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Seroprevalence of anti-SARS-CoV-2 antibodies in COVID-19 patients and healthy volunteers up to 6 months post disease onset

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SARS-CoV-2 has emerged as a human pathogen, causing clinical signs, from fever to pneumonia—COVID-19—but may remain mild or asymptomatic. To understand the continuing spread of the virus, to detect those who are and were infected, and to follow the immune response longitudinally, reliable and robust assays for SARS-CoV-2 detection and immunological monitoring are needed. We quantified IgM, IgG, and IgA antibodies recognizing the SARS-CoV-2 receptor-binding domain (RBD) or the Spike (S) protein over a period of 6 months following COVID-19 onset. We report the detailed setup to monitor the humoral immune response from over 300 COVID-19 hospital patients and healthcare workers, 2500 University staff, and 198 post-COVID-19 volunteers. Anti-SARS-CoV-2 antibody responses follow a classic pattern with a rapid increase within the first three weeks after symptoms. Although titres reduce subsequently, the ability to detect anti-SARS-CoV-2 IgG antibodies remained robust with confirmed neutralization activity for up to 6 months in a large proportion of previously virus-positive screened subjects. Our work provides detailed information for the assays used, facilitating further and longitudinal analysis of protective immunity to SARS-CoV-2. Importantly, it highlights a continued level of circulating neutralising antibodies in most people with confirmed SARS-CoV-2.

Keywords: SARS-CoV-2 · COVID-19 · neutralizing antibodies · Seroprevalence

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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[Correction added on 10 December 2020 after first online publication: the following wording of the abstract "a period of 5 months" was changed to "a period of 6 months"]

Introduction

SARS-CoV-2 infection causes a wide variety of disease symptoms, from fever, asthenia, or myalgia, to pneumonia and in most severe cases acute respiratory distress syndrome referred to as COVID-19. Yet, a large amount of SARS-CoV-2 infected patients remains asymptomatic. SARS-CoV-2 rapidly spread around the world and was declared a global pandemic in March 2020. It remains a continuing threat to health and socio-economic well-being. Despite the global number of infections reaching tens of millions, including almost one million fatalities, due to mitigation measures, the overall infection rate is relatively low with local infection hotspots. Although scientific progress is rapid, there remains a pressing need to understand the immune response that follows SARS-CoV-2 infection, including its role during disease and especially its potential long-term protective effects.

A prime immune target during coronavirus infections is the Spike (S) protein, closely associated with and targeted by neutralizing antibody responses and protective immunity, in contrast to most other viral proteins [1–4]. The S protein is responsible for the interaction of SARS-CoV-2 with the host cells via binding ACE2 [5–7]. It can be divided into two regions, S1 and S2. The extra-viral S1 region contains within its second domain the receptor-binding domain (RBD) [8]. The SARS-CoV-2 RBD sequence shows limited homology with seasonal coronaviruses or EMC/2012, the cause of the Middle East respiratory syndrome (MERS). In contrast, SARS-CoV-2 RBD shares 73% of its sequence with the RBD of SARS [3].

Attempts to curtail and control the SARS-CoV-2 virus rely on increasing inter-personal distance, including the closure of much social and economic activity, as well as testing for acute infection and personal hygiene measures. This was implemented early during the outbreak, with the University of Lisbon closing after March 13th, 10 days after the first recorded cases in Portugal. However, during the subsequent transition phase, restrictions have steadily been lifted. The gradual return to social and economic activity requires active surveillance to determine local outbreaks, contact tracing, and quarantine. In addition, those most vulnerable to COVID-19 will need to remain under enhanced protection. Important information is how protective immunity develops in the population at large and in specific groups such as healthcare professionals. A thorough assessment of the duration of protective immunity is critical to determine the measures that need to be taken to prevent and handle future waves of SARS-CoV-2. Such information will need to be gathered widely, in different locations around the world, reflecting local conditions, such as containment measures and their timing. The data obtained will need to be accurate and the methods used transparent and reproducible to enable comparisons between locations and countries. The recent SARS-CoV-2 outbreak brings limitations with respect to exposure time but also gives us the opportunity to acquire real-time data and develop reliable longitudinal follow-up studies.

To determine the cumulative rate of infection in communities and gaining insight into the potential protection against re-infection, serological assays are critical. Depending on the aims of the study, the setup of such assays can be used for the detection of exposure to SARS-CoV-2 as well as gaining insights into neutralization activity, since antibody titers for both the S protein and RBD have been shown to correlate well with neutralizing activity [3, 9–11]. We describe the detailed setup and versatility of a seroconversion assay to determine humoral immunity to SARS-CoV-2 that was used for screening hospital patients, healthy post-COVID-19 volunteers, and staff of the University of Lisbon. We report that in the acute phase men produce more antibodies than women do, but levels equilibrate during the resolution phase and are similar between the sexes in the months after SARS-CoV-2 infection. We show that antibodies against SARS-CoV-2 Spike and its RBD domain are readily detectable in the majority of cases, including in patients receiving immune suppressive or anti-retroviral therapy. In line with a classic immune response, SARS-CoV-2 antibodies in the blood peak around week 3 post-infection, and although antibody titers reduce, IgG antibodies remain detectable and show virus neutralization activity for at least 6 months post-SARS-CoV-2 infection.

Results

Seroconversion assay setup

The gold standard for antibody detection remains the ELISA, offering high flexibility and sensitivity, but limited scalability [11]. SARS-CoV-2 Spike is a prominent immunogenic antigen and its RBD is least conserved compared with other coronaviruses. Hence, the use of Spike and its RBD quickly became the focus of seroconversion assays. We chose for the present study the assay developed by Florian Krammer and his laboratory, a format that received FDA emergency approval in April 2020 and is described in detail [12].

Human sera pose a biological hazard to laboratory workers and can potentially contain not only SARS-CoV-2, but also other infectious viruses. Therefore, all ELISA steps were performed at biosafety level (BSL) 2, with BSL3 personal protective equipment. Inactivation procedures are recommended but can have uncertain effects on the accuracy of serological testing [13]. We tested three common procedures: (a) 1 h heat inactivation at 56°C, or (b) the addition of a non-ionic surfactant (0.1% Triton X-100), or (c) the combination of both, in comparison to neat serum. Serial dilutions of two chosen SARS-CoV-2 PCR-positive serum samples showed IgG detection following all three inactivation methods and were indiscriminate from untreated controls (Fig. 1A).

The S protein has a trimeric structure, while the in vitro expression of RBD results in the generation of monomeric and dimeric protein. However, when we tested the ability of RBD mono- and dimeric protein for antibody binding, both performed similar and comparable to the total protein fraction (Figure 1B). Additional parameters affecting the performance of ELISA assays, such as the coating time (o/n – 1 week at 4°C), serum incubation time and temperature, as well as the amount of tetramethylbenzidine (TMB) substrate and the development time (adjusted

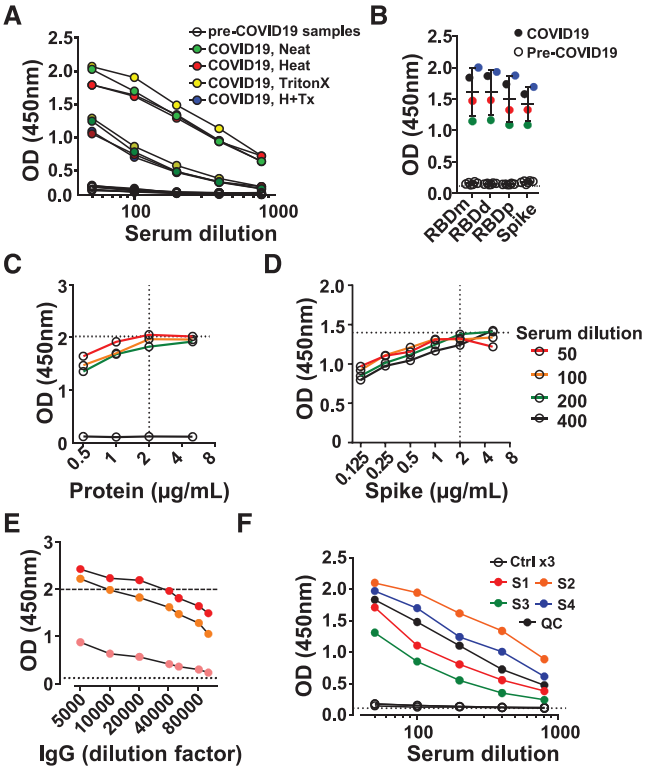

Figure 1. SARS-CoV-2 ELISA setup. SARS-CoV-2 IgG antibody detection in serum samples from SARS-CoV-2 PCR-positive subjects or pre-COVID-19 controls using Immulon 4HBX 96-well plates coated with RBD protein for ELISA. Absorbance (optical density, OD) was evaluated at 450 nm. (A) Three serum samples, pre-COVID-19 control, medium, and high titer were treated by the indicated methods to inactivate virus particles. (B) Isolated RBD monomer, RBD dimer, pooled RBD and Spike protein were used for coating at the same conditions, and four COVID-19 serum samples (colored) were tested versus four pre-COVID-19 (open symbols). (C and D) 96-well plate was coated with (C) RBD or (D) Spike protein at indicated concentrations and three COVID-19 (colored) and pre-COVID-19 (black) sera were tested. (E) Secondary antibody dilution titration anti-IgG, at indicated dilution on 96-well plate coated with 2 μg/ml RBD protein for three sera. (F) Quality control (QC) and sample serum serial dilution. QC sample is a mix of the serum samples tested from four healthcare workers (S1–S4). Dashed line indicates blank values. Data show individual sample values.

