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IL-6, IL-10, sFas, granulysin and indicators of intestinal permeability as early biomarkers for a fatal outcome in COVID-19

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ABSTRACT

The clinical presentation of coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), ranges between mild respiratory symptoms and a severe disease that shares many of the features of sepsis. Sepsis is a deregulated response to infection that causes life-threatening organ failure. During sepsis, the intestinal epithelial cells are affected, causing an increase in intestinal permeability and allowing microbial translocation from the intestine to the circulation, which exacerbates the inflammatory response. Here we studied patients with moderate, severe and critical COVID-19 by measuring a panel of molecules representative of the innate and adaptive immune responses to SARS-CoV-2, which also reflect the presence of systemic inflammation and the state of the intestinal barrier. We found that non-surviving COVID-19 patients had higher levels of low-affinity anti-RBD IgA antibodies than surviving patients, which may be a response to increased microbial translocation. We identified sFas and granulysin, in addition to IL-6 and IL-10, as possible early biomarkers with high sensitivity (>73 %) and specificity (>51 %) to discriminate between surviving and non-surviving COVID-19 patients. Finally, we found that the microbial metabolite D-lactate and the

Abbreviations: AUC, area under the curve; COVID-19, coronavirus disease 2019; CRP, C-reactive protein; HRP, horseradish peroxidase; ICU, intensive care unit; I-FABP, Intestinal fatty acid-binding protein; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NLR, neutrophil-to-lymphocyte ratio; PAMPs, pathogen-associated molecular patterns; PCA, principal component analysis; PCT, procalcitonin; RBD, receptor-binding domain; ROC, receiver operating characteristic; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; sFas, soluble Fas; sFasL, soluble Fas ligand; SOFA, Sequential Organ Failure Assessment; TLR, Toll-like receptor; TMB, 3,3′,5,5′-tetramethylbenzidine.

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tight junction regulator zonulin were increased in the serum of patients with severe COVID-19 and in COVID-19 patients with secondary infections, suggesting that increased intestinal permeability may be a source of secondary infections in these patients. COVID-19 patients with secondary infections had higher disease severity and mortality than patients without these infections, indicating that intestinal permeability markers could provide complementary information to the serum cytokines for the early identification of COVID-19 patients with a high risk of a fatal outcome.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused 603,711,760 cases of coronavirus disease 2019 (COVID-19) and 6,484,136 deaths worldwide (WHO, 2020a). A large proportion of the infected individuals are asymptomatic, and the clinical presentation of COVID-19 ranges between mild respiratory symptoms and a severe disease with pneumonia, acute respiratory distress syndrome and multiple organ failure. The immune response to SARS-CoV-2 is triggered when innate receptors recognize viral pathogen-associated molecular patterns (PAMPs) and induce the production of pro-inflammatory cytokines and type I interferons. NK cells eliminate virus-infected cells through perforin/granzyme-induced apoptosis and through the FasL/Fas pathway, and they have anti-SARS-CoV-2 activity. Cytotoxic CD8 T cells also induce apoptosis in virus-infected cells. In addition, SARS-CoV-2 infection induces the production of neutralizing antibodies, with the receptor-binding domain (RBD) of the S protein as their main target (Merad et al., 2022).

In many cases, the local inflammatory response is sufficient to clear the SARS-CoV-2 infection. However, in some individuals, the early type-I interferon response is impaired, which leads to an increased production of pro-inflammatory cytokines. These cytokines increase vascular permeability, which facilitates the recruitment of neutrophils, monocytes and T cells to the lung (Batah and Fabro, 2021). The levels of many pro-inflammatory cytokines (including IL-1β, IL-8, IFN-γ, G-CSF and TNF-α) correlate with disease severity in COVID-19, with IL-6 considered as a biomarker of disease severity. The increased and deregulated production of pro-inflammatory cytokines (hyperinflammation) can lead to tissue damage and organ failure (Henderson et al., 2020). In addition to hyperinflammation, patients with severe COVID-19 have lymphopenia, neutrophilia, thrombocytopenia and coagulopathies (Gustine and Jones, 2021). This clinical presentation includes many of the features of sepsis, which has led many authors to analyze the similarities between severe COVID-19 and bacterial sepsis (Loftus et al., 2021), and even to classify severe and critical COVID-19 as cases of viral sepsis (Alhazzani et al., 2020, Li et al., 2020a).

Sepsis is currently defined as a deregulated response to infection that causes life-threatening organ failure (Singer et al., 2016). Both the pro-inflammatory and the anti-inflammatory responses are activated systemically, leading to organ dysfunction and to a persistent immune-suppression. The immune-suppression is partially caused by lymphocyte exhaustion and apoptosis, and by a decreased response of many innate immune cells to Toll-like receptor (TLR) ligands. The immune-suppression increases the risk of secondary infections in these patients (Hotchkiss et al., 2016). In recent years, it has been recognized that the intestine is affected by the pro-inflammatory response during sepsis, which causes apoptosis of the intestinal epithelial cells and disrupts their tight junctions, leading to increased intestinal permeability. As a result, microorganisms and their products [such as lipopolysaccharide (LPS) and D-lactate] translocate from the intestinal lumen to the gut-associated lymphoid tissue and to the draining lymph nodes, and from these organs into the circulation (Haussner et al., 2019, Mittal and Coopersmith, 2014), further activating the innate immune system and providing a source of secondary infections.

Since its emergence as a pandemic in 2020, many molecules have been evaluated as possible biomarkers for the severity and the lethality of COVID-19. However, the utility of the proposed biomarkers remains limited. Here we evaluated the levels and affinities of anti-RBD IgG and IgA antibodies in patients with moderate, severe and critical COVID-19. We also measured a panel of cytokines associated with anti-viral responses and with NK cell and CD8 T cell responses, as well as pro-inflammatory cytokines, anti-inflammatory cytokines and intestinal permeability markers. These molecules are representative of the innate and adaptive immune responses to SARS-CoV-2, and they can also reflect the presence of systemic inflammation and the state of the intestinal barrier in these patients. Our results identify some of the mechanisms that could increase the intestinal permeability in patients with severe COVID-19, and also identify sFas and granulysin, in addition to IL-6 and IL-10, as possible biomarkers for the early identification of patients with increased risk of a fatal outcome.

2. Materials and methods

2.1. Patient selection criteria

This study was approved by the Ethics in Research Committee of the General Hospital of Mexico (CE/185/20), and was carried out in accordance with the code of ethics of the World Medical Association. All the individuals included in this study (or their family members) signed an informed consent form, according to the Helsinki declaration. We included 25 asymptomatic individuals that were household contacts of COVID-19 patients, 25 ambulatory patients with moderate COVID-19, 27 hospitalized patients with severe COVID-19 and 25 patients with critical COVID-19. The critical patients were treated in the intensive care unit (ICU) of the Hospital. The patients were classified according to the WHO COVID-19 clinical progression scale (WHO, 2020b). All the patients were diagnosed with a Panbio COVID-19 antigen rapid test (Abbott Laboratories, Chicago, IL) and a confirmatory Cobas SARS-CoV-2 RT-PCR test (Roche Diagnostics, Rotkreuz, Switzerland), and were treated in the Pneumology Service of the General Hospital of Mexico from June to November 2020. The contacts were not tested for SARS-CoV-2, but they were followed for 7 days after their blood sample was taken, to confirm that they had remained without symptoms during this period. None of the contacts or the patients had received any COVID-19 vaccines at the time of enrollment. Pregnant individuals, as well as individuals with HIV infection, cancer, autoimmune diseases, treatment with immunosuppressors or recent surgery (<3 months), were not included in this study. The presence of secondary infections in COVID-19 patients was determined according to clinical and radiological criteria, serum procalcitonin levels and microbial cultures. The Sequential Organ Failure Assessment (SOFA) score was determined, according to the Third International Consensus Definitions for Sepsis and Septic Shock (Singer et al., 2016).

2.2. Collection of blood samples

A single peripheral blood sample was taken from the contacts. A single peripheral blood sample was also taken from the moderate and severe COVID-19 patients, upon diagnosis confirmation and before any treatment. For the individuals with critical COVID-19, a first blood sample was taken (from the central venous catheter) upon admission to the ICU, and subsequent samples were taken 2, 4, 6, 8 and 10 days later. Three milliliters of blood were taken in BD Vacutainer tubes with EDTA (BD Biosciences, San José, CA) and were used for the complete blood

count, which was performed in a Sysmex XN-550 analyzer (Sysmex, Hyogo, Japan). Six milliliters of blood were taken in BD Vacutainer tubes without additives. The blood was left to clot for 45 min, and was then centrifuged at 1,000 xg for 20 min at 4 °C. The serum was stored in aliquots at −70 °C, until the quantification of antibodies, cytokines and intestinal permeability markers.

Creatinine, urea, L-lactate and lactate dehydrogenase were measured photometrically in an AU analyzer (Beckman Coulter, Brea, CA). C-reactive protein and ferritin were detected with latex-based immuno-agglutination assays (Cobas 6000 analyzer, Roche Diagnostics, Rotkreuz, Switzerland). Procalcitonin was detected with a colloidal gold immuno-chromatographic assay (Artron Laboratories, Burnaby, BC, Canada), and D-dimer was also detected with a latex-based immuno-agglutination assay (HemosIL D-dimer, Werfen, Bedford, MA, USA).

2.3. Determination of anti-RBD IgG and IgA antibodies

Serum samples of the patients were inactivated for 1 h at 56 °C and analyzed for anti-RBD IgG antibodies using an ELISA-based kit (UDISTEST-V2G®, UDIBI, Mexico City, Mexico), according to the manufacturer's instructions (Camacho-Sandoval et al., 2021). Briefly, a SARS-CoV-2 RBD-coated plate was washed with 220 μL per well of PBS with 0.1 % Tween 20, and blocked with 200 μL per well of PBS with 0.1 % Tween 20 and 3 % low-fat milk at room temperature. The serum samples were diluted (1:100) with blocking solution, and 100 μL of diluted sera were added to each well and incubated for 1 h at room temperature. The plate was then washed, and a horseradish peroxidase (HRP)-linked anti-human IgG antibody (dilution 1:15:000 in blocking solution) and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate were used for IgG determination. The reaction was stopped after 20 min by the addition of methanesulfonic acid. The absorbance was read at 450/570 nm using an automated SpectraMax M3 Multi-Mode Microplate reader (Molecular Devices, San Jose, CA, USA). A positive control (recombinant human IgG1 anti-SARS-CoV-2 RBD antibody) and a negative control (a non-related human IgG1 antibody) were also analyzed per analytical run, as described by the manufacturer. For the determination of anti-RBD IgA antibodies, 100 μL of inactivated sera (dilution 1:250 in blocking solution) were added to a SARS-CoV-2 RBD-coated plate, as described above. The plates were incubated for 1.5 h at room temperature and washed. An HRP-linked anti-human IgA antibody (Southern Biotech, Birmingham, AL, USA), diluted 1:5,000 in blocking solution, and TMB substrate were used for IgA determination. The absorbance was read as described above. A positive human serum and a negative human serum were used as internal controls per analytical run. The reference absorbances for the IgG and IgA assays were determined using SARS-CoV-2-positive or -negative human serum samples, as follows: positive control > 0.6, negative control < 0.1, internal positive control > 0.6, and internal negative control < 0.5 for IgG determination; internal positive control > 0.25 and internal negative control < 0.25 for IgA determination. The cutoff value was 0.5 for IgG determination, and 0.25 for IgA determination.

To evaluate the affinity of the anti-RBD antibodies, an additional step was added to this protocol. After incubating the sera and controls for 1 or 1.5 h, 100 μL of urea 7 M were added to each well. After 10 min at room temperature, the plates were washed 6 times before the addition of diluted secondary antibodies, and the protocol continued as described above. This strategy breaks the low-affinity interactions between antigens and antibodies (Moriyama et al., 2021).

2.4. Quantification of serum cytokines

IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IFN-α, IFN-β, IFN-λ1, IFN-λ2/3, IFN-γ, TNF-α, IP-10 (CXCL10), GM-CSF, soluble Fas (sFas), soluble Fas ligand (sFasL), granzyme A, granzyme B, perforin and granulysin were measured with bead-based immunoassays (LEGENDplex, BioLegend). At least 4,000 beads were acquired per sample, in a FACSAria lll flow cytometer (BD Biosciences, Franklin Lakes, NJ). Data were analyzed with LEGENDplex Data Analysis Software (BioLegend).

IL-22 was measured with an ELISA Max Deluxe Set (BioLegend), which uses a capture antibody, a biotinylated detection antibody, an avidin-HRP conjugate and TMB substrate to quantify IL-22 in serum samples. Absorbances at 450 nm, with 570 nm correction, were measured in a microplate reader. To determine IL-22 concentrations, corrected absorbances were interpolated in a standard curve (SigmaPlot 14.0, Systat Software, San Jose, CA).

2.5. Quantification of intestinal permeability markers

Intestinal fatty acid-binding protein (I-FABP, also known as FABP2) was measured by ELISA (Hycult Biotech, Wayne, PA), with a protocol that uses a capture antibody, a biotinylated detection antibody, a streptavidin-peroxidase conjugate and TMB substrate to quantify I-FAPB in 1:2 diluted serum samples. Zonulin was also measured by ELISA (MyBioSource, San Diego, CA), with a protocol that includes a capture antibody, a biotinylated detection antibody, an avidin-HRP conjugate and TMB substrate to quantify zonulin in serum samples. For both protocols, absorbances at 450 nm, with 570 nm correction, were measured in a microplate reader, and corrected absorbances were interpolated in a standard curve to determine the protein concentrations (SigmaPlot).

Serum D-lactate was measured with a colorimetric assay (Sigma-Aldrich, St. Louis, MO). Serum samples were diluted 1:2 or 1:3 with assay buffer, and a standard curve was prepared by diluting a D-lactate standard solution. 50 μL of diluted sera or diluted standard were added to each well of a 96 well flat-bottom plate, and 50 μL of reaction mix were added. The reaction mix contained a D-lactate hydrogenase, which specifically oxidizes D-lactate and generates a colored product. After 30 min at room temperature, absorbances at 450 nm, with 570 nm correction, were measured in a microplate reader, and corrected absorbances were interpolated in the standard curve to determine D-lactate concentrations (SigmaPlot).

2.6. Statistical analysis

All graphs represent individual data with medians. A Shapiro-Wilk normality test was applied to each dataset. One-way ANOVA with Tukey's multiple comparisons test, or t-tests, were used for normally distributed data; otherwise, Kruskal-Wallis tests with Dunn's multiple comparison test, or Mann-Whitney U tests, were used. For paired data (first and last samples from the same patient), paired t-tests were used. Spearman's rank correlation coefficient was used to analyze the statistical correlation between two variables. Receiver operating characteristic (ROC) curves were plotted for each cytokine or intestinal permeability marker. These statistical analyses were performed with GraphPad Prism 9.2.0 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant. