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Detection and Profiling of Human Coronavirus Immunoglobulins in Critically Ill Coronavirus Disease 2019 Patients

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OBJECTIVES: Coronavirus disease 2019 continues to spread worldwide with high levels of morbidity and mortality. We performed anticoronavirus immunoglobulin G profiling of critically ill coronavirus disease 2019 patients to better define their underlying humoral response.

DESIGN: Blood was collected at predetermined ICU days to measure immunoglobulin G with a research multiplex assay against four severe acute respiratory syndrome coronavirus 2 proteins/subunits and against all six additionally known human coronaviruses.

SETTING: Tertiary care ICU and academic laboratory.

SUBJECTS: ICU patients suspected of being infected with severe acute respiratory syndrome coronavirus 2 had blood collected until either polymerase chain reaction testing was confirmed negative on ICU day 3 (coronavirus disease 2019 negative) or until death or discharge if the patient tested polymerase chain reaction positive (coronavirus disease 2019 positive).

INTERVENTIONS: None

MEASUREMENTS AND MAIN RESULTS: Age- and sex-matched healthy controls and ICU patients who were either coronavirus disease 2019 positive or coronavirus disease 2019 negative were enrolled. Cohorts were well-balanced with the exception that coronavirus disease 2019 positive patients had greater body mass indexes, presented with bilateral pneumonias more frequently, and suffered lower Pao_2:FIO_2 ratios, when compared with coronavirus disease 2019 negative patients ($p < 0.05$). Mortality rate for coronavirus disease 2019 positive patients was 50%. On ICU days 1–3, anti–severe acute respiratory syndrome coronavirus 2 immunoglobulin G was significantly elevated in coronavirus disease 2019 positive patients, as compared to both healthy control subjects and coronavirus disease 2019 negative patients ($p < 0.001$). Weak severe acute respiratory syndrome coronavirus immunoglobulin G serologic responses were also detected, but not other coronavirus subtypes. The four anti–severe acute respiratory syndrome coronavirus 2 immunoglobulin G were maximal by ICU day 3, with all four anti–severe acute respiratory syndrome coronavirus 2 immunoglobulin G providing excellent diagnostic potential (severe acute respiratory syndrome coronavirus 2 Spike 1 protein immunoglobulin G, area under the curve 1.0, $p < 0.0005$; severe acute respiratory syndrome coronavirus receptor binding domain immunoglobulin G, area under the curve, 0.93–1.0; $p \leq 0.0001$; severe acute respiratory syndrome coronavirus 2 Spike proteins immunoglobulin G, area under the curve, 1.0; $p < 0.0001$; severe acute respiratory syndrome coronavirus

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2 Nucleocapsid protein immunoglobulin G area under the curve, 0.90–0.95; $p \leq 0.0003$). Anti–severe acute respiratory syndrome coronavirus 2 immunoglobulin G increased and/or plateaued over 10 ICU days.

CONCLUSIONS: Critically ill coronavirus disease 2019 patients exhibited anti–severe acute respiratory syndrome coronavirus 2 immunoglobulin G, whereas serologic responses to non–severe acute respiratory syndrome coronavirus 2 antigens were weak or absent. Detection of human coronavirus immunoglobulin G against the different immunogenic structural proteins/subunits with multiplex assays may be useful for pathogen identification, patient cohorting, and guiding convalescent plasma therapy.

KEY WORDS: coronavirus disease 2019; humoral response; immunoglobulins; intensive care unit; multiplex

Human coronaviruses are common and usually cause mild to moderate upper-respiratory tract illnesses (1). Highly lethal coronaviruses have recently emerged and include severe acute respiratory syndrome coronavirus (SARS-CoV) (2003), Middle East respiratory syndrome coronavirus (2012), and SARS-CoV-2 (coronavirus disease 2019 [COVID-19]). When infected with the latter, a person may be asymptomatic or suffer a spectrum of viral pulmonary symptoms from mild to severe. Patients with severe COVID-19 are admitted to the ICU for increased monitoring and potential life-saving interventions, where the mortality rate can be high (2). COVID-19 induces an innate immune response (3) that includes increased interferons, tumor necrosis factor (TNF), bradykinin, and serine proteases (4–6). COVID-19 mortality has been attributed in part to microvascular disease (7), which is associated with the formation of pulmonary microthrombi (8).

A humoral immune response follows the innate reaction, with production of coronavirus-specific antibodies or immunoglobulins (9). Only a few studies have investigated the intensity and duration of the SARS-CoV-2 humoral response (10, 11), with both the response rate and the time to seroconversion being variable depending on the targeted antigen, the immunoglobulin isotype investigated, and the assay platform used (12). Typically, an immunoglobulin M response is detected early after infection, and an immunoglobulin G (IgG) response begins shortly thereafter (13).

Human coronavirus express four structural proteins: spike (S), nucleocapsid (N), membrane, and envelope (1). The main immunogens for all seven human coronavirus strains, including those causing severe respiratory syndromes such as SARS-CoV-2, are the "S" and "N" proteins (14). Functionally, the "S" glycoprotein homotrimers on the surface of coronaviruses promote host attachment and fusion of the viral and cellular membranes for entry. The "S" protein consists of two subunits (S1 and S2), whereas the essential receptor binding domain (RBD) lies within the S1 subunit of the protein (15). The "N" protein is also involved in essential functional activities as well as in the proliferation of the virus; it is not only the most abundant viral protein but also has the highest homology within the coronavirus family displaying up to 90% amino acid identity to the other coronavirus strains. Even for the four endemic coronavirus strains that are widespread all over the world, an up to 42% amino acid homology is described (16) for these two immunogenic proteins or their protein subunits currently used in all common SARS-CoV-2 serologic assays. Thus, the choice or combination of antigens is essential for serologic assay specificity.

As coronavirus serologic studies in critically ill patients are few and limited by insufficient sampling time points (17), human coronavirus IgG profiling of suspected COVID-19 ICU patients over time was the aim of this study. Specifically, our objectives were: 1) to determine and compare human coronavirus IgG responses with a novel multiplex research tool between COVID-19 positive (COVID-19+) ICU patients and either healthy control subjects or COVID-19 negative (COVID-19–) ICU patients and 2) to determine which SARS-CoV-2 antibody specificities dominate the serologic responses in COVID-19+ patients.

