicted as the reciprocal AUC (Fig. 5). Once again, the neutralization activity of patient-derived antibodies was quite diverse, with high neutralization activity observed for a small number of patients. However, high NL63 neutralizing activity was not detected in samples from patients with severe COVID-19 (score of 5-7) patients. However, the overall difference between the neutralization activities of patients with clinical score 1 and 2 compared to score 5-7 patients was significant (Fig. 5).

Discussion

The analysis of 143 COVID-19 patients from the University Hospital Frankfurt/Main revealed that most patients had mild disease (78%). However, severe cases were more frequent with older patients, particularly males. The clinical score was positively correlated with SARS-CoV-2 neutralizing activity in serum samples from the patients. This was reflected by the observation, that male patients not only had increased levels of neutralizing antibodies, but also more severe disease. In addition, IgG or IgA directed against the SARS-CoV-2 spike protein or RBD and, less significantly to the N protein, correlated with disease severity. The less significant correlation shown by antibodies directed against the N protein might reflect different sensitivities in the detection assays or the transient nature of the antibody responses. Similar correlations have been observed previously by others [13], [14], [15], [16].

Among the patients with a clinical score of 2, a decrease in S- and N-directed IgG antibodies was observed in blood samples drawn at later time points after infection. This might indicate the waning of SARS-CoV-2 specific antibodies that has been described previously [17], [18]. Longitudinal analysis of individual patients is needed to fully confirm a decline in antibody titer. Similar observations of declining antibody levels have been described for human coronaviruses. A study of individuals experimentally infected with hCoV-229E showed that levels IgG and IgA antibodies directed against the virus waned to background levels within 11 weeks to 1 year [19].

Overall, the neutralizing titers of patients with mild disease were very low and higher titers were only detected in patients with severe disease. A reciprocal AUC value of higher than 8 corresponds to high neutralizing activity and was reached by only 5.4% of the samples. Thereby we confirm previous observations that most convalescent plasma samples obtained from individuals who recover from COVID-19 do not contain high levels of neutralizing activity [13], [20]. Transfusion of

convalescent plasma is being considered and evaluated as a therapeutic option for the treatment of COVID-19, but the identification of suitable plasma donors is hampered by this substantial variability in levels of neutralizing antibodies in convalescent patients.

Interestingly, patients with severe disease (scores of 5-7) had no high level NL63-neutralizing antibodies, which might be related to the small number of patients that were analyzed. However, the small number of SARS-CoV-2 naïve individuals analyzed, had high NL63-neutralizing antibodies and a high seroprevalence. Serological data about NL63 infections is mainly available from children and indicate that HCoV-NL63 infections are common during childhood [21]. It is tempting to speculate that preexisting immunity to NL63 or other common cold coronaviruses might reduce the risk of severe disease. Neutralizing antibodies might only be a surrogate for this immunity, since preexisting SARS-CoV-2 T cell immunity has been described in naïve individuals [11], [12]. Conserved peptides, with low homology among beta-coronaviruses have been recognized before and infection with human CoVs might induce a pan-coronavirus T cell immunity [11].

Currently vaccine development mainly relies on the assumption that antibodies will be essential for protection. Protection of individuals with anti-SARS-CoV-2 antibodies has recently been described [22]. However, the minimal threshold level for protection is currently unknown. Here, we characterized the humoral immune responses of COVID-19 patients; however, the correlation of preexisting immune responses with protection is necessary for the identification of correlates of protection that will aid and accelerate vaccine development.

Acknowledgments

We thank Heike Baumann and Julia Gerbeth for excellent technical assistance.

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Figure legend

Figure 1

Distribution of clinical scores of COVID-19 patients stratified by age and gender.

The age of female (f) and male (m) COVID-19 patients is indicated for patients with mild to moderate disease (score 1-3) and patients with severe disease (score 4-7). The mean age is depicted above the columns. The p-values indicate significant differences between the samples and were calculated using the Student's *t*-test and the GraphPad Prism 7.04 software. Ns= not significant * $P \leq 0.05$; *** $P \leq 0.001$.

Figure 2

SARS-CoV-2 neutralizing activity of serum samples from COVID-19 patients.

The SARS-CoV-2 neutralizing activity of serum samples was determined with SARS-CoV-2 pseudotyped lentiviral vectors.

(A) Reciprocal area under the curve (AUC) plotted against the corresponding clinical score. The p-values indicate significant differences from 7 naïve samples and were calculated using the Student's *t*-test and the GraphPad Prism 7.04 software.

