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SARS-CoV-2 Antibody Responses Are Correlated to Disease Severity in COVID-19 Convalescent Individuals

Cecilie Bo Hansen,*† Ida Jarlhelt,*† Laura Pérez-Alós,*‡ Lone Hummelshøj Landsy,† Mette Loftager,‡ Anne Rosbjerg,* Charlotte Helgstrand,‡ Jais Rose Bjelke,‡ Thomas Egebjerg,‡ Joseph G. Jardine,§ Charlotte Svaerk Jorgensen,¶ Kasper Iversen,** Rafael Bayarri-Olmos,*† Peter Garred,*‡ and Mikkel-Ole Skjoedt*†¶

Globally, the COVID-19 pandemic has had extreme consequences for the healthcare system and has led to calls for diagnostic tools to monitor and understand the transmission, pathogenesis, and epidemiology, as well as to evaluate future vaccination strategies. In this study, we have developed novel, to our knowledge, flexible ELISA-based assays for specific detection of human SARS-CoV-2 Abs against the receptor-binding domain, including an Ag sandwich ELISA relevant for large population screening and three isotype-specific assays for in-depth diagnostics. Their performance was evaluated in a cohort of 350 convalescent participants with previous COVID-19 infection, ranging from asymptomatic to critical cases. We mapped the Ab responses to different areas on protein N and S and showed that the IgM, A, and G Ab responses against receptor-binding domain are significantly correlated to the disease severity. These assays and the data generated from them are highly relevant for diagnostics and prognostics and contribute to the understanding of long-term COVID-19 immunity. The Journal of Immunology, 2021, 206: 109–117.

Coronaviruses (CoVs) are zoonotic pathogens primarily targeting the human respiratory system (1). Although most human CoV infections are mild, three CoVs have...
higher affinity (12), which may explain the higher transmission rate of COVID-19.

Precise diagnosis of COVID-19 with RT-PCR detection of SARS-CoV-2 nucleic acids remains crucial to identify symptomatic infections to secure correct treatment and as a tool in quarantine strategies to limit the infection rate in asymptomatic infections. Serological detection of specific SARS-CoV-2 Abs is a useful tool to identify convalescent individuals that have developed immunity and thereby might be protected against reinfection, although this issue is still not resolved (13). Moreover, serological testing is necessary to understand the transmission, pathogenesis, and epidemiology of SARS-CoV-2, providing critical data to inform public health authorities for controlling the spread of COVID-19 and eventually reopening societies. Another question that has arisen is whether an overwhelming Ab response may even aggrivate COVID-19 infection in patients (14).

Because of the urgency and demand, many serological tests have been developed rapidly and made commercially available with only limited validation on clinical samples (R. Lasaunie, A. Frische, Z.B. Harboe, A.C.Y. Nielsen, A. Fomsgaard, K.A. Krogefält, and C.S. Jørgensen, manuscript posted on medRxiv, DOI: 10.1101/2020.04.09.20056325). We have developed a flexible ELISA-based platform for rapid and specific detection of SARS-CoV-2 Abs. The platform includes an indirect RB sandwich ELISA (S-ELISA) for pan Ig detection suitable for large-scale Ab surveillance and direct ELISAs for in-depth analyses of the IgM, IgA, and IgG isotype Ab responses toward RBD and protein N. Moreover, we set out to characterize the Ab response levels in relation to symptom characteristics and disease severity to elucidate the immunological response in COVID-19 convalescent individuals.

Materials and Methods

Ethics statement

All procedures involving the handling of human samples are in accordance with the principles described in the Declaration of Helsinki and ethically approved by the Regional Ethical Committee of the Capital Region of Denmark (H-20028627).

Buffers

The following buffers were used: PBS (10.1 mM Na2HPO4, 1.5 mM KH2PO4, 137 mM NaCl, 2.7 mM KC1), PBS-Tween (PBS-T), PBS, 0.05% Tween 20 (8218/4050; Merck, Darmstadt, Germany) and PBS-T-EDTA plus skin milk (dilution buffer) (PBS, 0.05% Tween 20, 5 mM EDTA [EDS-500G; Merck], 5% skin milk [70166; Merck]).