METHODS

This study was approved by the Western University, Human Research Ethics Board (HREB). Given the unprecedented pandemic situation and the restricted hospital access for substitute decision-makers, waived consent was approved for a short, defined period of time (Research Ethics Board [REB] number 1670;

issued March 20, 2020). In keeping with the Society for Critical Care Medicine statement on "Waiver of Informed Consent in Emergency Situations" (18), the following criteria were considered relevant for HREB approval of waived consent: the subjects were admitted to the ICU with a life-threatening condition; the subjects had impaired decisional capacity; the research staff encountered significant obstacles and delays when attempting to contact the absent substitute decision-makers; the study risk was minimal; the research knowledge gained on this new, lethal disease offered an eventual chance of benefit; and community consultation had been implemented. Given the pandemic circumstances and the waived consent model applied, no further attempts were made to contact the surviving patients and/or substitute decision-makers. The last patient enrolled under waived consent was May 1, 2020.

Study Participants and Clinical Data

We enrolled consecutive patients who were admitted to our level-3 academic ICUs at London Health Sciences Centre (London, ON) and were suspected of having COVID-19 based on standard hospital screening procedures (19). Blood sampling began on ICU admission for up to 3 days in COVID-19– patients or up to 7 days in COVID-19+ patients followed by every 3 days until death or discharge. COVID-19 status was confirmed as part of standard hospital testing by detection of two SARS-CoV-2 viral genes using polymerase chain reaction (20). Patient baseline characteristics were recorded at admission and included age, sex, comorbidities, medications, hematologic laboratories, creatinine, Pao_2:Fio_2 ratio, and chest radiograph findings. We calculated Multiple Organ Dysfunction Score (21) and Sequential Organ Failure Assessment Score (22) for both COVID-19+ and COVID-19– patient groups to enable objective comparison of their illness severity. Both patient groups were characterized as having confirmed or suspected sepsis diagnosis using Sepsis 3.0 criteria (22). We also recorded clinical interventions received during the observation period including use of antibiotics, antiviral agents, systemic corticosteroids, vasoactive medications, venous thromboembolism prophylaxis, antiplatelet or anticoagulation treatment, renal replacement therapy, high-flow oxygen therapy, and mechanical ventilation (invasive and noninvasive). Final participant groups were constructed by age- and sex-matching COVID-19+ patients with COVID-19– patients and healthy control subjects without disease, acute illness, or prescription medications (REB number 6963; https://translationalresearchcentre.com/) (23, 24).

Blood Draws

Standard operating procedures were used to ensure all samples were treated rapidly and equally. Blood was obtained in the morning from critically ill ICU patients via indwelling catheters using vacuum serum separator tubes and placed immediately on ice. If a venipuncture was required, research blood draws were coordinated with a clinically indicated blood draw. In keeping with accepted research phlebotomy protocols for adult patients, blood draws did not exceed maximal volumes (25). Once transferred to a negative pressure hood, blood was centrifuged and sera isolated, aliquoted at 250 μL and frozen at –80°C. All samples remained frozen until use, and freeze/thaw cycles were avoided.

Multiplex Immunoassay Panels

Immunoglobulin levels against 10 human coronavirus antigens were determined in human plasma using a multiplexed immunoassay screening kit according to manufacturer's instructions (Invitrogen ProcartaPlex Coronavirus Ig Total 11-plex Panel; Cat. No. EPX110-16000-901; Thermo Fisher Scientific, Vienna, Austria), which use Luminex xMAP fluorescent bead-based technology (Luminex, Austin, TX). The assay plate was run according to the manufacturer's instructions and read on a compatible Luminex system (Bio-Plex 200 system; Bio-Rad Laboratories, Hercules, CA). Data were expressed as the mean fluorescence intensity (MFI) of the raw data values, with cut off values converted to concentration in U/mL.

Population Statistics

Medians (IQRs) and frequency (%) were used to report ICU patient baseline characteristics for continuous and categorical variables, respectively; continuous variables were compared using Mann-Whitney *U* tests (or Kruskal-Wallis tests, as appropriate), and categorical variables were compared using Fisher exact chi-square. *p* values less than 0.05 were considered statistically significant for demographic and clinical data,

whereas p less than 0.01 was used for IgG measurements to control for repeated measures. Receiver operating characteristic (ROC) curves were conducted to determine sensitivity and specificity of individual IgG ratios for predicting a binary outcome. Area under the curve (AUC) was calculated as an aggregate measure of IgG ratio performance across all possible classification thresholds. The Youden's J Score was used to determine optimal cut off values. All analyses were conducted using SPSS Version 26 (IBM, Armonk, NY).

Machine Learning

Coronavirus IgG data were visualized with a nonlinear dimensionality reduction on the full data matrix using the t-distributed stochastic nearest neighbor embedding (t-SNE) algorithm (26). t-SNE attempts to find an optimal nonlinear projection of the observed data onto a manifold with complex geometry, but low dimension, embedded in the higher dimensional space of the raw observations. A random forest classifier was also trained on the variables to predict COVID-19 status. A random forest is a set of decision trees that can be interrogated to identify the features that have the highest predictive value. To control overfitting, multiple COVID-19 classifiers were trained and tested using a seven-fold cross validation with a random forest of 10 trees with a max depth of three (27).

RESULTS

We investigated 14 COVID-19+ patients (median = 61.0 yr old; interquartile range [IQR] = 54.0–67.0 yr old), 14 age- and sex-matched COVID-19– patients (median = 58.5 yr old, IQR = 52.5–63.0 yr old), and 14 age- and sex-matched healthy controls (median = 57.5; IQR = 53.3–63.0 yr old; p = 0.812). Baseline demographic characteristics, comorbidities, laboratory values, and chest radiograph findings are reported in **Table 1**. The COVID-19+ patients had a higher body mass index (p = 0.011) and a higher rate of bilateral pneumonia (p < 0.001), whereas COVID-19– patients were more likely to have unilateral pneumonia (p = 0.013). COVID-19+ patients had lower admission Pao_2:FIO_2 ratios when compared with COVID-19– patients (p = 0.015) and were more likely to be trialed on high-flow oxygen therapy (p = 0.013). Sepsis was "confirmed" by infectious pathogen identification in only 28.6% of COVID-19– patients, with sepsis "suspected" in the remaining 71.4%. The mortality rate was 50% for COVID-19+ patients.

Human anticoronavirus IgG measurements were performed over multiple ICU days (**Table 2**). Blood IgG levels against the four SARS-CoV-2 specific antigens were higher in COVID-19+ patients versus either COVID-19– patients or healthy control subjects (p < 0.001). The anti–SARS-CoV S1 protein IgG level was slightly elevated in COVID-19+ patients relative to both COVID-19– patients and healthy control subjects on ICU day 3 only (p < 0.01).