to 10 min) were optimized (Supporting Information Fig. S1A, data not shown). Coated plates were stable for a week and incubation of 1 or 2 h at room temperature or 37°C was indistinguishable. To ensure that the ELISAs runs at non-saturating conditions, we performed a full titration of the capture antigens (from 0.125 to 10 μg/mL) and the secondary antibodies (1:5000–1:100 000) used for antibody detection. SARS-CoV-2 proteins were titrated and assessed using individual patient sera (high, medium, and low titers). Simultaneously, the secondary antibodies were titrated, such as anti-IgG, but also anti-IgM, anti-IgA, and anti-total Ig, whereby OD of 2.0 was used as an upper limit to avoid saturation of the assay (Fig. 1C–E; Supporting Information Fig. S1B–G).

In order to prepare for diagnostic use, the generation of quality control (QC) serum is critical to validate each assay run. Sera from exposed patients is most desirable as it will contain antibodies with a range of avidities and isotypes, providing more stable binding properties. However, sufficient volume needs to be obtained to ensure that there is enough material to complete the ELISA validation process and the study or series of studies to be undertaken. Sera from four SARS-CoV-2 exposed but healthy volunteers were assessed and pooled to serve as quality control for subsequent assays (Fig. 1F). Ultimately, antibody signals should diminish in a dose-dependent way using serial dilutions of the sera, enabling the accurate determination of antibody titers (Supporting Information Fig. S1H).

Seroconversion assay validation

We used 100 pre-COVID-19 sera from healthy volunteers collected between October 2012 and November 2017 as negative controls (Table 1). Furthermore, we obtained 19 sera from PCR positive hospital healthcare workers with mainly mild symptoms, just over 30 days since the first symptoms and the positive SARS-CoV-2 PCR result (Table 2). Seroconversion was detected in the sera of all SARS-CoV-2 PCR-positive patients using the RBD part of SARS-CoV-2 S antigen and 18 of 19 using the full-length SARS-CoV-2 S protein by probing for IgG (Figure 2A). Receiver operating characteristic (ROC) curve determined sensitivity and specificity and the assay´s cutoff at 0.4171 and 0.4816 for RBD and S protein, respectively, corresponding to 100% specificity and 99% sensitivity for RBD and 94.74% specificity and 98% sensitivity for Spike in this initial analysis (Fig. 2B and C and Table 3).

In order to increase pre-COVID-19 sample size and reflect a broader spectrum of the population, we obtained 61 samples of individuals with food allergies and 20 samples from individuals with bee and wasp allergies, because these contain increased levels of antibodies [14]. Serum from allergic subjects increased the observed background on both RBD and Spike proteins (Figure 2D). Of importance, increased reactivity to one protein was often not observed on the second SARS-CoV-2 protein (Figure 2E), substantiating the two-step process of screening for RBD and subsequently, those sera found positive for Spike [12].

Seroconversion screening of COVID-19 hospitalized patients

We subsequently analyzed 307 samples from hospitalized patients who tested positive for SARS-CoV-2 by PCR for the presence of anti-SARS-CoV-2 IgG. Samples were acquired between April 6 and August 12, 2020 and at different times after the development of COVID-19 symptoms. The patients demonstrated a variety of symptoms and underlying medical conditions (Table 1). Seroconversion screening resulted in varied OD measurements when including all samples assessed (Fig. 3A). Taking into account the number of days taken by adaptive cellular immunity to be initiated, we further separated the samples into 14 days post-onset, excluding those that were asymptomatic, which revealed a robust seroconversion in 73 of 73 (100%) samples on RBD and 71 of 73

Table 1. Demographics of patient participants, disease severity categories and symptoms, underlying conditions, and medication

Clinical data (%, *n*)	COVID-19		COVID-19 (<Day7)		Pre-COVID-19		Pre-COVID-19 Allergies		Asymptomatic	
Age (years)	63.23	(20-93)	68.77	(20-98)	54.73	(20-88)	30.59	(2-71)	62.85	(22-98)
Male	48%	91	54%	42	49%	49	51%	41	55%	22
Female	52%	98	46%	36	51%	51	49%	40	45%	18
Sample collection date	6/4/2020 - 12/8/2020		6/4/2020 - 12/8/2020		31/10/2012 - 10/11/2017		03/01/2019 - 29/10/2019		6/4/2020 - 12/8/2020	
SARS-CoV-2 PCR+	100%		100%		N/A		N/A		100%	
Seroconversion (Spike)	82%	(155/189)	50%	(39/78)	2.0%	(2/100)	6.2%	(5/81)	50%	(20/40)
Seroconversion (RBD)	84%	(159/189)	51%	(40/78)	0.0%	(0/100)	7.4%	(6/81)	50%	(20/40)
Disease Severity										
Mild	18%	31	16%	13	N/A		N/A		0%	
Moderate	73%	127	73%	60	N/A		N/A		0%	
Severe	10%	17	11%	9	N/A		N/A		0%	
Symptoms										
Cough	60%	105	61%	50	N/A		N/A		N/A	
Myalgia	30%	53	18%	15	N/A		N/A		N/A	
Fever	60%	105	59%	48	N/A		N/A		N/A	
Anosmia	9%	15	2%	2	N/A		N/A		N/A	
Dyspnea	35%	62	49%	40	N/A		N/A		N/A	
Diarrhea	13%	23	12%	10	N/A		N/A		N/A	
Odynophagia	1%	1	0%	0	N/A		N/A		N/A	
Hypogeusia	3%	6	0%	0	N/A		N/A		N/A	
Headache	19%	33	10%	8	N/A		N/A		N/A	
Rhinorrhea	1%	1	0%	0	N/A		N/A		N/A	
Asthenia	33%	57	27%	0	N/A		N/A		N/A	
Days Post Symptom onset at collection	15.53	(3-52)	3.88	(1-7)	N/A		N/A		N/A	
PCR+ day to serum collection	12.07	(1-63)	4.04	(0-15)	N/A		N/A		10.74	(-1-53)
Underlying conditions										
None	21.71%	38	6.10%	5	N/A		N/A		22.22%	6
Chronic kidney disease	10.86%	19	17.07%	14	N/A		N/A		40.74%	11
Diabetes	25.71%	45	25.61%	21	N/A		N/A		33.33%	9
Hypertension	50.29%	88	57.32%	47	N/A		N/A		77.78%	21
Heart disease	6.29%	11	17.07%	14	N/A		N/A		7.41%	2
Alzheimer	0.00%	0	1.22%	1	N/A		N/A		0.00%	0
Cerebrovascular disease	3.43%	6	7.32%	6	N/A		N/A		0.00%	0
Dementia/Parkinson	14.29%	25	21.95%	18	N/A		N/A		11.11%	3
Asthma	1.14%	2	4.88%	4	N/A		N/A		0.00%	0
Cancer	10.29%	18	7.32%	6	N/A		N/A		40.74%	11
COPD	2.86%	5	4.88%	4	N/A		N/A		0.00%	0
HIV	2.86%	5	4.88%	4	N/A		N/A		11.11%	3
SLE	1.14%	2	0.00%	0	N/A		N/A		0.00%	0
Medication										
None	89.71%	157	85.37%	70	N/A		NA		55.56%	15
Midostaurin	0.57%	1	0.00%	0	N/A		NA		0.00%	0
Aciclovir	0.57%	1	0.00%	0	N/A		NA		0.00%	0
Tacrolimus	2.29%	4	2.44%	2	N/A		NA		0.00%	0
Prednisolone	4.57%	8	6.10%	5	N/A		NA		0.00%	0
Chemotherapy (Paclitaxel)	0.00%	0	0.00%	0	N/A		NA		7.41%	2
Methotrexate	0.00%	0	1.22%	1	N/A		NA		0.00%	0
Raltegravir	0.57%	1	1.22%	1	N/A		NA		3.70%	1
Darunavir/Cobicistat	0.57%	1	1.22%	1	N/A		NA		3.70%	1
Exemestane	1.14%	2	0.00%	0	N/A		NA		0.00%	0

(*Continued*)