SARS-CoV-2 Ags

The coding sequence for protein S RBQ (QJC53204.1, aa R319-S593) was synthesized by GenScript and cloned into a pCDNA3.4 expression vector with an N-terminal human VH2-2 signal peptide and a C-terminal 10xHis tag followed by an AviTag (-GSG-HHHHHHHHHHHH-GSG-GLNDIFEAQKIEWHE). The sequence of protein N (QLD29192.1) was optimized as described elsewhere (15) and synthesized by GeneArt (Thermo Fisher Scientific, Waltham, MA) into a pCDNA3 vector with the human serum albumin signal peptide and a C-terminal 8xHis tag (-GSG-HHHHHHHHHH). Both constructs were produced by transient transfection using the Exp293 Expression System Kit (Life Technologies, Thermo Fisher Scientific) according to the manufacturer’s recommendations and grown shaking in suspension in a humidified incubator at 37°C and 8% CO2. On day 5 after transfection, the supernatants were harvested by centrifugation and sterile filtered. The recombinant proteins were purified using a two-step automated purification method setup on an AKTAExpress chromatography system with an immobilized metal affinity HisTrap excel column and a size exclusion Superdex200 column (chromatography system and columns were from Cytiva, Marlborough, MA). The purified proteins were stored in a buffer composed of 20 mM HEPES, 150 mM NaCl (pH 7.4). A portion of the RBQ was specifically biotinylated in the AviTag sequence using a BirA500 Kit (Avidity, Aurora, CO).

Convalescent individuals’ samples and healthy individuals’ controls

A total of 350 recovered individuals previously tested RT-PCR-positive for SARS-CoV-2 were included in the study. The Department of Cardiology at Herlev University Hospital in Denmark recruited the participants. The RT-PCR-positive participants are comprised of males and females aged from 18 to 86, and the course of disease ranged from asymptomatic to critically ill. All participants were invited to complete a health screening questionnaire providing additional information about symptom onset, characteristics, and disease severity. The participants had to categorize the severity of COVID-19 illness using the following predefined descriptions (translated from Danish): 1) I had no symptoms; 2) I was feeling well at home with symptoms; 3) I was bedridden; 4) I was admitted to the hospital; or 5) I was admitted to hospital and needed mechanical ventilation. The study we have named the beforementioned groups as follows: 1) asymptomatic, 2) mild, 3) moderate, 4) severe, or 5) critical. Characteristics of the study participants are detailed in Table I. Serum and EDTA plasma samples were stored in aliquots and kept frozen at ~80°C until used. A total of 580 EDTA plasma samples collected from healthy blood donors at four time points (May 2017, February 2018, September 2018, and February 2019) were used as negative controls for SARS-CoV-2. All SARS-CoV-2–positive samples and controls, a total of 930 samples, were subjected to Ab detection. The samples were then stored at −80°C.

ELISA-based assays

RBD S-ELISA: in-house–produced assay measuring a total Ig Ab response against SARS-CoV-2. Nunc MaxiSorp flat-bottom 96-well plates (442404; Thermo Fisher Scientific) or Clear Flat-Bottom Immuno Nonsterile 384-Well Plates (464718; Thermo Fisher Scientific) were coated with 0.5 µg/mL RBD in PBS overnight (ON) at 4°C. Within the 384-well setup, plates were blocked with WellChampion blocking solution (4900; Kementec, Taastrup, Denmark) following the manufacturer’s instructions. Within the 96-well setup, plates were blocked with PBS-T for 1 h. Samples were diluted 1:10 (96-well plate) or 1:100 (96-well plate) in PBS-T overnight (ON) at 4°C. A total of nine pre–COVID-19 pandemic samples from individuals obtained 4 wk after recovery from endemic human CoVs 229E, OC43, and NL63 were further analyzed to determine possible interference.