(B) SARS-CoV-2 neutralizing activity of serum samples of patients with clinical score 2 plotted against patient gender (m=male; f=female).

Ns= not significant $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$

Figure 3

SARS-CoV-2 binding antibodies.

(A) SARS-CoV-2 binding antibodies were determined by different ELISA assays. The p-values indicate significant differences in binding compared to sera from score 1 patients and were calculated using the Student's *t*-test and the GraphPad Prism 7.04 software.

(A) In-house ELISA with RBD as antigen and detection of IgG as OD values. Naïve sera had a mean OD value of 0.16.

(B) In-house ELISA with RBD as antigen and detection of IgA as OD values. Naïve sera had a mean OD value of 0.31.

(C) Liaison SARS-CoV-2 S1/S2 IgG ELISA for quantitative detection of anti-spike antibodies. Values below 15 AU are considered negative.

(D) Architect SARS-CoV-2 IgG ELISA for the detection of N directed antibodies. Values below 1.4 units are considered negative.

Ns= not significant; P > 0.05; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001; **** P ≤ 0.0001

Figure 4

Antibody titers of score 2 patient samples at different time points after a positive PCR test.

SARS-CoV-2 binding antibodies were determined by different ELISA assays. The p-values indicate significant differences in binding compared to initial samples obtained at the earliest time points and were calculated using the Student's *t*-test and the GraphPad Prism 7.04 software.

(A) Architect SARS-CoV-2 IgG ELISA (Abbott GmbH, Wiesbaden, Germany) for the detection of N directed antibodies.

(B) In-house ELISA with RBD as antigen and detection of IgG as OD values.

(C) In-house ELISA with RBD as antigen and detection of IgA as OD values.

Ns= not significant; P > 0.05; * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001; **** P ≤ 0.0001

Figure 5

HCoV-NL63-neutralizing activity of serum samples from COVID-19 patients.

The NL63-neutralizing activity of serum samples was determined with NL63-pseudotyped lentiviral vectors and the reciprocal area under the curve (AUC) was plotted against the indicated clinical scores. Significant differences were detected using the Student's *t*-test and were calculated using the GraphPad Prism 7.04 software. Ns= not significant; P > 0.05; * P ≤ 0.05; **

Figure 1

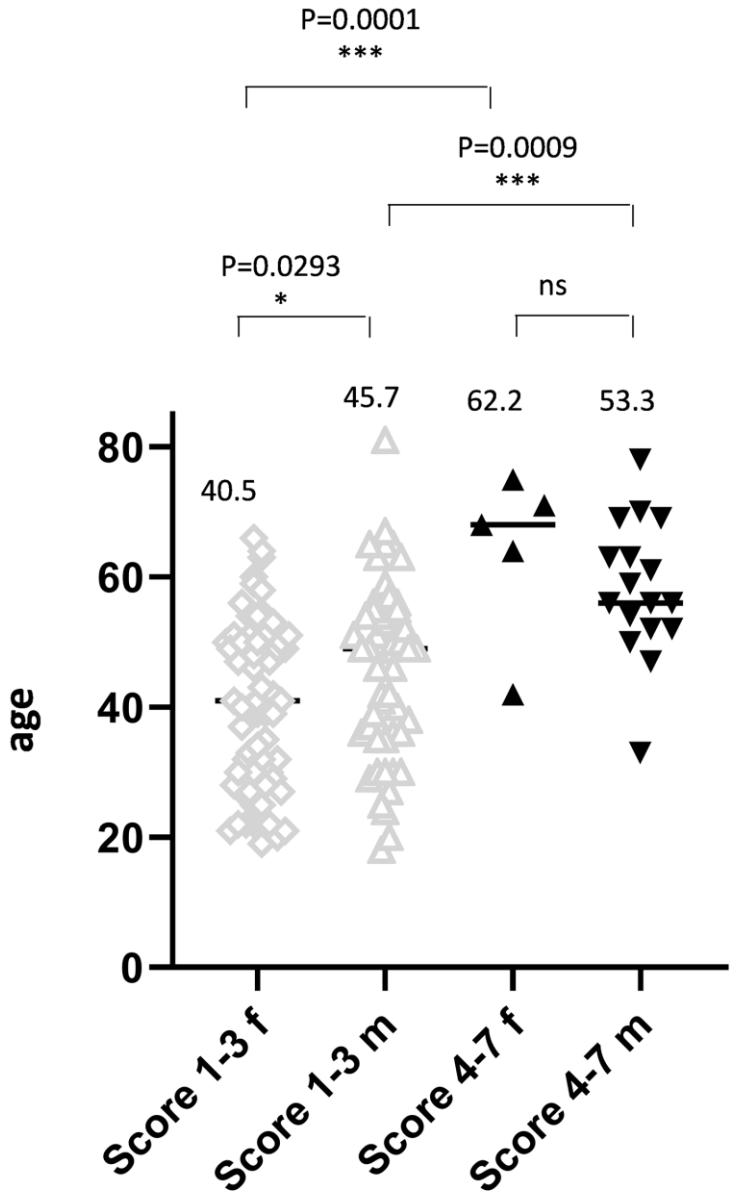
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Figure 2