Table 1. Continued

Clinical data (%, n)	COVID-19		COVID-19 (<Day7)		Pre-COVID-19	Pre-COVID-19 Allergies	Asymptomatic	
Azathioprine	1.14%	2	1.22%	1	N/A	N/A	0.00%	0
Dexamethasone	0.00%	0	0.00%	0	N/A	N/A	3.70%	1
Tamoxifen	0.57%	1	1.22%	1	N/A	N/A	0.00%	0
Abacavir	0.57%	1	1.22%	1	N/A	N/A	7.41%	2
Lamivudina	0.57%	1	1.22%	1	N/A	N/A	7.41%	2
Micofenolate	1.14%	2	1.22%	1	N/A	N/A	0.00%	0
Entecavir	0.57%	1	0.00%	0	N/A	N/A	0.00%	0
Dolutegravir	0.57%	1	0.00%	0	N/A	N/A	7.41%	2

(97.3%) on Spike protein (Fig. 3B). Although antibody responses take time to mature, around half of the samples obtained in the first-week post-symptoms showed robust IgG seroconversion within the first week of symptoms, 40 of 78 (51%) and 39 of 78 (50%) on RBD and Spike, respectively (Fig. 3C). Follow up samples from 68 patients showed that those who had seroconverted in the first week of symptoms maintained high levels of IgG 1 week later (27/27, 100%). From all symptomatic subjects, those who did not have an IgG response within the first week, 30 of 41 (73%) showed a robust response seven days later (days 9-14). The remaining 11 of 41 (27%) patients had their second sample analyzed in the second week, pre-day 14 after onset of symptoms, still did not show an IgG response. However, although some patients did not show an IgG response in the second or even third week after onset of symptoms, those that were tested again 1 week later all seroconverted (10/10) (Fig. 3D). From those who did not seroconvert within week 2 of COVID-19 symptoms (11/41, 27%), some had underlying conditions, such as systemic lupus erythematosus (SLE) (1), lymphoma (1), chemotherapy (1), or immunosuppressive medication (3).

Table 2. Demographics of healthcare participants, disease severity categories, and symptoms

Clinical data (%, n)	Healthcare workers	
Age (years)	41.84	(25-61)
Male (%)	16%	3
Female (%)	84%	16
Sample collection date	6/4/2020 - 27/5/2020	
SARS-CoV-2 PCR+	100%	
Seroconversion (Spike)	94.70%	(18/19)
Seroconversion (RBD)	100%	(19/19)
Disease Severity		
Asymptomatic	5%	1
Mild	84%	16
Moderate	11%	2
Severe	0%	0
Symptoms		
Cough	74%	14
Myalgia	79%	15
Fever	68%	13
Anosmia	37%	7
Dyspnea	11%	2
Diarrhea	16%	3
Odynophagia	5%	1
Hypogeusia	21%	4
Headache	63%	12
Rhinorrhea	5%	1
Asthenia	32%	6
Days Post Symptom onset at collection	34.5	(3-55)
PCR+ day to serum collection	33.05	(13-63)

Some patients who were SARS-CoV-2 PCR positive within an average of 8.7 days (-1 - 53) after blood was taken, did not show any classic COVID-19 symptoms (Table 1). Of these, 20 of 40 (50%) patients showed seroconversion for anti-SARS-CoV-2 IgG (Figure 3E). Since no symptoms were reported, it remained unclear at what stage of the infection these patients were. Those who were IgG negative may have been within the first days of infection, or antibody levels were very low. However, repeated sampling from several patients seven days later revealed that only 3/11 (27%) patients seroconverted, although OD remained modest, while in six of 11 (55%) anti-SARS-CoV-2 RBD IgG levels remained below the assay´s cutoff (Figure 3F). This suggests a limited or much-delayed seroconversion response.

Effect of demographics, immunomodulatory, and antiviral medication on seroconversion

IgG seroconversion, 14 days after onset of symptoms, was detected equally well between female and male patients, independently of age, and for both RBD and Spike protein (Fig. 4A and B). Seroconversion detection or the antibody response, since patients were assayed on average 8.5 days after being SARS-CoV-2 PCR positive, were reduced or delayed in those asymptomatic for COVID-19 compared with those experiencing COVID-19 symptoms (Fig. 4C). In line with an adaptive immune response taking several days to develop, the main factor influencing seroconversion was time since the onset of COVID-19 symptoms (Figure 4D).

Within the hospital patient cohort, two groups were of special interest, those on immunosuppressive therapy and those receiving antiviral medication due to infections with either HIV or hepatitis B virus (HBV) (Table 4). Within the patient cohort receiving immunosuppressive medication, seven of 29 (24%)

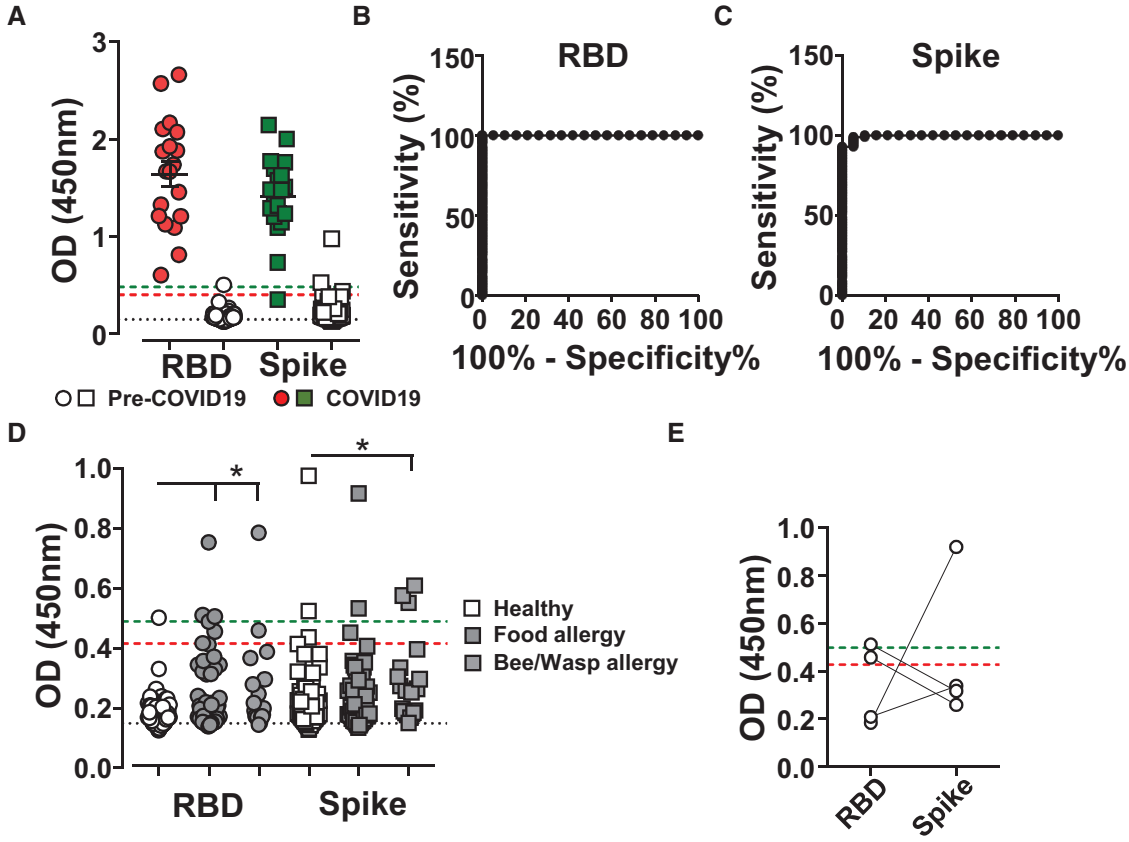

Figure 2. SARS-CoV-2 ELISA testing. SARS-CoV-2 IgG antibody detection in serum samples from SARS-CoV-2 PCR-positive subjects (colored) or pre-COVID-19 controls (open) using Immulon 4HBX 96-well plates coated with RBD (circles) or Spike (squared) proteins for ELISA. Absorbance was evaluated at 450 nm. (A) Serum at 1/50 dilution from 19 SARS-CoV-2 PCR-positive healthcare workers were assessed for anti-SARS-CoV-2 RBD and Spike IgG and compared with 100 pre-COVID-19 sera. Bars indicate mean ± SD. (B and C) ROC analysis, plotting sensitivity against the specificity of (B) RBD or (C) Spike samples as shown in (A). D) Serum at 1/50 dilution from pre-COVID-19 cohorts, healthy (100 donors, open symbols), food allergies (61 donors, dark grey symbols), and bee/wasp allergies (20 donors, light grey symbols), were tested for RBD and Spike protein reactivity. (E) Example of cross-reactivity on RBD or Spike protein from pre-COVID-19 serum as used in (D). Statistical analysis was performed using Kruskal–Wallis test with Graphpad Prism software. $^{*}p < 0.05$, $^{***}p < 0.001$. Dashed lines indicate Black, blank values; Red, RBD cutoff; Green, Spike cutoff. Data show individual sample values.

were asymptomatic for COVID-19. The average seroconversion rate in both groups was below those seen in the collective patient cohort or the healthcare workers, at 9/29 (31%) (Table 4).

Within the hospital cohort, we obtained 29 sera from patients who received immunosuppressive drugs, including 13 of 29 (40%) receiving the glucocorticoids Prednisolone or Dexamethasone. Sera of these patients were collected between days 2 and 35 post-COVID-19 symptom onset. In this cohort, all patients tested SARS-CoV-2 positive by PCR, 20 of 29 (69%) did not seroconvert (Fig. 4E). Out of these 20, seven (35%) were asymptomatic, and three (15%) were tested within 7 days of symptoms. In line with required T cell help for isotype switching, three of five (67%) patients on the calcineurin inhibitor Tacrolimus, which was combined with Prednisolone did not seroconvert. The use of corticosteroids had an inhibitory effect on antibody production, with those patients that seroconverted showing a low signal. The patient on chemotherapy (Paclitaxel) did not show an anti-SARS-CoV-2 RBD IgG response. Three (3/29, 10%) patients receiving

Table 3. ROC analysis of RBD or Spike protein after the initial screening of pre-COVID-19 serum and sera from healthcare workers who were SARS-COV-2 PCR-positive

RBD					Spike				
	Sensitivity		Specificity			Sensitivity		Specificity	
Cut off	%	CI (%)	%	CI (%)	Cut off	%	CI (%)	%	CI (%)
0.4171	99.00	94.55- 99.97	100.00	82.35- 100.00	0.4816	98.00	92.96- 99.76	94.74	73.97- 99.87
0.5532	100.00	96.38- 100.00	100.00	82.35- 100.00	0.6302	99.00	99.55- 99.97	94.74	73.97- 99.87

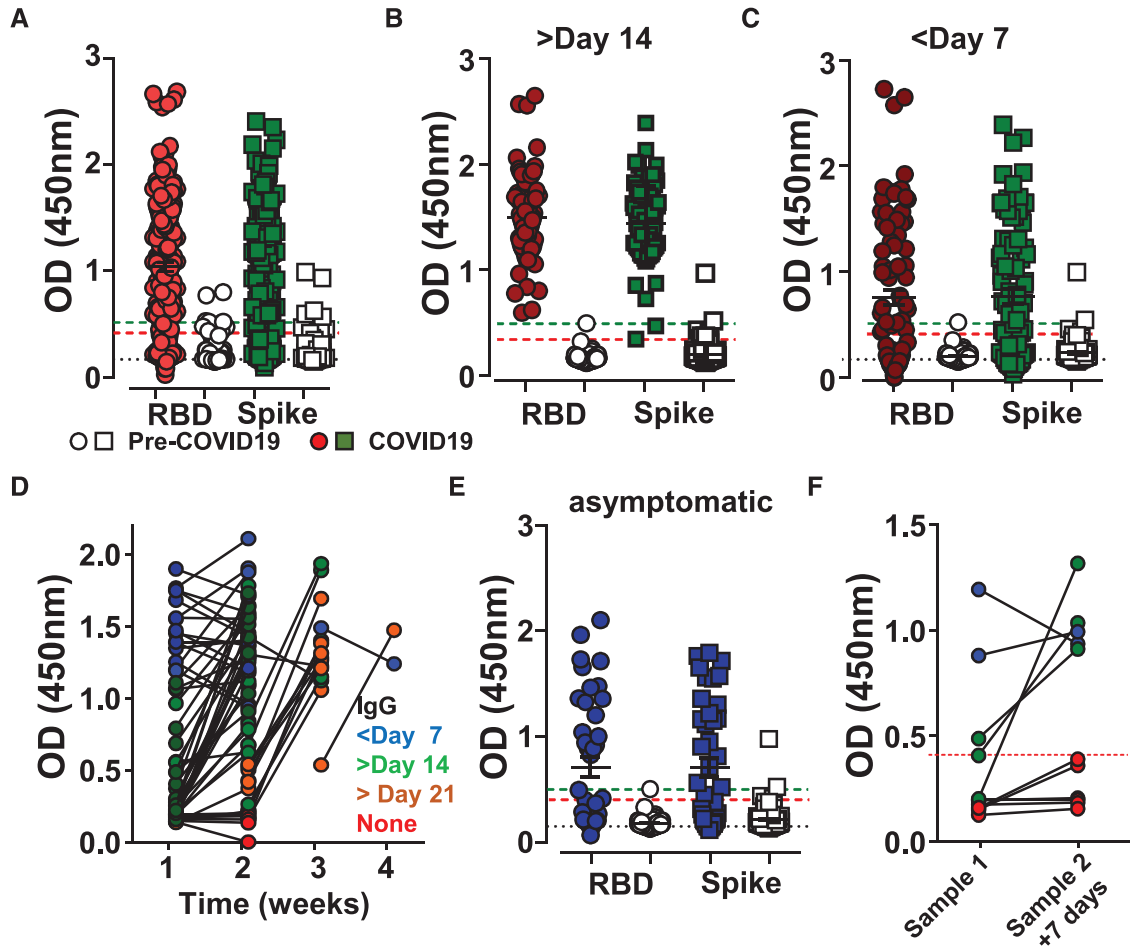

Figure 3. Seroconversion in hospitalized patients. SARS-CoV-2 IgG antibody detection in serum samples from SARS-CoV-2 PCR-positive hospital patients (colored) or pre-COVID-19 controls (open) using Immulon 4HBX 96-well plates coated with RBD (circles) or Spike (squared) proteins for ELISA. Absorbance was evaluated at 450 nm. (A) Overview of all over 300 SARS-CoV-2 PCR-positive tested subjects from Hospital Santa Maria and accumulated 181 pre-COVID-19 controls. (B) Selected samples presented in (A) post-day 14 of the initial reported COVID-19 symptoms ($n = 73$ COVID-19 donors, $n = 100$ pre-COVID-19 healthy controls). (C) Selected samples presented in (A) pre-day 7 of the initial reported COVID-19 symptoms ($n = 78$ COVID-19 donors, $n = 100$ pre-COVID-19 healthy controls). (D) Longitudinal follow-up of patients sampled at the indicated week of COVID-19 symptom onset and re-sampling 7 days later ($n = 76$ COVID-19 donors). Blue indicates continued high signal, Green those that seroconverted at the second sampling in week 2, Orange those that seroconverted at the second sampling past week 2, Red those in which no seroconversion was detected in first and second sampling. (E) Selected samples presented in (A) without reported COVID-19 symptoms ($n = 40$ COVID-19 donors, $n = 100$ pre-COVID-19 healthy controls). (F) Longitudinal follow-up of asymptomatic patients sampled in the first week of COVID-19 symptom onset and re-sampling 7 days later, colors as used as in (D) ($n = 10$ donors). Dashed lines indicate Black, blank values; Red, RBD cutoff; Green, Spike cutoff. Data show individual sample values, bars indicate mean ± SD.

immunomodulatory drugs showed a similar anti-SARS-CoV-2 IgG response compared with patients without such medication (days 5 to 13). Two of these received either Prednisolone or Methotrexate. Although the number of patients tested was modest, these findings indicate that immunosuppressive medications inhibit seroconversion upon SARS-CoV-2 infection.

Twelve samples were from patients who previously contracted HIV1 or HIV2 and were treated with antiretroviral therapy (Raltegravir, Darunavir/Cobicistat, Lamivudine, and Dolutegravir) and one with HBV receiving Entecavir. All patients were male and SARS-CoV-2 seroconversion was analyzed within 11 days of symptom onset. Most patients (9/12, 75%) seroconverted (Fig. 4F). Of the remaining three HIV patients, two were asymptomatic and one was an early sample taken only 3 days after COVID-19 symptoms. These results suggest that anti-retroviral medication does not interfere with SARS-CoV-2 seroconversion.

Large seroconversion screen shows limited SARS-CoV-2 exposure

The University of Lisbon decided to close for all non-essential work early during the COVID-19 outbreak, starting from midnight March 13. Since the initial outbreak, reported infection levels in Portugal have remained modest compared with nearby European countries, with 5200 cases per million of the population reported (Johns Hopkins, Worldometer, August 2020). To determine the seroprevalence in University staff, we screened 2571

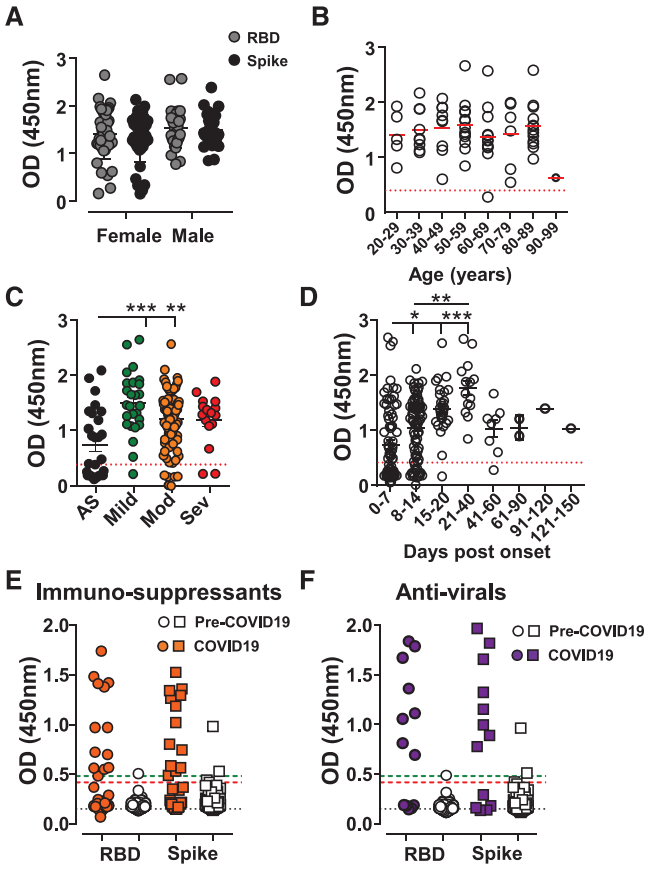

Figure 4. Seroconversion in subgroups and time. SARS-CoV-2 IgG antibody detection in serum samples from SARS-CoV-2 PCR-positive hospital patients or pre-COVID-19 controls using Immulon 4HBX 96-well plates coated with RBD (circles) or Spike (squared) proteins for ELISA. Absorbance was evaluated at 450 nm. (A and B) IgG OD signals were plotted of (A) female ($n = 42$) and male ($n = 28$) or (B) by age at the time of blood sampling, of those subjects 14-days post first COVID-19 symptoms. Red line marks the mean. (C and D) IgG OD signals of all subjects were plotted by (C) severity of symptoms or (D) over time since the day of first symptoms. (E and F) Hospital patients receiving (E) immunomodulatory medication (orange, $n = 31$) or (F) antiviral medication (purple, $n = 12$) who tested SARS-CoV-2 PCR-positive were assessed for IgG antibodies and compared with pre-COVID-19 controls (open symbols, $n = 100$). Statistical analysis was performed using Kruskal–Wallis test using Graphpad Prism software. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data show individual sample values, bars indicate mean ± SD.

employees, across all divisions, with serum obtained between May 13 and July 10, 2020. As mentioned, our assay is based on Stadlbauer et al. [12] that received emergency FDA approval utilizing a two-step method. With the expectation of a low infection prevalence, we first screened staff in a single dilution using the setup as depicted in Figure 5A. We detected 68 samples with an OD above the cut off value of 0.41 (2.6%) (Fig. 5B). These samples were subsequently reassessed, using both RBD and Spike protein as well as two serum dilutions, 1/50 and 1/150, as depicted in Figure 5C. Of the 68 tested samples, 38 (56%) were confirmed positive during the second assay (Fig. 5D and E), resulting in an infection prevalence of 1.5%. Samples with an intermediate signal for RBD, often just above the cutoff, frequently failed the second assay on Spike or even RBD (Fig. 5E and F) and some samples with a robust RBD signal did not respond to Spike protein at all. As previously observed (Fig. 2), signals between RBD and Spike protein were often comparable, with only a few samples showing a stronger response against Spike. Samples providing a robust signal for RBD often responded similarly with Spike (Fig. 5G). To ensure ODs between plates are comparable, the assays inter-plate variation was determined using two dilutions of a QC serum sample or monoclonal antibody (QC_{hi} and QC_{lo}) run in each plate. Although day-to-day plate variability is present, this is of very modest amplitude (Fig. 5H). The average and SD of QC_{hi} OD values for the first 12 plates performed were calculated and taken into consideration to validate the following diagnostic plates. The same was done for QC_{lo} values.

Antibody titers follow a classic immune response pattern

To determine antibody responses accurately, we performed serum titrations using RBD protein to assay the anti-SARS-CoV-2 IgM, IgG, and IgA responses. In agreement with earlier results, not all subjects assessed early (pre-day 7) after the onset of COVID-19 or those asymptomatic show seroconversion, but anti-RBD antibodies rise swiftly during the first days of infection. This is the case for all three isotypes assessed. As with many reported antibody responses, including SARS [15], the anti-SARS-CoV-2 response follows a classic pattern with high antibody responses at the start of the immune response (Supporting Information Fig. S3A–C).

In addition to early responses from healthcare workers and hospitalized patients, we analyzed 209 potential plasma donors for convalescent plasma therapy via the Portuguese Blood and Transplantation Institute (IPST). The volunteers were predominantly male (67%) and on average 38 years old. All were reported to be SARS-CoV-2 PCR positive, on average 107 days prior to serum sample collection (Table 5). COVID-19 symptoms varied from asymptomatic to mild and moderate. At the time of collection, 184/209 (88%) of the potential plasma donors had readily detectable IgG anti-SARS-CoV-2 RBD antibodies. In line with a characteristic immune response, antibody titers assessed in volunteers were reduced compared with titers found during the early immune response in COVID19 patients and healthcare workers, especially IgM and IgA (Supporting Information Fig. S3D–F).

Analysis of the IgM, total IgG, and IgA responses confirmed a rapid and near-simultaneous response of the three isotypes tested during the first weeks of SARS-CoV-2 infection (Fig. 6A–C). Antibody responses peaked around three weeks after the first symptoms, after which the circulating antibody levels were reduced. IgM, IgG, and IgA peaked at days 15-21 with geometric antibody titer means of 1915, 10695, and 5212, respectively. From the second month after disease onset IgG and IgA antibody levels remained readily detectable in most people up to 6 months after first symptoms (Fig. 6A–C; Supporting Information Fig. S3G). Characteristically for antibody response, IgM titers were low (≤1/200) in 130 of 182 (72%) of IgG-positive potential

plasma donors (Fig. 6A–C). Geometric means of IgM, IgG, and IgA titers at days 91–120 were 96, 533, and 141, respectively. Early anti-SARS-CoV-2 RBD antibody levels (day 40) were higher in men, with significantly higher titers for all three antibody isotypes, but at late time points (Day 40-150) no differences between men and women were observed (Figure 6D-I). The increased antibody level observed in men was not explained by the severity of COVID-19, with the overall increase in antibodies observed independently of disease symptoms (Supporting Information Fig. S3H). Furthermore, stratifying subjects within the first 40 days after COVID-19 by the severity of symptoms highlighted that increased severity correlates well with increased antibody titers at early stages (Fig. 6J–L).

Since titers 2 months after COVID-19 reduced, we next determined the antibodies' potential to neutralize SARS-CoV-2. We found neutralization activity in all tested sera in which anti-SARS-CoV-2 IgG was determined, across 2–5 months after initial SARS-CoV-2 PCR-positive testing (Fig. 6M). The level of SARS-CoV-2 neutralization activity was found to be proportional to the anti-SARS-CoV-2 RBD IgG titer determined, but not IgM and only a trend for IgA (Fig. 6N; Supporting Information Fig. S3I–J). Collectively the data highlight that a sustained level of antibodies circulate in the blood for at least 6 months after COVID-19, which shows SARS-CoV-2 neutralizing activity in line with the level of anti-SARS-CoV-2 RBD IgG titers.

Discussion

For many pathogens and vaccines, antibody titers have been established over the past decades, accumulating detailed knowledge of average antibody responses. However, every microorganism is different, and level, neutralization activity, and longevity of antibody responses can be different between pathogens, vaccines as well as between individuals. Therefore, it remains important to acquire data for each novel infection, especially those posing a threat to human health such as SARS-CoV-2. In addition, initial antibody levels after vaccination, generally correlate well with a significantly reduced risk of (re-)infection and pathology. Thus, the global collection of data from many different geographic locations, patient cohorts, and under different local conditions will contribute to a holistic understanding of the new pandemic.

We explain the setup of an ELISA system in detail, as described previously [9], to facilitate implementation in other places and comparison with published results. Although the Spike protein used in the assay is not the only immune-reactive SARS-CoV-2 protein, it was selected because provides additional correlative insights with respect to potential neutralizing antibodies present [3, 4, 16]. The assay was set up using samples with high, medium, and low titers, and an OD of 2.0 was selected to avoid saturating signals. Although serum samples for the assay setup were determined with the initial assays prior to full optimization, this did not affect their subsequent use.

We show that neither frequently used methods of viral inactivation nor the complexity of RBD, monomeric, dimeric, or a mixture of both affects antibody determination. Although the reported presence of SARS-CoV-2 is limited in blood [17], the inactivation of serum contributes to reduced risk handling human material. Using the whole RBD fraction from mammalian expression systems will reduce costs due to its superior yield. The higher levels of expression using the relatively small RBD, compared with the full-length Spike protein, makes the use of RBD more economical. We show that individual samples can show differences in signal for either RBD or Spike and that some limited cross-reactivity is observed with both proteins. However, there is no disadvantage using RBD compared with the full-length Spike protein, resulting in high specificity and sensitivity. Care should be taken when using serum samples from patients with increased antibody levels. These can be observed in allergies and autoimmune conditions, which may increase the background signal compared with otherwise healthy controls. Most subjects who encountered SARS-CoV-2 seroconverted, although some showed delayed kinetics. Only a few patients did not show an IgG response that was not explained by early sampling (<day 7) or an underlying condition that required the use of immunosuppressive drugs. Most of the non-responders were asymptomatic, which may point to a very modest immune response upon encountering a low viral load, or, since SARS-CoV-2 PCRs also generate false-positives, these participants may not have been infected.

Within the patient cohort, there were additional groups of interest. With SARS-CoV-2 being a positive-stranded RNA virus, the use of anti-retroviral medication had no major effect on antibody responses against the virus. The mode of action of these drugs is not known to interfere with RNA viruses or antibody production. Our data show the successful management of the eleven patients previously infected with HIV, with all of those patients showing a robust SARS-CoV-2 antibody response after the second week of COVID-19 symptoms, in line with previous works [18, 19]. The use of immunomodulatory drugs had an inhibitory effect on SARS-CoV-2 seroconversion. This is in line with the action mechanism of these drugs, inhibiting the activation or production of lymphocytes. Although this will inhibit the clearance of SARS-CoV-2, the use of these immune-inhibitory drugs such as Dexamethasone could be beneficial in cases of severe immune response against SARS-CoV-2, resulting in cytokine storm and immunopathology [20].

We and others [4] found higher antibody titres in men compared with women. This is surprising since women on average have more B cells and produce more antibodies [21]. Higher antibody titres in men, only observed during the acute stage, correlates well with men showing more severe symptoms and increased fatality, as reported [22, 23]. Innate antiviral responses, such as those mediated via toll-like receptor-7, are enhanced in women [24], which may explain their increased resistance against SARS-CoV-2, similarly to influenza virus [25].

In many countries implementing mitigation strategies, infection prevalence remained modest at the time of sampling, May–June 2020, with a low frequency of infection [26–29]. This increases the proportional contribution of any false positives to the result. The ELISA assay as used was approved by the FDA

Table 4. Demographics of patient participants under immunomodulatory or antiviral medication, disease severity categories and symptoms, underlying conditions and medication

Clinical data (%, n)	Immunomodulatory		Antiviral	
Age (years)	60.21	(20–87)	51.87	(43–60)
Male (%)	31%	9	100%	12
Female (%)	69%	20	0%	0
Sample collection date	6/4/2020 - 12/8/2020		6/4/2020 - 12/8/2020	
SARS-CoV-2 PCR+	100%		100%	
Seroconversion (Spike)	28%	(8/29)	75%	(9/12)
Seroconversion (RBD)	31%	(9/29)	75%	(9/12)
Disease Severity				
Asympomatic	24%	7	25%	3
Mild	14%	4	17%	2
Moderate	59%	17	58%	7
Severe	3%	1	0%	0
Symptoms				
Cough	45%	13	0%	0
Myalgia	10%	3	17%	2
Fever	41%	12	58%	7
Anosmia	7%	2	0%	0
Dyspnea	31%	9	42%	5
Diarrhea	0%	0	17%	2
Odynophagia	0%	0	0%	0
Hypogeusia	0%	0	0%	0
Headache	7%	2	17%	2
Rhinorrhea	0%	0	0%	0
Asthenia	28%	8	8%	1
Days Post Symptom onset at collection	10.96	(2-35)	9.00	(3-11)
PCR+ day to serum collection	6.43	(1-13)	6.17	(1-11)
Underlying conditions				
None	0.00%	0	0.00%	0
Chronic kidney disease	17.24%	5	25.00%	3
Diabetes	17.24%	5	16.67%	2
Hypertension	34.48%	10	16.67%	2
Heart disease	6.90%	2	0.00%	0
Alzheimer	0.00%	0	0.00%	0
Cerebrovascular disease	3.45%	1	0.00%	0
Dementia/Parkinson	17.24%	5	0.00%	0
Asthma	0.00%	0	0.00%	0
Cancer	37.93%	11	0.00%	0
COPD	0.00%	0	0.00%	0
HIV	0.00%	0	91.67%	11
SLE	6.90%	2	0.00%	0
Medication				
None	0.00%	0	0.00%	0
Midostaurin	3.45%	1	0.00%	0
Aciclovir	3.45%	1	0.00%	0
Tacrolimus	17.24%	5	0.00%	0
Prednisolone	41.38%	12	0.00%	0
Chemotherapy (Paclitaxel)	6.90%	2	0.00%	0
Methotrexate	3.45%	1	0.00%	0
Raltegravir	0.00%	0	25.00%	3
Darunavir/Cobicistat	0.00%	0	25.00%	3
Exemestane	6.90%	2	0.00%	0
Azathioprine	10.34%	3	0.00%	0
Dexamethasone	3.45%	1	0.00%	0
Tamoxifen	6.90%	2	0.00%	0

(*Continued*)

Table 4. Continued

Clinical data (%, n)	Immunomodulatory		Antiviral	
Abacavir	0.00%	0	33.33%	4
Lamivudina	0.00%	0	33.33%	4
Micofenolate	6.90%	2	0.00%	0
Entecavir	0.00%	0	8.33%	1
Dolutegravir	0.00%	0	25.00%	3

as a two-step method (https://www.fda.gov/media/137029/download). We show that using only one protein for a large population screen picks up some false positives (30/2571; 1.2%). Especially when the infection rate is low, the two-step method is highly beneficial and strongly recommended [27]. Furthermore, the introduction of an additional dilution step ensures robustness, resulting in 1.5% (38/2571) of the staff members that seroconverted. Overall, there were limited differences between RBD and Spike reactivity, with a few samples responding more robustly to Spike, possibly reflecting the increased amount of epitopes available in the larger protein.

The question of long-lasting and protective immunity against SARS-CoV-2 is a focus of current research. We show that the initial antibody response against SARS-CoV-2 raises the three main isotypes in close concert as previously reported for SARS as well as SARS-CoV-2 [15, 30]. The kinetics of the response follows