Direct ELISA: in-house–produced assay measuring SARS-CoV-2 IgM, IgA, and IgG Ab levels. Nunc MaxiSorp flat-bottom 96-well plates or Nunc MaxiSorp flat-bottom nonsterile 384-well plates were coated with 1 µg/mL RBD or protein N in PBS ON at 4°C. Positive and negative samples were diluted 1:400 in dilution buffer, diluted 3-fold to 1:1200 and 1:3600 and incubated 1 h at room temperature. EDTA plasma from a recovered COVID-19 patient with high titers of IgM, IgA, and IgG was used as a calibrator and applied in a 2-fold serial dilution and incubated as stated above. Abs bound to SARS-CoV-2 Ags were detected using 0.5 µg/mL labeled RBD or protein N in PBS 1:1000 in PBS-T premixed with 0.5 µg/mL biotin-labeled RBD before adding to the ELISA plate and then it was incubated for 2 h at room temperature. TMB One (4380A; Kementec) was used as a substrate and allowed to react for 5 min. The reaction was stopped with 0.3 M H2SO4, and the OD of the samples was measured at 450–620 nm using a Tecan Magellan reader for 96-well plates (Tecan, Männedorf, Switzerland) and a Multiskan FC reader for 384-well plates (Thermo Fisher Scientific). Plates were washed four times with PBS-T between the steps mentioned above. All 384-well plates were handled by the Tecan Fluent Automation Workstation.

Assay development and validation: specificity of the signal, limit of detection, variation, parallelism, and clinical performance

The RBD S-ELISA and the direct ELISAs were subjected to optimization with regards to dilution range, the use of blocking buffers, and variations in incubation times. The final conditions were chosen based on signal-to-noise...
(S/N) ratios. Specificity and sensitivity were calculated based on a receiver operating characteristic (ROC) curve representation and selection of the most appropriate cutoff by prioritizing the specificity. Assay sensitivity regarding the limit of detection was determined by the concentration given by interpolating the OD value of the cutoff. The calibrator was prepared by spiking 100 \( \mu \text{g/ml} \) of recombinant human monoclonal IgG Ab against SARS-CoV-2 Spike1 (A02038; GenScript, Piscataway, NJ) into normal human serum and diluting in serum into a 2-fold dilution. Samples were treated as the same as patient samples and further diluted 1:100 in the S-ELISA and 1:1000 in the direct ELISA in PBS-T following by incubation as stated above. Intra-assay variation was evaluated by calculating the coefficient of variation (CV) of an individual CVs for all the duplicates in a total of 40 samples. Interassay variation was evaluated by calculating the CV of two samples run in duplicates in eight separate plates on three different days. The parallelism between serum and plasma samples was evaluated by comparing 137 pairs of serum and plasma samples using Spearman rank correlation tests. To evaluate whether the Ab levels correlated with the disease severity and/or the days after symptom onset, sample absorbances were logistically transformed, and a four-parameter logistic curve fitting was applied to calibrate the Ab levels into arbitrary units (A.U./ml). The appropriate dilution for each sample was chosen based on the best fit in the linear range of the calibrator. The interpolated value in A.U./ml was corrected by the dilution factor (400, 1200, or 3600). A sample OD value below 0.06 was automatically given the value of 0.001 A.U./ml.

Heatmap

A total of 12 different SARS-CoV-2 protein fragments on the protein S1 and S2 and protein N coupled via an N-terminal cysteine and maleimide conjugation to recombinant human serum albumin (AlbIX; Novozymes, Bagsværd, Denmark); two short peptides from the RBD of protein S, and full-length protein S, protein N, and RBD were analyzed for immunogenicity capacity on the direct ELISA. The peptides (produced by chemical synthesis; TAG Copenhagen, Frederiksberg, Denmark) were chosen based on previously published linear B cell epitopes for common regions with SARS-CoV (16) and by manually screening the available crystal structures of protein N (6W35) and protein S (6VSB) (12). Protein details are illustrated in Fig. 2A. Nunc MaxiSorp flat-bottom nonsterile 384-well plates were coated with 1 \( \mu \text{g/ml} \) of the proteins in PBS ON at 4˚C. A total of 90 RT-PCR–positive samples with high titers of IgG and IgM and/or IgA and six negative controls were diluted 1:20 in dilution buffer and incubated as mention above. Detection and development procedures were followed, as described in the subsection Direct ELISA: in-house–produced assay measuring SARS-CoV-2 IgM, IgA, and IgG Ab levels.

Statistics

Statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA) and R (version 4.0.2 for Windows, R Foundation for Statistical Computing, Vienna, Austria). Measurements of samples and the calibrator were performed in duplicates. Data were analyzed for normal distribution using the Anderson–Darling test. Estimation of levels of IgM, IgA, and IgG were interpolated by regression analysis using a four-parameter logistic curve fitting, and results were given in A.U./ml (in a 1:200 dilution, the calibrator was defined to contain 2 A.U./ml). Statistical differences between disease severity and symptoms onset groups were analyzed using one-way ANOVA (Kruskal–Wallis test) with a Dunn multiple comparison test. Spearman rank correlation tests were used to determine the correlation between COVID-19 symptoms and Ab levels. Multiple regression models were used to assess the relationship between the Ab levels and the independent variables of disease severity, age, and sex. Data are represented as the average of the sample duplicate and the median. Significance levels are as follows: \( p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; p < 0.05 \) was considered statistically significant.

Results

Development of specific ELISA-based assays for the detection of Abs against SARS-CoV-2

The assays were optimized with a specific focus on diminishing nonspecific binding, and the final setup was chosen based on the OD of the absorbance signal and S/N ratio between the positive sample and the negative quality control. The developed ELISA-based assays proved to be suitable for automation and were used in high-throughput setups with both 96-well and 384-well formats, correlating significantly (Supplemental Fig. 1A–D). No differences were observed when serum or plasma was employed for the detection of human Abs against SARS-CoV-2 (Supplemental Fig. 1E–G).

Heatmap

To provide a better insight into Ab seroconversion during SARS-CoV-2 infection and reactivity against different locations on protein
S and protein N, we conducted IgM, IgA, and IgG detection in 90 positive samples against 14 protein fragments and short peptides located on the protein S and protein N structures, full-length RBD, protein S, and protein N (Fig. 2A). The heatmap shown in Fig. 2B indicates a different reactivity of IgM, IgA, and IgG toward the 17 proteins/peptides analyzed. Fig. 2C represents a simplified overview of the percentage of individuals with Abs recognizing each protein/fragment. A clear tendency toward a higher prevalence of IgG responses against the S1 subunit part of protein S and the middle part of protein N was observed. Parts of the S2 subunit and most of the N-terminal part of RBD showed little immunogenicity. The cutoff was calculated as the average of the negative controls plus three times the SD.

Ab detection in convalescent plasma

Levels of Abs against SARS-CoV-2 were measured from plasma samples of 350 recovered individuals with COVID-19. The RBD S-ELISA measures total anti-RBD Igs present in the samples using a single dilution (Fig. 3A), offering a sensitive and specific assay suitable for screening of the population. The direct ELISAs offer a more detailed characterization of the Ab types and levels (Fig. 3B–D) and the possibility to estimate Ab titers by interpolation. A total of 335 of the individuals previously infected with SARS-CoV-2 had mounted a detectable Ab response (Fig. 3A), IgG being the most abundant isotype (present in the 330 individuals; Fig. 3B). In comparison, responses of IgM and IgA isotypes were detected in 215 and 222 of the individuals, respectively (Fig. 3C, 3D).