Figure 1 shows three t-SNE plots corresponding to the complete SARS-CoV-2 IgG response on the first 3 ICU days and could distinguish COVID-19+ versus healthy control subjects with increasing accuracy: ICU day-1, 82%; ICU day-2, 93%; and ICU day-3, 100%. Feature ranking provided the importance of the IgG levels against each of the four different SARS-CoV-2–specific antigens for determining COVID-19+ status from healthy control subjects on ICU day-3: anti-S1 protein IgG 33.0%, anti-RBD IgG 32.6%, anti-S protein IgG 31.5%, and anti-N protein IgG 2.9%.

Figure 2 shows three t-SNE plots corresponding to the complete SARS-CoV-2 IgG response on the first 3 ICU days and could distinguish COVID-19+ versus COVID-19– patients with increasing accuracy: ICU day-1, 86%; ICU day-2, 89%; and ICU day-3, 99%. Feature ranking provided the importance of the IgG levels against each of the four different SARS-CoV-2–specific antigens for determining COVID-19+ status from COVID-19– patients on ICU day-3: anti-S1 protein IgG 34.6%, anti-S protein IgG 30.8%, anti-RBD IgG 30.6%, and anti-N protein IgG 4.0%.

Figure 3 illustrates the temporal SARS-CoV-2 IgG responses for COVID-19+ patients versus COVID-19– patients and healthy control subjects. The IgG levels against each of the four different SARS-CoV-2–specific antigens increased over the 10 ICU days. ROC curve analyses demonstrated excellent diagnostic potential for each of the SARS-CoV-2 antigen-specific IgGs on ICU day 3 with all AUCs greater than or equal to 0.90 (p < 0.0005) (**Fig. 4**). To declare COVID-19 serology positive, the following ICU day 3 cut off values in MFI (approximate plasma concentration) were calculated for the four SARS-CoV-2 antigens: S1 protein IgG, 34 (0.41 U/mL); S protein IgG, 120 (1.24 U/mL); RBD IgG, 85 (0.37 U/mL); and N protein IgG, 187 (0.95 U/mL). Based on these cut off values, the percentage

TABLE 1.
Subject Demographics and Clinical Data

Variables	Coronavirus Disease 2019 Positive Patients	Coronavirus Disease 2019 Negative Patients	Healthy Controls	*p*
n	14	14	14	1.000
Age, yr, median (interquartile range)	61.0 (54.0–67.0)	58.5 (52.5–63.0)	57.5 (53.3–63.0)	0.812
Sex	8 female:6 male	8 female:6 male	8 female:6 male	1.000
Body mass index, median (interquartile range)	30.5 (27.1–41.8)	25.8 (21.1–29.7)		**0.011**
Multiple Organ Dysfunction Score, median (interquartile range)	4.0 (3.0–5.5)	6.0 (3.0–8.0)		0.286
Sequential Organ Failure Assessment Score, median (interquartile range)	4.5 (2.0–9.3)	6.0 (4.3–10.5)		0.204
Comorbidities, *n* (%)				
Hypertension	7 (50.0)	9 (64.3)		0.445
Diabetes	5 (35.7)	5 (35.7)		1.000
Chronic kidney disease	2 (14.3)	1 (7.1)		1.000
Cancer	2 (14.3)	1 (7.1)		1.000
Chronic obstructive pulmonary disease	1 (7.1)	3 (21.4)		0.596
Heart disease	2 (14.3)	2 (14.3)		1.000
Chronic heart failure	0 (0)	2 (14.3)		0.481
Baseline laboratories, median (interquartile range)				
WBC count	8.5 (6.9–16.1)	15.3 (11.1–20.5)		0.056
Neutrophils	7.3 (5.6–12.6)	12.2 (8.6–15.7)		0.062
Lymphocytes	0.7 (0.6–1.0)	1.3 (0.5–1.8)		0.093
Platelets	206 (134–294)	202 (164–260)		0.872
Hemoglobin	122 (102–135)	124 (102–138)		0.818
Creatinine	82 (58–187)	75 (54–113)		0.448
Chest radiograph findings, *n* (%)				
Bilateral pneumonia	13 (92.9)	2 (14.3)		**< 0.001**
Unilateral pneumonia	1 (7.1)	8 (57.1)		**0.013**
Interstitial infiltrates	0 (0)	1 (7.1)		1.000
Normal	0 (0)	3 (21.4)		0.222

(*Continued*)

TABLE 1. (*Continued*).
Subject Demographics and Clinical Data

Variables	Coronavirus Disease 2019 Positive Patients	Coronavirus Disease 2019 Negative Patients	Healthy Controls	*p*
Pao_2:Fio_2 ratio, median (interquartile range)	107 (66–162)	172 (138–312)		**0.015**
Lactate, median (interquartile range)	1.5 (1.0–2.0)	1.2 (0.9–1.6)		0.233
Sepsis diagnosis, *n* (%)				
Suspected	0 (0)	10 (71.4)		**< 0.001**
Confirmed	14 (100)	4 (28.6)		**< 0.001**
Interventions during study, *n* (%)				
Antibiotics	14 (100)	14 (100)		1.000
Antivirals	3 (21.4)	2 (14.3)		1.000
Steroids	3 (21.4)	5 (35.7)		0.678
Vasoactive medications	11 (78.6)	8 (57.1)		0.420
Renal replacement therapy	2 (14.3)	1 (7.1)		1.000
High-flow nasal cannula	8 (57.1)	1 (7.1)		**0.013**
Noninvasive mechanical ventilation	6 (42.9)	8 (57.1)		0.450
Invasive mechanical ventilation	10 (71.4)	11 (78.6)		1.000
Survived, *n* (%)	7 (50.0)	12 (85.7)		0.103

Boldface values indicate statistically significant differences.

of COVID-19+ patients that were serology positive on ICU days 1, 2, and 3 were as follows: anti-S1 protein IgG, 79%, 93%, and 100%; anti-S protein IgG, 93%, 93%, and 100%; anti-RBD IgG, 79%, 93%, and 100%; and anti-N protein IgG, 71%, 79%, and 86%.

Our final analyses directly compared IgG levels between COVID-19+ ICU patients who survived or died (Mann-Whitney *U* test; $n = 7$/group). For all four different SARS-CoV-2–specific antigens, as well as the six non–SARS-CoV-2 antigens, the measured IgG levels at all time points (ICU day 1, 2, 3, 7, and 10) were similar (data not shown).