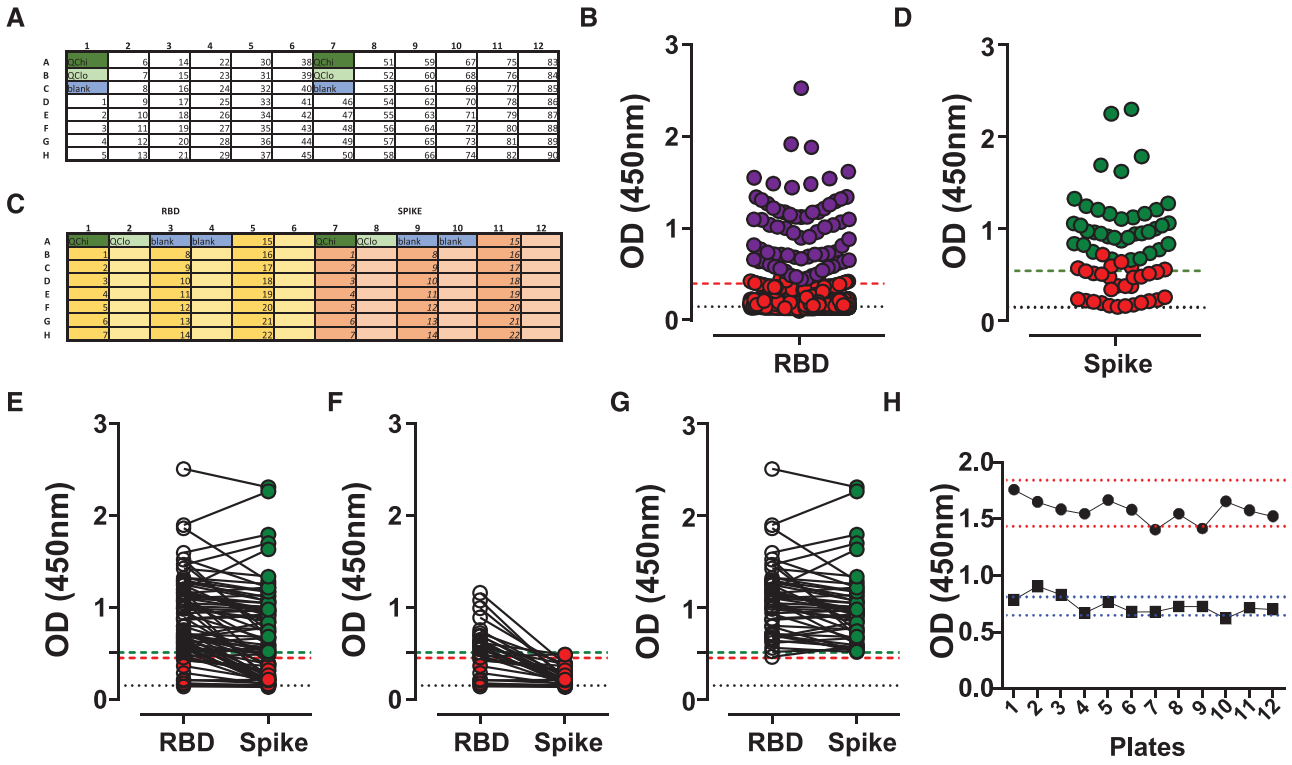

Figure 5. Largescale seroconversion testing of Lisbon University staff. SARS-CoV-2 IgG antibody detection in 1/50 diluted serum samples from University of Lisbon staff using Immulon 4HBX 96-well plates for ELISA. Absorbance was evaluated at 450 nm. (A) Schedule of screening plates, coated with 2 μg/ml RBD, accommodating 90 samples/plate, and includes two blanks, two quality control at high concentration (QC_{hi}), and two at low concentration (QC_{lo}). B) Overview of tested staff from Lisbon University ($n = 2571$ donors). Red symbols indicate negative scores, purple symbols indicate ODs above the cutoff. (C) Schedule of re-screening plates, coated with 2 μg/ml RBD (left) or Spike (right), accommodating 21 samples/plate, and includes two dilutions per sample (1/50 and 1/150), two blanks, two $QC_{hi,}$ and two QC_{lo} per protein used. (D) Screening results from the re-screen ($n = 68$ donors), showing samples tested on Spike protein. Green depicts those samples above the cut off for RBD and Spike at both 1/50 and 1/150 dilution ($n = 38$); Red indicates those samples below the cut off on the second screen for either RBD or Spike ($n = 30$). (E–G) Showing RBD and Spike protein signals for the re-assessed samples and an additional 10 negative samples, (E) all samples, (F) samples assessed negative (open to red symbols) and an additional 10 that were originally negative (red to red symbols), (G) samples assessed as positive (open to green symbols). (H) Quality control signals for 12 sequential plates, showing QC_{hi} (circles) and QC_{lo} (squares). Dotted lines indicate average signal ± SD for QC_{hi} (red, 200ng/ml) and QC_{lo} (blue (10 ng/ml)) of human anti-SARS-CoV-2. Data show individual sample values.

Table 5. Demographics of potential plasma donor participants and determined IgG titres

Potential plasma donors		
Age (years)	37.91	(18-58)
Male (%)	67%	139
Female (%)	33%	70
Sample collection date	8/6/2020 - 30/9/2020	
SARS-COV2 PCR+	100%	
Seroconversion (RBD)	88%	(184/209)
PCR+ day to serum collection	107	(47-199)
PCR- day to serum collection	81	(15-183)
Titer IgG	**n**	**%**
No titer	23	11%
Low (50–300)	31	15%
Medium (300–900)	135	65%
High (>900)	20	10%

a well-known pattern with antibody levels peaking around 3 weeks after symptoms and declining thereafter. Late responses are characterized by low or sometimes undetecTable levels of IgM, modest IgA, but at least until 199 days post-PCR-positive reaction, mostly a robust IgG response. Between days 40 and 199, we found 90% of previous SARS-CoV-2-PCR-positive subjects (198/221), healthcare workers, and potential plasma donors, to carry antibodies, 75% of which had medium to high titers (>300). In addition, we found that in subjects with detectable anti-SARS-CoV-2 IgG, neutralization activity was in accordance with the determined IgG titer level. This is in agreement with a recent report [16]. This and the strong correlation between RBD IgG titers and neutralizing activity as well as protective immunity [16, 31], suggests that most people infected with SARS-CoV-2 will have circulating protective immunity for many months after COVID-19. In addition, recent reports of T cell responsiveness [32–35] show a robust T cell response. Since the SARS-CoV-2 response is in line with well-known and detailed studied immune responses resulting in lymphocyte memory, it is very likely that SARS-CoV-2 protective immunity, reducing disease severity, will last for at least a few years.

Materials and methods

SARS-CoV-2 RBD and Spike protein constructs

RBD and Spike protein constructs were obtained from Dr. Florian Krammer, Icahn School of Medicine at Mount Sinai, New York, USA.

Production and purification of antigen recombinant proteins

Production and purification of recombinant proteins were performed at Instituto de Biologia Experimental e Tecnológica (iBET) Oeiras, Portugal as part of the Serology4COVID consortium as previously described by Stadlbauer et al. [12]. Briefly, Spike or RBD antigen containing His-tag is produced by transient transfection of Expi293F™ cells (Thermo Fisher Scientific) with plasmids suitable for mammalian cell expression (pCAGGS), harboring Spike gene or RBD gene, respectively. All purification steps were performed at 4°C. At 3 days post-transfection, cultures are centrifuged and supernatants are collected and filtered through Sartopore MidiCaps. The clarified supernatants are concentrated and dialyzed with binding buffer by tangential flow filtration, using 10 kDa or 30 kDa membranes, for RBD or Spike purification, respectively. The final dialyzed and concentrated sample is filtered through a 0.22 μm membrane and loaded into His-Trap HP columns, equilibrated with binding buffer. Proteins are eluted with a linear gradient up to 500 mM Imidazole. The fractions containing Spike or RBD are concentrated to 1–2 mg/ml using Vivaflow 200 crossflow devices. Removal of imidazole and exchange to PBS buffer is performed by diafiltration with 10 volumes of PBS. Protein concentration is determined by A280nm combined with the specific extinction coefficient. The concentrated and formulated products are filtered through 0.22 μm membrane, aliquoted, snap-frozen in liquid nitrogen, and stored at -80°C.

Human samples collection

Upon informed consent, blood was taken by vein puncture and two BD Vacutainer CPT tubes of blood and one serum tube were collected per patient. For serum collection, tubes were centrifuged at 2200 rpm, 10 min at 4°C, and upper 6 × 0.25 ml of serum placed into six cryotubes. Samples are stored in a temperature-controlled -80°C ultra-low freezer at the iMM Biobank until analysis.

Serum samples were obtained from the iMM biobank COVID-19 collection and pre-pandemic control sera from two allergy collections. Patients were recruited from the COVID-19 unit and the Immunoallergology Department of Hospital de Santa Maria, Centro Hospitalar Lisboa Norte. The COVID-19 collection and scientific use were approved by the Lisbon Academic Medical Center Ethics Committee (Ref. no. 155/20) as was the staff screening (Ref. no. 181/20). The allergy studies were approved with reference 116/13. Potential plasma donors registered voluntarily via the IPST website. Criteria for registration were a diagnosis of COVID-19 documented by a positive PCR test for SARS-CoV2 followed by two negative or one negative PCR tests 14 or 28 days prior to collection, respectively. Medical interviews were conducted to ensure that the criteria for apheresis plasma donation were fulfilled and that a full recovery from COVID-19 had been achieved.

Signed informed consent was obtained from all volunteers. All data were treated confidentially and anonymous, according to (EU) 2016/679 of 27 April 2016 (General Data Protection Regulation). A professional obliged to confidentiality with guarantee appropriate information security measures carried out the data

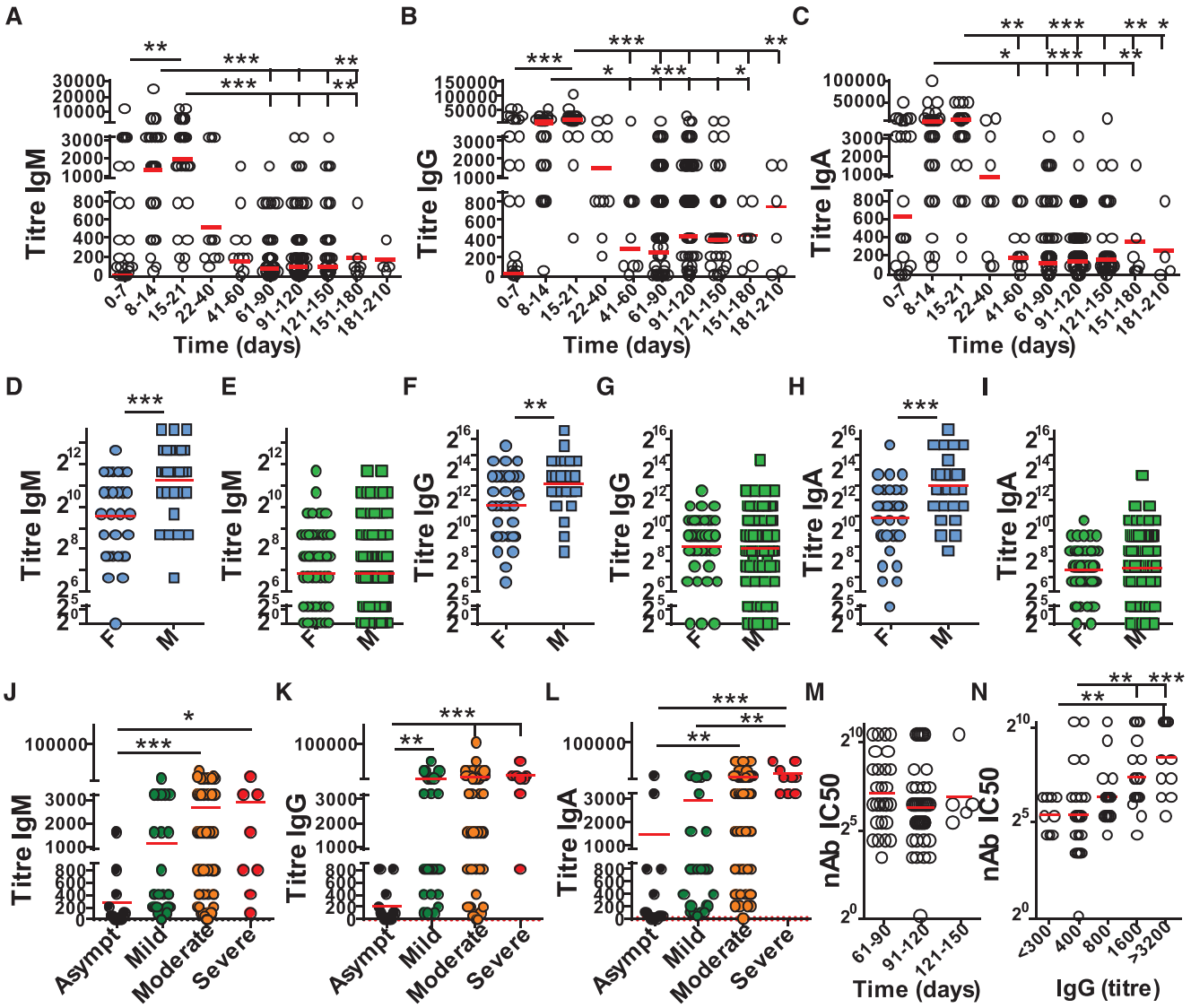

Figure 6. Longitudinal SARS-CoV-2 antibody titers. SARS-CoV-2 antibodies were assessed in 1/50 diluted serum samples from donors in Portugal using Immulon 4HBX 96-well plates for ELISA. Absorbance was evaluated at 450 nm. (A–C) Anti-SARS-CoV-2 RBD antibody titers plotted over time for (A) IgM, (B) IgG, and (C) IgA ($n = 356$ total donors). Red line marks the geometric mean. (D–I) Anti-SARS-CoV-2 antibody titers for males and females during (D, F, and H) early, days 7–40 (females $n = 32$, males $n = 29$) or (E, G, and H) late response, days 40–150 (females $n = 60$, males $n = 114$) for (D and E) IgM, (F and G) IgG or (H and I) IgA. Red line marks the mean. (J–L) Anti-SARS-CoV-2 RBD antibody titers plotted by severity of COVID-19 symptoms experienced for (J) IgM, (K) IgG, and (L) IgA. Red line indicates the mean (Asymptomatic $n = 13$, Mild $n = 28$, moderate $n = 54$, severe $n = 8$). (M and N) SARS-CoV-2 neutralizing activity was determined in sera ($n = 84$ total donors) and plotted against (M) time since SARS-CoV-2 PCR+ or N) IgG titer. Red lines indicate geographic mean. Statistical analysis was performed using Mann–Whitney *U*-test (d–i) or Kruskal–Wallis test using Graphpad Prism software. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data show individual sample values.

collection under the terms of the GDPR paragraph 2, article 29 Law no. 58 /2019, 8 August.

Virus inactivation

To reduce risk from any potential residual virus in the serum, three different virus inactivation methods were tested: incubation for 1 h at 56°C (heat inactivation), the addition of 0.1% Triton X-100 (a non-ionic surfactant), or the combination of both (H+ Tx).

Antibody measurements

Anti-SARS-CoV-2 ELISAs were performed as described previously [12]. Briefly, flat-bottom 96-well plates (Immulon 4 HBX; Thermo Scientific) were coated with recombinant protein RBD or Spike prepared in PBS at a concentration of 2 μg/ml (50 μl/well) overnight at 4°C. Coated plates were washed with PBS-0.05%Tween (PBS-T) using a Well-wash 1x8 (Thermo Fisher Scientific) or Aquamax200, 3× for IgG detection, and 10× for IgM analysis. Plates were blocked with 200 μl/well of 3%

non-fat milk powder in PBS-1%T for 1 h at room temperature and then washed with PBS-T 3× or 10×, as described previously. Serum samples were diluted in PBS-0.1%T + 1% non-fat milk powder, added (100 μl/well) and incubated for 1–2 h at room temperature, washed with PBS-T 3× or 10×. Hereafter several antibody isotypes, namely Total Ig, IgG, IgM, and IgA anti-SARS-CoV2 were detected using HRP-labeled goat anti-human IgG+IgM+IgA (Abcam, ab102420), IgG Fc (Abcam, ab97225), IgM mu chain (Abcam, ab97205), IgA alpha chain (Abcam, ab97215), respectively. Secondary antibodies were diluted in PBS-0.1%T + 1% non-fat milk powder (50 μl/well) and added for 1 h at room temperature, washed with PBS-T 3× or 10×, and developed with TMB substrate solution (TMB Substrate Reagent Set, BD OptEIA™, 555214), 100 μl/well for 10 min. The reaction was stopped with 1M sulphuric acid (50 μl/well) and OD at 450nm was measured via Infinite M200 (TECAN) plate reader. Each plate contained a Quality control (QC) sample, composed of a pool of positive samples or monoclonal antibody, tested in a high and low dilution. For material details, see Methods supplement.

Neutralization assays

Anti-SARS-CoV-2 ELISAs were performed as described in detail recently [36]. SARS-CoV-2 pseudo particles (pp) were kindly provided by Dr Benhur Lee, Icahn School of Medicine at Mount Sinai, New York, USA. Briefly, 24 h prior to infection, Vero CCL81 cells grown in DMEM supplemented with 10% FBS were seeded at 20 000 per well in a 96-well plate. SARS-CoV-2pp and serial dilutions of sera (1/10 in DMEM with 10% FBS, and further twofold dilutions) were incubated at room temperature for 30 min. Media from Vero cells was substituted with the SARS-CoV-2pp/serum mix; plates were spinoculated by centrifugation at 1250 rpm for 1 h at 37°C. After overnight incubation at 37°C, culture medium was removed, and Renilla luciferase production was assessed on Tecan 2 plate reader using the Dual-luciferase Reporter Assay system (Promega), according to manufacturer instructions. IC50 were determined as the last serum dilution at which the titration curve matches inhibition equal or above 50% of the 100% assay.

Statistical analyses

A Kruskal–Wallis test (non-parametric test) was done to compare the geometric ratios between groups with a significance level of 0.05 (Dunn's multiple comparisons test), Student's *t*-test or two-way ANOVA were used as stated in the figure legends, calculated using GraphPad Prism 6.0 software.

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Author contributions: P.F.-C. and B.B. setup the ELISA, tested it and run many samples. C.M. and P.G. provided blood samples from COVID19 patients, A.G. and M.S. run all antibody titrations, S.A., C.C. and J.P.S. setup and ran the neutralisation assays, H.N.-C. and A.M. generated sera from COVID19 patients, M.C.P.-S. provided allergy sera, F.R. and Â.A. catalogued all samples and generated sera, J.C., M.A.E., M.S. and E.V. coordinated potential plasma donor blood sample collections, M.R. and J.M.-C. coordinated staff blood collection, M.V. coordinated the work, secured funding and wrote the manuscript.

Conflict of interest: The authors have no commercial or financial conflict of interest.

Data availability statement: All data will be made available upon reasonable request.

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Abbreviations: **COVID-19**: Coronavirus Disease 2019 · **ELISA**: Enzyme-linked immunosorbent assay · **Ig**: Immunoglobulin · **OD**: Optical density · **PCR**: Polymerase chain reaction · **QC**: Quality control · **RBD**: Receptor binding domain · **ROC**: Receiver operating characteristic · **SARS-CoV-2**: Severe acute respiratory syndrome coronavirus 2 · **S protein**: Spike protein

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