Ab titers against SARS-CoV-2 are correlated with disease severity, symptom onset, age, and symptoms

A dynamic range of Ab titers expressed as A.U./ml was obtained when OD values were interpolated by regression analysis using a four-parameter logistic curve fitting. The convalescent individuals were classified according to disease severity (Fig. 4A–C), and symptom onset was calculated as the time from the first self-perceived symptom related to COVID-19 to the moment of blood sampling (Fig. 4D–F). We observed a highly significant difference in the Ab titers between the disease severity groups (Fig. 4A–C) (p < 0.0001), with a clear association between increasing Ab levels and more severe disease symptoms for all three Ab isotypes. IgG shows the most significant increase between the severity groups (Fig. 4C). In contrast, asymptomatic individuals appeared not to follow the same severity tendency. This could be explained by the low number of individuals in this group; thus it may not be representative. When we assessed the difference in Ab titers between groups based on the time of symptom onset (Fig. 4D–F), we observed a significant increase in IgG levels continuously over the time of sampling (Fig. 4F), whereas IgM (Fig. 4D) and IgA levels (Fig. 4E) did not change significantly.
Table III illustrates multiple regression analysis for IgG, IgM, and IgA Ab titers in relation to disease severity, age, and sex. All three Ab isotype titers were significantly positively correlated to age and disease severity (grouped as mild, moderate, and severe). Neither the IgG nor IgM Ab titers were significantly correlated to sex; however, the analysis revealed a positive correlation between IgA titers and male sex. Table IV depicts the correlation between self-perceived COVID-19 symptoms and the IgM, IgA, and IgG titers. Symptoms such as fever, shortness of breath, and lack of appetite were significantly and positively correlated with IgM and IgG levels. In contrast, IgA levels were significantly negatively correlated with the loss of sense of smell/taste and headache.

**Discussion**

We have developed an ELISA-based platform for detection of SARS-CoV-2 Abs, comprising an indirect RBD S-ELISA for pan Ig detection and direct ELISAs for in-depth analyses of the IgM, IgA, and IgG isotype responses toward RBD and protein N. RBD was chosen as the primary Ag for the screening and estimation of Ab titers for several reasons. It is regarded to be a sufficiently representative part of protein S to induce an immunogenicity response (17), and we did not detect additional sensitivity improvements by employing the full protein S as a ligand target in the direct ELISA (to be described in detail elsewhere). In a recent phase 1 trial, Ab responses against a vaccine candidate (S-2P Ag) and the RBD were assessed, finding similar Ig responses in pattern and magnitude between both Ags (18). One of the advantages of using RBD instead of full-length protein S is the more efficient production and higher stability of recombinant RBD because of its reduced size and simple tertiary structure. The relatively unique primary sequence of the SARS-CoV-2 RBD (19) also reduces the risk of cross-reactive Ab signals derived from prior B cell responses against other types of CoVs (Supplemental Fig. 3). It is, however, important to annotate that this setup is highly flexible, making it possible to substitute the Ag upon a change in demand or in case of viral mutations. The use of protein N as a target Ag for serological screening has several theoretical pitfalls: the location of protein N makes it less accessible for BCR interaction on the naïve B cell and probably requires a viral membrane degradation. Furthermore, protein N shows higher sequence conservation (90.5%) and thereby increases the risk of false positive detection (20). Nevertheless, large commercial providers have chosen to use protein N as the serological target in different types of SARS-CoV-2 Ab assays (21).

In our study, the detection of IgM Abs toward protein N or its fragments was weaker than IgG, suggesting a fast seroconversion of IgM into IgG. Furthermore, around 5–10% of the convalescent individuals did not mature any detectable Ab response, which is in good agreement with a previous study (22). The direct ELISA setup allows the use of different SARS-CoV-2 proteins in their full-length, shortened variants, or fragmented immunogens, offering a useful tool to study the different reactivity patterns of IgM, IgA, and IgG toward specific exposed areas on the viral Ags. We measured several different Ag areas on protein N and S to establish a heatmap of the Ab landscape. Based on the results, we could demonstrate a tendency toward immune-dominating areas in the S1 unit and the central part of protein N. However, it is important to highlight that the heatmap does not represent the full sequences of the CoV Ags and that the dissection of Ags into shorter fragments could have a significant impact on the Ab reactivity. It could, however, in a more extensive setup, provide valuable information toward a targeted vaccine strategy to elucidate B cell epitopes relevant for the development of synthetically designed vaccine epitopes and select the best candidates based on the Ab titer upon clinical vaccine trials.

The indirect Ag RBD S-ELISA has applicatory value as a screening tool because of easy implementation, low reagent cost, and a clear distinguished difference between positive and negative values. Serological surveys are the ideal tool for tracking the spread of an infectious disease within a population, particularly in the presence of asymptomatic cases or incomplete ascertainment of those with symptoms. The given specificity and sensitivity (99.7 and 94.9%, respectively) of the RBD S-ELISA indicate that the Ab test performed with high accuracy and can determine whether an individual has been infected with SARS-CoV-2. The direct ELISA setups, in contrast, provide the best applications in a diagnostic setup for an in-depth examination of Ab-positive individuals. They allow quantification of IgM, IgA, and IgG Ab levels, rendering more detailed information about the immunological response to the viral infection. The specificities of the direct assays were 99.1, 99.3, and 99.5% for IgM, IgA, and IgG, respectively. The assay sensitivities of the direct ELISAs are, especially for IgM and IgA, profoundly affected by the biological response, as a number of the convalescent individuals did not have measurable IgM levels or matured an IgA response. A total of 111 individuals were negative for both IgM and IgA. Twenty-four individuals were negative for IgM but positive for IgG. In contrast, 91 individuals were negative for IgA but positive for IgM. This suggests that a majority of individuals are lacking an early response from IgA rather than IgM. We have compared our results with a previously published report (23), in which the sensitivity for IgA and IgM for protein N are substantially higher than our results. These discordances might be explained by the different cohort samples employed in both reports. Although we have used 350 samples from convalescent individuals, the above-mentioned report has tested 48 hospitalized individuals, indicating an increased disease severity and consequently an expected higher Ab response. In contrast, our cohort includes a wider severity course of the disease, suggesting that we have analyzed a more representative cohort for Ab determination. Nevertheless, when the three direct ELISAs were combined in a logistic regression analysis and ROC curve analysis, the model predicts with 99.5% specificity and 94.3% sensitivity, which is very comparable with the RBD S-ELISA.

We show that IgG levels increase during the first 11 weeks after symptom onset, whereas the IgM and IgA levels remain stable for the same period of time. This was surprising, as we would have expected a more pronounced decrease in IgM levels over time. This observation could indicate an importance of the two isotypes, reinforced by the fact that severity is correlated with high levels of Abs of all three isotypes. It has previously been shown that both IgG independently and total Ab levels correlate with disease severity in patients during hospitalization (13), but to our knowledge, the prolonged clear correlation between IgM, IgA, and IgG titers and disease severity have not been reported before. The data illustrate that individuals with mild symptoms during infection with SARS-CoV-2 will, in general, mount a lower Ab response compared with individuals with moderate and severe symptoms several months after recovering from a COVID-19 infection. This observation gives an essential insight into the immunological response regarding clinical disease presentation, which further highlights the demand for more quantitative assays.

In this study, all three Ab isotypes correlate positively with age, which can be explained by the fact that severe COVID-19 infection is more pronounced in the elderly population (24). We show that the Ab titers, especially IgG levels, are correlated with specific symptom characteristics, including fever, pharyngalgia, shortness of breath, and nausea, which again shows the link between clinical...
FIGURE 2. Differences in Ab seroconversion during SARS-CoV-2 infection and reactivity. (A) Location of the 14 protein fragments and short peptides on the protein S and protein N structures. The protein fragments are mapped on the atomic model of a partially open SARS-CoV-2 S trimer (6VSB) and the peptides in the monomer for clarity purposes. Protein N–derived short peptides are mapped on the freely oriented N-terminal RNA binding domain (6WKP) and the C-terminal dimerization domain (6WJI). Dashed lines represent approximate peptide locations not covered in the coordinate files. (B) Heatmap representation of OD of IgM, IgA, and IgG Abs binding to the 14 protein fragments and short peptides and protein S, protein N, and RBD. (C) Simplified representation of positive bindings to the different proteins, protein fragments, and short peptides. The cutoff was stated by the mean of the negative controls plus three times the SD. A total of 90 positive samples and six negative controls were analyzed.
manifestations and the immunological response. In contrast, the IgA level was negatively correlated with loss of the sense of taste/smell and surprisingly showed no other correlation to the symptoms of the upper respiratory tract in this study. The role of IgA, which is considered the predominant Ab involved in mucosal immunity, remains to be fully understood. IgA is suggested to mediate antiviral defense functions at different anatomic levels in relation to mucosal epithelium (25). However, the mechanisms behind this remain unknown and often gain limited attention during infectious studies. In this respect, it could be interesting to examine the IgA levels in mucosal tissue during SARS-CoV-2 infection and determine whether the mucosa-associated IgA plays a protective role during SARS-CoV-2 infection or whether the IgA response exacerbates the disease.

**FIGURE 3.** Ab detection in convalescence plasma. OD levels of total Ig (A), IgM (B), IgA (C), and IgG (D) in convalescent individuals and negative controls. Samples were measured in a 1:10 dilution (A) or titrated thrice in a 1:400, 1:1200, and 1:3600 dilution (B–D). Dashed line represents the cutoff for each ELISA. DF, Dilution factor.

**FIGURE 4.** Ab titers are correlated with disease severity and symptom onset. (A–C) Dynamic range represented in A.U./ml of IgM (A), IgA (B), and IgG (C) levels in convalescent individuals stratified according to disease severity. (D–F) Dynamic range represented in A.U./ml of IgM (D), IgA (E), and IgG (F) levels in convalescent individuals stratified according to symptoms onset. Dashed line represents the cutoff for each ELISA. In panel (B), the solid diamond represents groups of 35 individual samples, the solid circle represents groups of 38 individual samples, and the solid square represents groups of 40 individual samples with A.U./ml value equal to 0.4. In panel (E), the solid diamond represents groups of 13 individual samples, the solid circle represents groups of 24 individual samples, and the solid square represents groups of 40 individual samples with A.U./ml value equal to 0.4. A p value <0.05 was considered significant. *p < 0.05, **p < 0.01, ****p < 0.0001 by Kruskal–Wallis test.
Our findings provide support to the notion that Abs toward SARS-CoV-2 represent a double-edged sword. Abs are important in viral neutralization but also in Fc receptor–mediated phagocytosis, Ab-dependent cellular cytotoxicity, complement-dependent cellular cytotoxicity, and subsequent elimination of pathogens. However, it is known that particularly Ab-dependent cellular cytotoxicity and complement-dependent cellular cytotoxicity can drive harmful and systemic proinflammatory responses that can have severe pathophysiologic consequences. Thus, based on our findings and others, it may be suggested that an unwanted immune response toward SARS-CoV-2 may be one of the mechanisms causing hyperactivation of macrophages and monocytes, leading to the deadly cytokine storm, which seems to be a hallmark of COVID-19 (26).