DISCUSSION

In this study, we used a human coronavirus multiplex research assay panel to measure IgG titers against 10 coronavirus antigens from ICU patients, both COVID-19+ and COVID-19– and healthy control subjects. Four of the IgG measured were specific for SARS-CoV-2, whereas the remaining six IgG represented the other known human coronavirus for discrimination purposes. Given the number of the antigens used and corresponding IgG levels measured, we analyzed the data with state-of-the-art machine learning, as well as conventional statistics. Our data demonstrate SARS-CoV-2 serologic responses evident in all COVID-19+ patients by ICU day 3. The multiplexed assay was both sensitive and specific for SARS-CoV-2, with only minor interference by SARS-CoV on ICU day 3. Despite the exploratory nature of our study, the data generated suggest that critically ill COVID-19 patients develop significant serologic responses.

Our COVID-19+ ICU patients were similar to those reported in earlier cohorts (28–31) with respect to demographic, comorbidities, and clinical presentation. In contrast to COVID-19– ICU patients, our COVID-19+ ICU patients had a higher occurrence rate

of bilateral pneumonia and lower Pao_2/Fio_2 ratios. A trend toward lower leukocyte counts was observed in COVID-19+ ICU patients, as opposed to COVID-19–. Previous functional enrichment analysis demonstrated a shift in the circulating leukocyte population in COVID-19+ ICU patients with a transcriptional enrichment for CD14+ monocytes and CD33+ myeloid cells (6), accentuating the relative lymphopenia in these COVID-19+ patients. Previous work by our study group, investigating many of these same patient samples, determined a unique inflammatory profile characterized by elevated TNF and serine proteases (5) and

TABLE 2.

Immunoglobulin G Measurements From Age- and Sex-Matched Healthy Control Subjects and Critically Ill ICU patients (Days 1–3), Either Coronavirus Disease 2019 Negative or Coronavirus Disease 2019 Positive

Immunoglobulin	Healthy Control	Coronavirus Disease 2019 Negative	Coronavirus Disease 2019 Positive	*p*
ICU day 1				
SARS-CoV-2 S1 protein IgG	8 (5.1–12.3)	4.4 (3.3–6.6)	169.1 (31.6–255.9)[a]	**< 0.0001**
SARS-CoV-2 S protein IgG	11.5 (8.3–15.2)	7.0 (4.1–10.5)	696.3 (55.1–830.5)[a]	**< 0.0001**
SARS-CoV-2 RBD IgG	27.3 (22.5–48.1)	20.2 (14.4–25.8)	799.4 (85.4–1,170.9)[a]	**< 0.0001**
SARS-CoV-2 Nucleocapsid protein IgG	28.9 (22.2–48.3)	28.3 (14.6–33.1)	1,101.7 (117.6–3,476.9)[a]	**0.0008**
SARS-CoV S1 protein IgG	8.1 (5.5–10.1)	5.3 (4.1–10.9)	12.5 (7.9–21.7)	0.024
HCoV-NL63 IgG	118.9 (75.1–158.0)	74 (33.9–118.9)	69.9 (53.8–80.8)	0.110
HCoV-HKU1 IgG	255.5 (176.0–570.3)	237.7 (83.1–362.0)	108.4 (46.2–278.8)	0.150
HCoV-229E IgG	203.7 (163.0–425.5)	101.9 (49.7–251.8)	147.5 (68.2–193.5)	0.104
MERS S1 protein IgG	7.5 (5.9–11.9)	6.8 (5.1–9.2)	13.4 (7.3–20.8)	0.099
HCoV-OC43 IgG	285.9 (119.5–473.0)	470.3 (130.2–795.8)	159.5 (81.6–227.6)	0.099
ICU day 2				
SARS-CoV-2 S1 protein IgG	8 (5.1–12.3)	4.4 (3.1–4.9)	126.6 (39.1–629.5)[a]	**< 0.0001**
SARS-CoV-2 S protein IgG	11.5 (8.3–15.2)	5.5 (4.5–6.7)	595.5 (172.7–1,822.2)[a]	**< 0.0001**
SARS-CoV-2 RBD IgG	27.3 (22.5–48.1)	16.3 (12.3–24.3)	584.5 (114.4–2,381.6)[a]	**< 0.0001**
SARS-CoV-2 Nucleocapsid protein IgG	28.9 (22.2–48.3)	22.5 (15.6–29.0)	1,314.7 (138.8–5,209.7)[a]	**< 0.0001**
SARS-CoV S1 protein IgG	8.1 (5.5–10.1)	4.8 (3.0–8.3)	14.6 (8.7–21.8)	0.023
HCoV-NL63 IgG	118.9 (75.1–158.0)	67.2 (33.2–91.1)	58.6 (33.0–90.8)	0.041
HCoV-HKU1 IgG	255.5 (176.0–570.3)	201.3 (86.1–367.9)	124.8 (51.7–242.9)	0.098
HCoV-229E IgG	203.7 (163.0–425.5)	90.6 (42.1–197.0)	121.3 (57.5–178.1)	0.035
MERS S1 protein IgG	7.5 (5.9–11.9)	6.5 (4.3–8.3)	11.2 (9.0–18.5)	0.045
HCoV-OC43 IgG	285.9 (119.5–473.0)	395.1 (164.3–667.2)	162.3 (89.3–224.1)	0.087

(*Continued*)

TABLE 2. (*Continued*).

Immunoglobulin G Measurements From Age- and Sex-Matched Healthy Control Subjects and Critically Ill ICU patients (Days 1–3), Either Coronavirus Disease 2019 Negative or Coronavirus Disease 2019 Positive

Immunoglobulin	Healthy Control	Coronavirus Disease 2019 Negative	Coronavirus Disease 2019 Positive	*p*
ICU day 3				
SARS-CoV-2 S1 protein IgG	8 (5.1–12.3)	4.3 (2.4–5.8)	617.8 (124.5–1,579.6)[a]	**< 0.0001**
SARS-CoV-2 S protein IgG	11.5 (8.3–15.2)	5.2 (4.0–7.6)	1,913.0 (619.5–4,286.5)[a]	**< 0.0001**
SARS-CoV-2 RBD IgG	27.3 (22.5–48.1)	18.7 (10.7–23.0)	2,383.3 (401.6–5,141.9)[a]	**< 0.0001**
SARS-CoV-2 Nucleocapsid protein IgG	28.9 (22.2–48.3)	22.2 (15.0–30.6)	4,261.3 (517.6–7,003.8)[a]	**< 0.0001**
SARS-CoV S1 protein IgG	8.1 (5.5–10.1)	5.3 (3.6–7.1)	19.9 (12.8–34.0)[b]	**< 0.001**
HCoV-NL63 IgG	118.9 (75.1–158.0)	69.2 (45.2–106.5)	70.8 (55.9–80.9)	0.082
HCoV-HKU1 IgG	255.5 (176.0–570.3)	174.5 (73.4–322.3)	115.8 (48.0–230.1)	0.094
HCoV-229E IgG	203.7 (163.0–425.5)	113.7 (39.3–242.5)	141.2 (60.1–181.9)	0.077
MERS S1 protein IgG	7.5 (5.9–11.9)	6.8 (3.9–8.9)	16.7 (7.6–26.8)	0.033
HCoV-OC43 IgG	285.9 (119.5–473.0)	444.2 (121.5–666.2)	139.2 (88.1–231.0)	0.200

HCoV = human coronavirus, IgG = immunoglobulin G, MERS = Middle East respiratory syndrome, RBD = receptor binding domain, S = spike, SARS-CoV = severe acute respiratory syndrome coronavirus.

[a]$p < 0.01$, coronavirus disease 2019 positive (COVID-19+) > both Healthy Controls and coronavirus disease 2019 negative (COVID-19–).

[b]$p < 0.01$, COVID-19+ > COVID-19–.

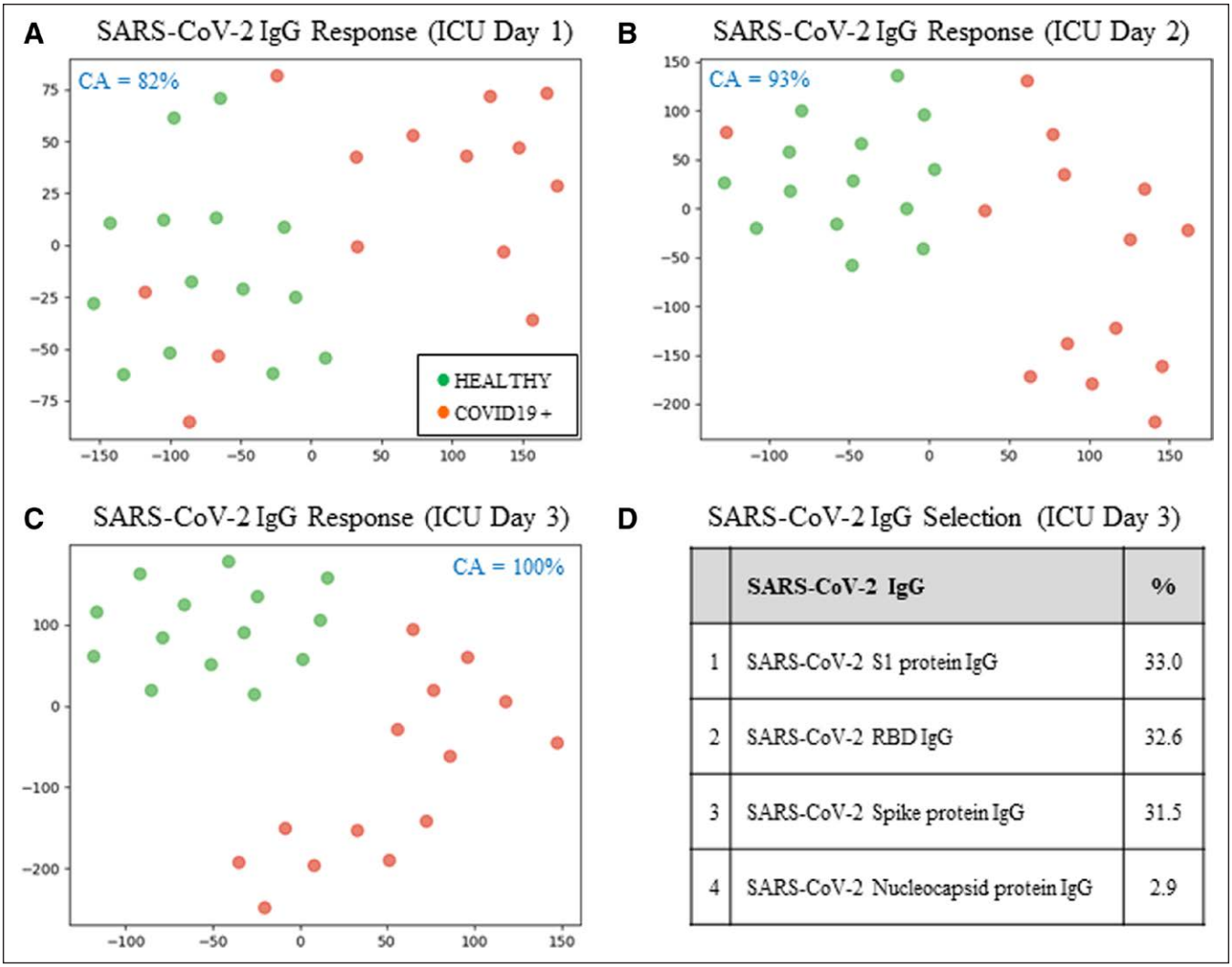
Data are presents as median (interquartile range) and analyzed with a Kruskal-Wallis test and a Dunn's post hoc ($n = 14$/group). A $p < 0.01$ was considered significant to control for repeated measures.

Boldface values indicate statistically significant differences.

a thrombotic profile associated with endothelial activation and glycocalyx degradation (7). By employing targeted proteomics and metabolomics, we identified novel biomarkers that accurately predict COVID-19 mortality (32, 33). Taken together with the data from this study, COVID-19 represents a severe illness with a unique pathophysiologic signature, as well as a high mortality rate. Indeed, in this "first-wave" cohort of COVID-19 patients, ICU death was 40–50% with standardized ICU care.

A ROC curve analysis for each of the four anti–SARS-CoV-2 IgG yielded an AUC higher than 0.90, with the majority of comparisons providing an AUC equal to 1.00, indicating an excellent discrimination between COVID-19+ patients and either COVID-19– patients or healthy control subjects. In fact, the assays used in this study performed well when compared with other commercially available serologic assays (34). Although the patients in our study were critically ill, future studies can use a similar approach to establish whether the combination of one or more SARS-CoV-2 specific antigens may be useful in discriminating COVID-19 serologic positive patients with minimal or no symptoms. Furthermore, our multiplex approach that targets different antigens with a broader epitope coverage may be important for future epidemiologic studies examining seroprevalence of populations with high sensitivity and specificity. To this end, a similar recent study used a combination of up to four separate serologic tests to gain a comparable holistic set of data (35). In addition, this multiplexing assay could be useful to assess future vaccine's seroconversion rates, as well as to cohort patients with different coronavirus strain infections, thereby modifying infection control practices.