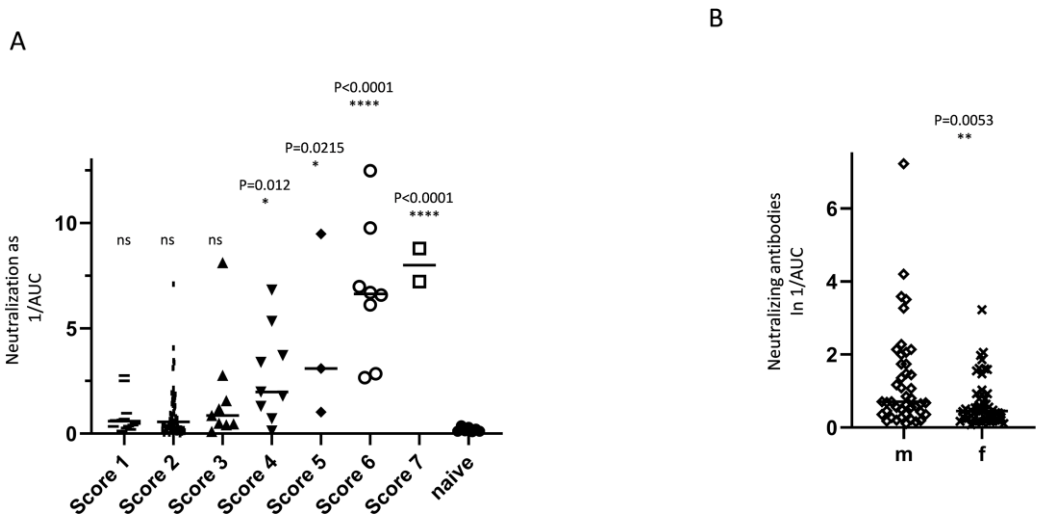
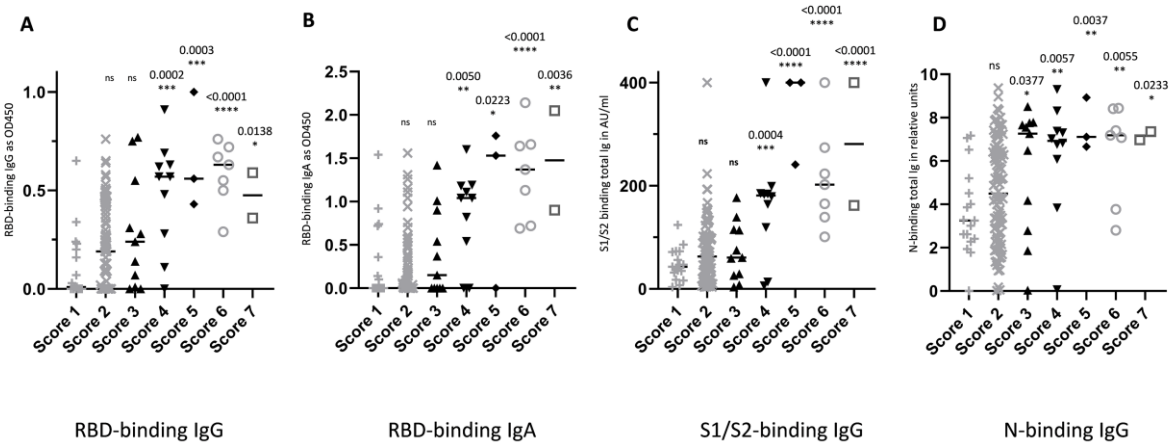
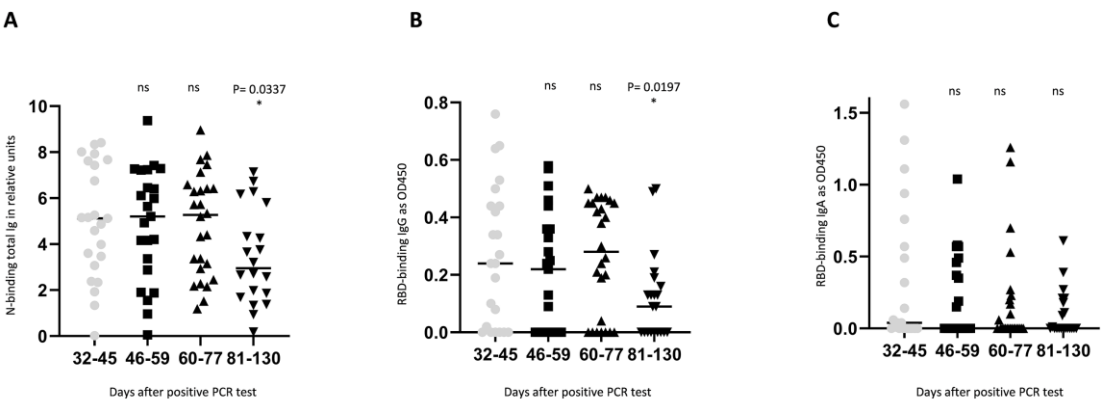