Whether a previously infected individual can expect stable long-term protection against reinfection with SARS-CoV-2 remains unknown. A recent study found a correlation between the production of neutralizing Abs against RBD and elevated IgG titers in convalescence COVID-19 individuals (27), reinforcing the use of RBD as the candidate to analyze for neutralizing Abs in these individuals. The durability of neutralizing Abs (primarily IgG) against SARS-CoV-2 has yet to be defined, but persistence for up to 40 days from symptom onset has been described (Ref. 13 and B. Zhang, X. Zhou, C. Zhu, F. Feng, Y. Qiu, J. Feng, Q. Jia, Q. Song, B. Zhu, and J. Wang, manuscript posted on medRxiv, DOI: 10.1101/2020.03.12.20035048). In comparison, when following infection with SARS-CoV, concentrations of IgG remained high for ~4–5 months before subsequently declining slowly during the next 2–3 years (28). It is uncertain whether an individual with low Abs titers, mainly IgG, has a higher risk of reinfection compared with an individual with high levels of Abs postdisease. It has recently been suggested that, in the absence of Ab levels, an

### Table III. Multiple regression analysis for IgG, IgM, and IgA Ab levels of previously infected individuals with SARS-CoV-2

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### Table IV. Correlation between COVID-19 symptoms and Ab levels of previously infected individuals with SARS-CoV-2

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<th>IgM</th>
<th>IgA</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>R²</td>
<td>p Value</td>
<td>R²</td>
<td>p Value</td>
</tr>
<tr>
<td>No symptoms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>-0.0334</td>
<td>0.5334*</td>
<td>-0.0034</td>
</tr>
<tr>
<td>Nasal congestion</td>
<td>0.1730</td>
<td>0.0012**</td>
<td>0.0620</td>
</tr>
<tr>
<td>Pharyngalgia</td>
<td>-0.0995</td>
<td>0.0633</td>
<td>-0.0534</td>
</tr>
<tr>
<td>Coughing</td>
<td>0.0227</td>
<td>0.6722</td>
<td>0.0764</td>
</tr>
<tr>
<td>Headache</td>
<td>0.1287</td>
<td>0.0161**</td>
<td>0.0645</td>
</tr>
<tr>
<td>Muscle pain and joint pain</td>
<td>0.0298</td>
<td>0.5785</td>
<td>0.0303</td>
</tr>
<tr>
<td>Fatigue</td>
<td>0.0383</td>
<td>0.4759</td>
<td>0.0008</td>
</tr>
<tr>
<td>Lack of appetite</td>
<td>0.1488</td>
<td>0.0320*</td>
<td>0.0345</td>
</tr>
<tr>
<td>Nausea</td>
<td>0.0101</td>
<td>0.8511</td>
<td>0.0723</td>
</tr>
<tr>
<td>Vomiting</td>
<td>0.0122</td>
<td>0.8204</td>
<td>0.0238</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>0.0040</td>
<td>0.9402</td>
<td>0.0556</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>0.0438</td>
<td>0.4142</td>
<td>0.0408</td>
</tr>
<tr>
<td>Loss of sense of taste/smell</td>
<td>-0.0573</td>
<td>0.2853</td>
<td>-0.1195</td>
</tr>
</tbody>
</table>

A p value <0.05 was considered significant. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by Spearman rank correlation analysis. n = 349 participants.
individual could be protected against reinfections by the presence of memory T cells (29). Furthermore, the major proportion of convalescent individuals included in this study did indeed show a dominating IgG response, suggesting that both affinity maturation, isotype class switching, and B cell memory response has occurred. These B cell populations could, together with the memory CD4+ and CD8+ cells, secure a fast and efficient response to a secondary exposure of SARS-CoV-2.

This study is limited by relying on participants’ retrospectively self-reported symptoms and symptom debut, which allows for an unknown amount of misclassification. Moreover, with this design, we could not monitor the Ab response concerning survival. However, because of the detailed analysis of the Ab responses and the clear associations in spite of the retrospective design, we assume that the associations would be even stronger in a carefully conducted prospectively designed study.

In conclusion, we have established robust, flexible, and specific ELISA-based platforms for detection of SARS-CoV-2 Abs and presented novel, to our knowledge, insight into the link between Ab responses and COVID-19 severity.

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Disclosures

The authors have declared that no conflict of interest exists. The assays were developed in a noncommercial collaboration between Rigshospitalet and Novo Nordisk A/S.

References