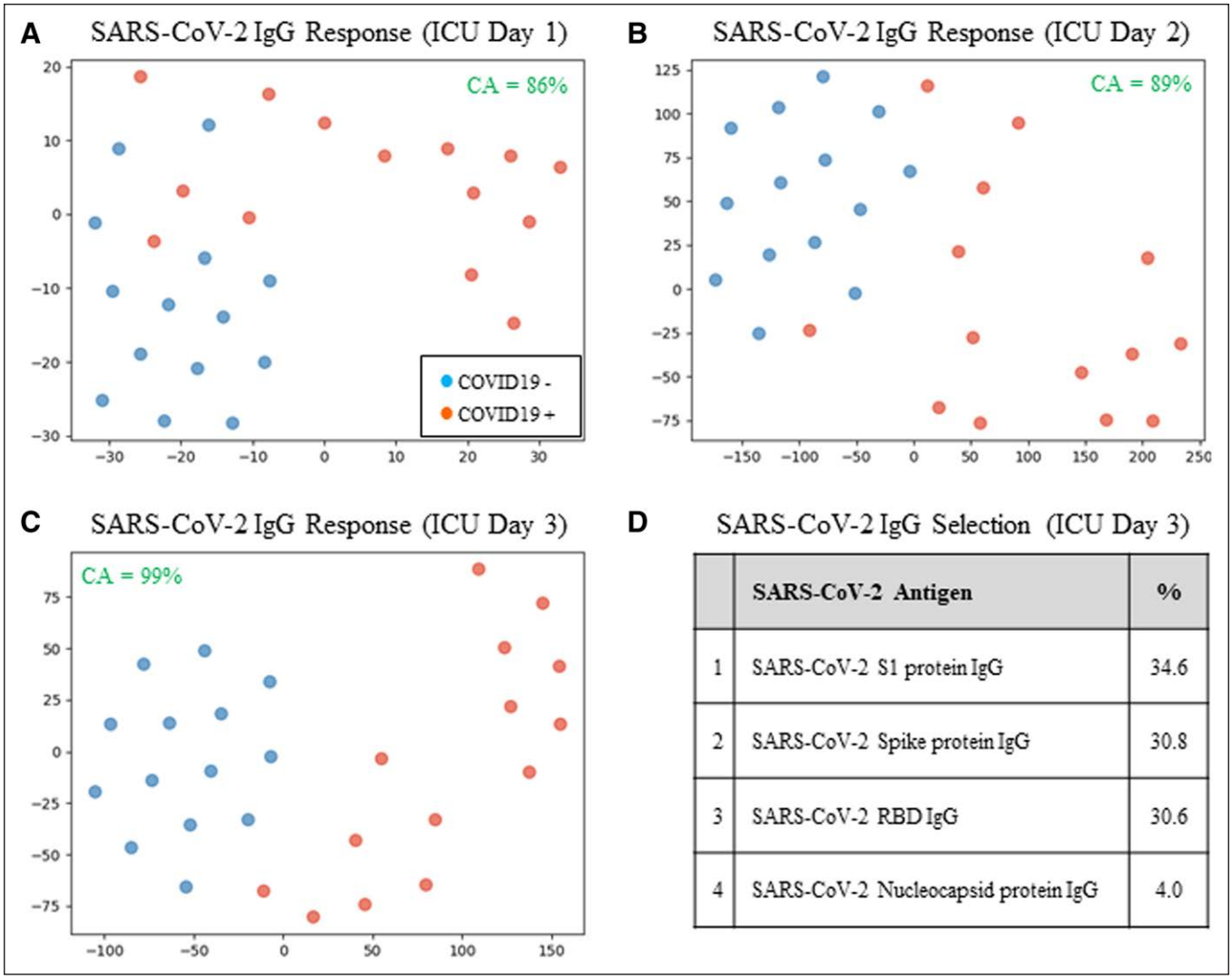
Our data showed that all COVID-19+ patients demonstrated a robust serologic response on or before



	SARS-CoV-2 IgG	%
1	SARS-CoV-2 S1 protein IgG	33.0
2	SARS-CoV-2 RBD IgG	32.6
3	SARS-CoV-2 Spike protein IgG	31.5
4	SARS-CoV-2 Nucleocapsid protein IgG	2.9

Figure 1. T-distributed stochastic neighbor embedding plots comparing complete plasma severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) immunoglobulin G (IgG) responses between Healthy Controls (*green*) and coronavirus disease 2019 positive (COVID-19+) (*red*) patients on different ICU days (the axes are dimension less). **A**, Subjects plotted in 2D following dimensionality reduction of their respective SARS-CoV-2 IgG responses on ICU Day 1. Classification accuracy (CA) was 82%. The dimensionality reduction shows some mixing of Healthy Controls with COVID-19+ patients. **B**, Subjects plotted in 2D following dimensionality reduction of their respective SARS-CoV-2 IgG responses on ICU Day 2. CA was 93%. The dimensionality reduction shows minimal mixing of Healthy Controls and COVID-19+ patients. **C**, Subjects plotted in two dimensions following dimensionality reduction of their respective SARS-CoV-2 IgG responses on ICU Day 3. CA was 100%. The dimensionality reduction shows complete separation of Healthy Controls and COVID-19+ patients. **D**, A list indicating the relative importance of each SARS-CoV-2 IgG for determining differences between groups. RBD = receptor binding domain.

ICU day 3; however, we do not know the precise day of infection or symptom onset. In contrast, none of the age- and sex-matched COVID-19– ICU patients had a measurable serologic response. In the COVID-19+ patients, the temporal assessment demonstrated increasing concentrations of anti–SARS-CoV-2 IgGs, albeit with substantial variability (12, 13). Our temporal data are consistent with published data describing a 38.3% antibody response during the first 7 days of infection and an 89.6% antibody response during the second week of SARS-CoV-2 infection (11). Indeed, 100% of COVID-19 patients expressed IgG by 17–19 days after symptom onset (10). Published data suggest that the magnitude of SARS-CoV-2 antibody production after infection was similar between COVID-19 patients with variable disease severity before postinfectious day 12; however, antibody levels diverged to reflect disease severity by 14 days after infection (11). These aforementioned studies, while compatible with our human coronavirus multiplex results, may underlie



	SARS-CoV-2 Antigen	%
1	SARS-CoV-2 S1 protein IgG	34.6
2	SARS-CoV-2 Spike protein IgG	30.8
3	SARS-CoV-2 RBD IgG	30.6
4	SARS-CoV-2 Nucleocapsid protein IgG	4.0

Figure 2. T-distributed stochastic neighbor embedding plots comparing complete plasma severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) immunoglobulin G (IgG) responses between coronavirus disease 2019 negative (COVID-19–) (*blue*) and coronavirus disease 2019 positive (COVID-19+) (*red*) patients on different ICU days (the axes are dimension less). **A**, Subjects plotted in 2D following dimensionality reduction of their respective SARS-CoV-2 IgG responses on ICU Day 1. Classification accuracy (CA) was 86%. The dimensionality reduction shows some mixing of COVID-19– with COVID-19+ patients. **B**, Subjects plotted in 2D following dimensionality reduction of their respective SARS-CoV-2 IgG responses on ICU Day 2. CA was 89%. The dimensionality reduction shows minimal mixing of COVID-19– and COVID-19+ patients. **C**, Subjects plotted in two dimensions following dimensionality reduction of their respective SARS-CoV-2 IgG responses on ICU Day 3. CA was 99%. The dimensionality reduction shows complete separation of COVID-19– and COVID-19+ patients. **D**, A list indicating the relative importance of each SARS-CoV-2 IgG for determining differences between groups.

the IgG variation determined with fluorescent intensity that we report at multiple ICU day time points.

As a final observation, our data suggest that a blunted serologic response may not contribute to COVID-19 mortality, as 50% of our COVID-19+ ICU patients died despite having similar serologic responses as those who survived. COVID-19 mortality may reflect increased viral load (36) that was associated with greater cytokine production (5), endothelial damage (7), microvascular thrombus formation (8), and lung injury (37), suggesting that inhibitors of cytokines, such as TNF antagonists (38) and platelet aggregation (7), may be therapeutically beneficial.

Recent studies examining convalescent plasma therapy for critically ill COVID-19 patients have yielded mixed results (39, 40). A major problem with this intervention is that neither the ideal patient candidates nor the optimal timing for convalescent plasma therapy are known (41). Future studies might employ a multiplexing approach with different antigens and a

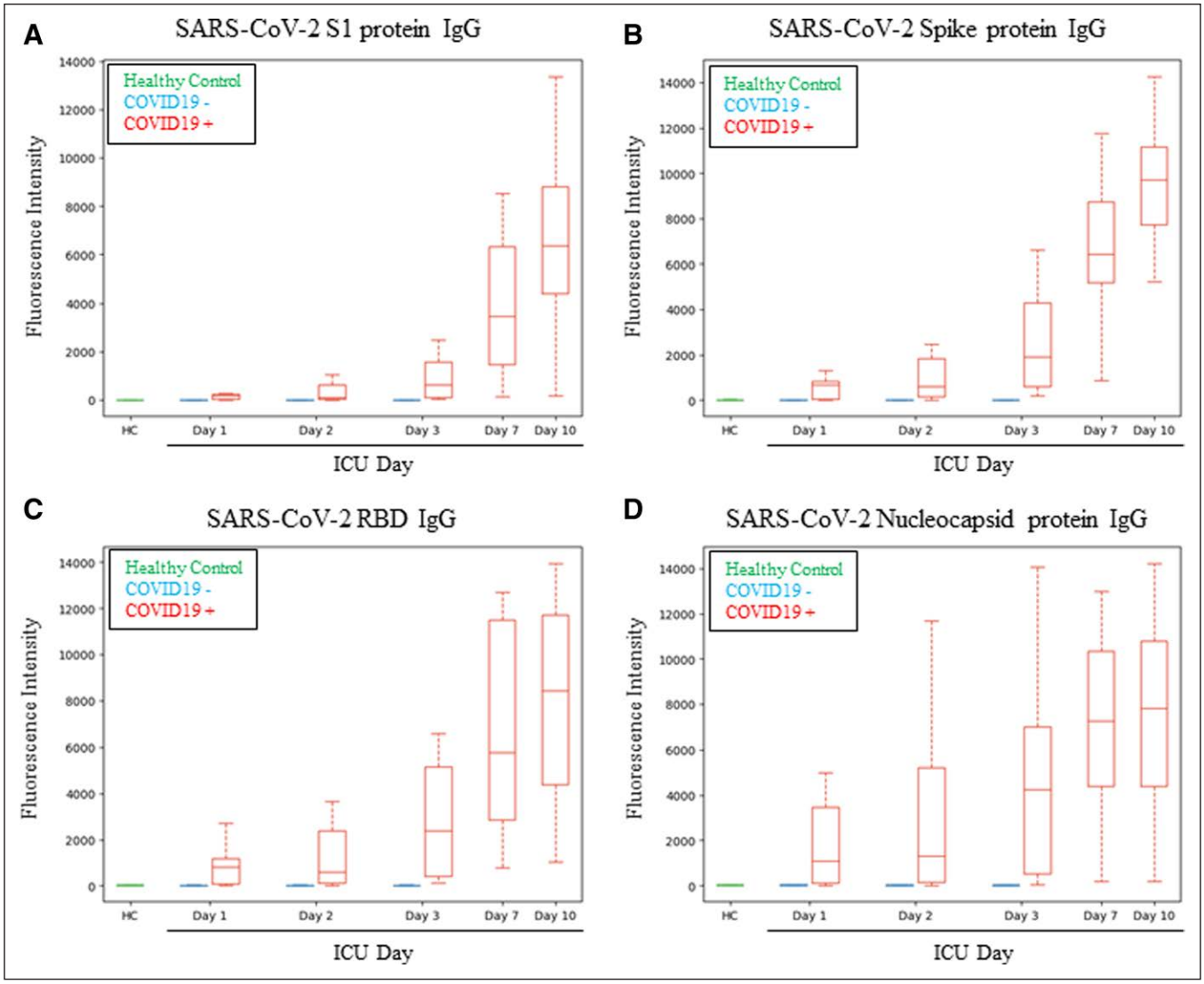


Figure 3. Box plots demonstrating fluorescence intensity for plasma severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) immunoglobulin G (IgG) for Healthy Controls (*green*), coronavirus disease 2019 negative (COVID-19–) ICU patients (*blue*; ICU days 1–3) and coronavirus disease 2019 positive (COVID-19+) ICU patients (*red*; ICU Days 1–3, 7, and 10). **A**, A box plot demonstrating the temporal responses of the SARS-CoV-2 S1 protein IgG. **B**, A box plot demonstrating the temporal responses of the SARS-CoV-2 S protein IgG. **C**, A box plot demonstrating the temporal responses of the SARS-CoV-2 receptor binding domain (RBD) IgG. **D**, A box plot demonstrating the temporal responses of the SARS-CoV-2 Nucleocapsid protein IgG. S = spike.

broad epitope coverage for highest sensitivity and specificity, as we have done in this study, to help aid patient selection and timing of convalescent plasma administration. This precision medicine approach described above would be especially welcomed in ICUs (42), where inherent heterogeneity of patients and failure to use biologic characteristics to selectively enroll patients with high likelihood of a response to an intervention have yielded many negative trials (e.g., usually fewer than 5% of trials in critically ill patients are positive).