Figure 3



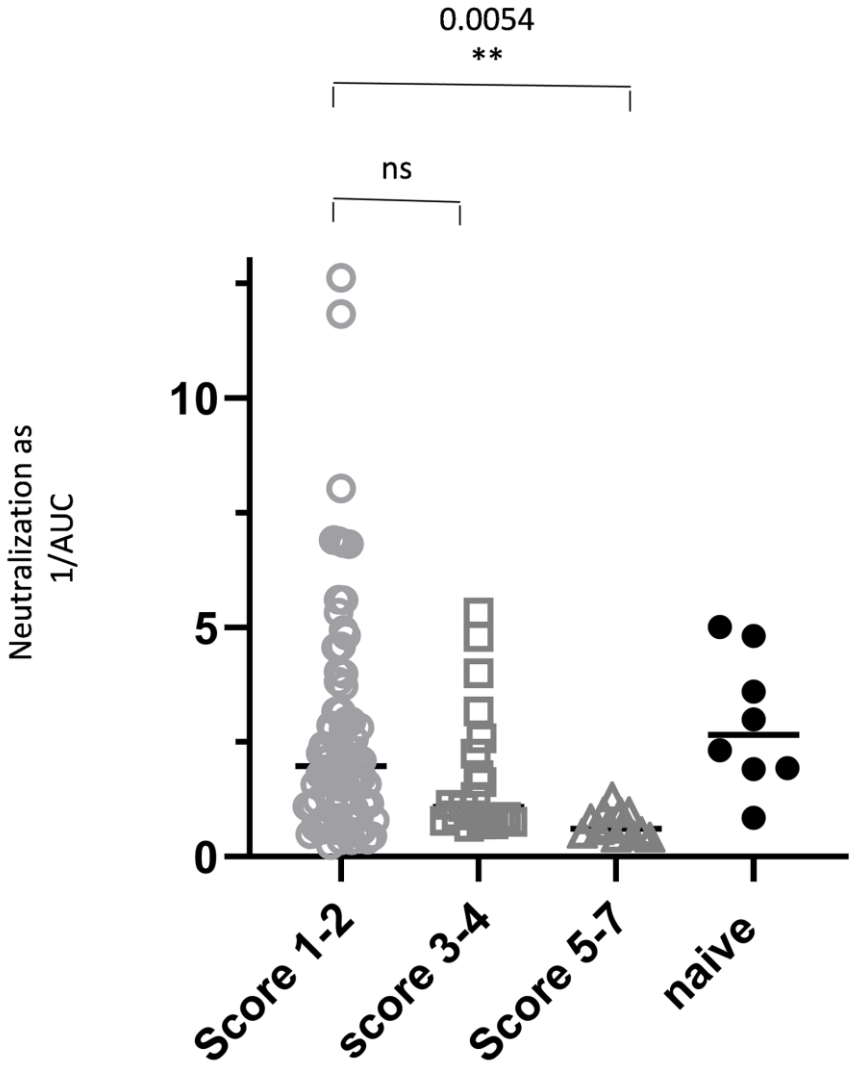
RBD-binding IgG | RBD-binding IgA | S1/S2-binding IgG | N-binding IgG

Figure 4



N-binding IgG RBD-binding IgG RBD-binding IgA

Figure 5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