Despite the novelty of our serologic profiling in critically ill COVID-19 patients, our study is exploratory and had several limitations. First, we only studied critically ill patients, and we cannot determine the disease onset and symptom duration. Nonetheless, our ICU is representative of ICUs across North America with similar admission criteria. Second, although our study identified serologic responses, our overall COVID-19 study population was limited in number and pathologies highlighting the urgent need for future studies on larger patient cohorts. Future studies with larger sample sizes can explore whether serologic responses correlate with additional clinical outcomes such as functional status in survivors.

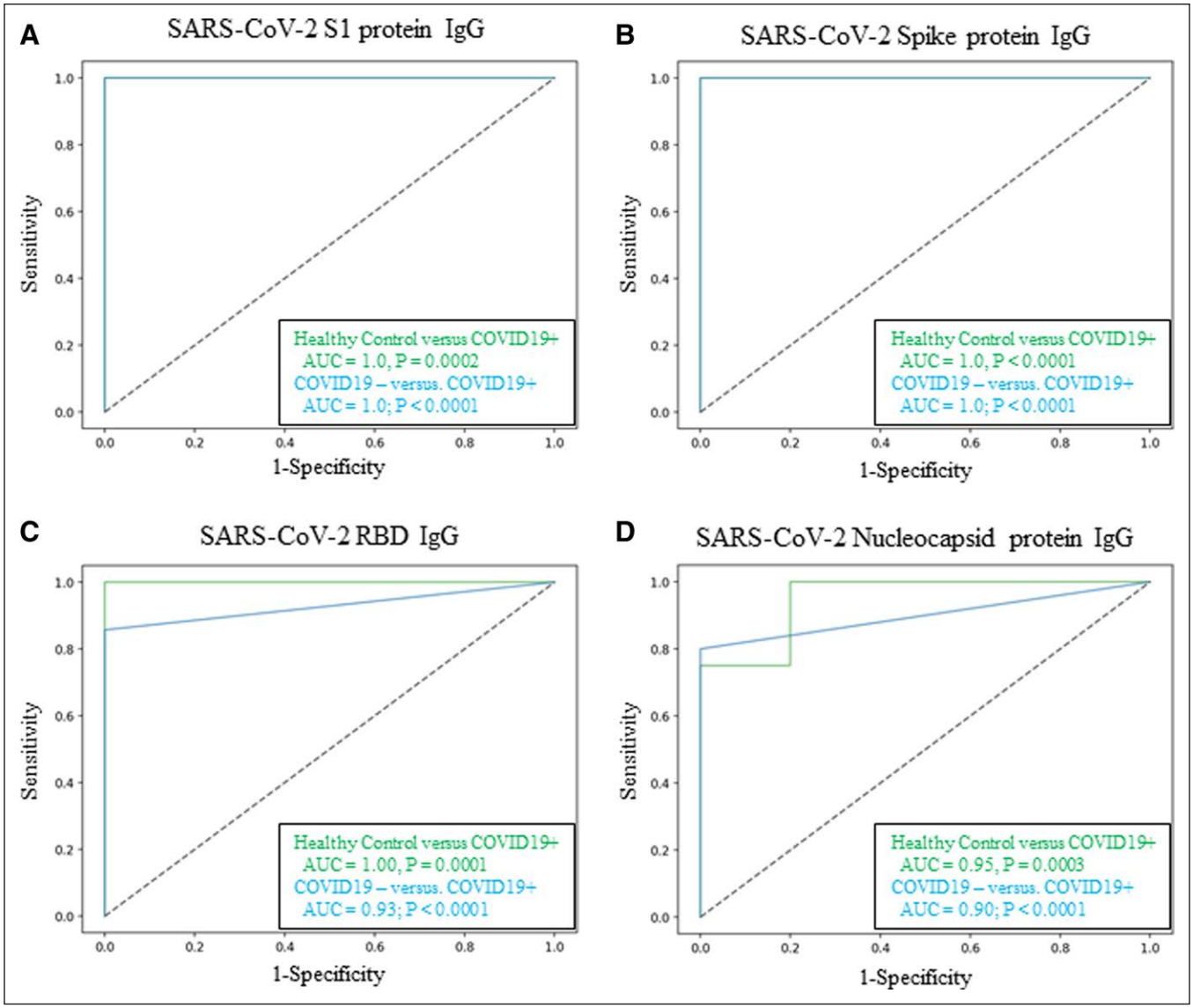


Figure 4. Receiver operating characteristic (ROC) curves demonstrating area under the curves (AUCs) for plasma severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) immunoglobulin G (IgG) subtypes on ICU Day 3 for Healthy Controls versus coronavirus disease 2019 positive (COVID-19+) ICU patients (*green*) and coronavirus disease 2019 negative (COVID-19–) versus COVID-19+ ICU patients (*blue*). **A**, ROC curves demonstrating the excellent diagnostic potential of the SARS-CoV-2 S1 protein IgG (AUCs = 1.0). **B**, ROC curves demonstrating the excellent diagnostic potential of the SARS-CoV-2 S protein IgG (AUCs = 1.0). **C**, ROC curves demonstrating the excellent diagnostic potential of the SARS-CoV-2 receptor binding domain (RBD) IgG (AUCs = 0.93, 1.0). **D**, ROC curves demonstrating excellent diagnostic potential of the SARS-CoV-2 Nucleocapsid protein IgG (AUCs = 0.90, 0.95). S = spike.

CONCLUSIONS

In summary, we report anti–SARS-CoV-2 serologic responses with a novel human coronavirus multiplex research assay in COVID-19+ ICU patients, with the expected temporal responses for IgGs. Although exploratory, our study filled a knowledge gap on the specificity of human coronavirus IgG measurements from critically ill COVID-19 patients and demonstrated the potential future use of multiplex technology as a human coronavirus serologic diagnostic tool. Given the rapid spread of COVID-19, our data may be important for refining future SARS-CoV-2 serologic assays in milder cases and after vaccination, as well as patient cohorting during viral seasons.

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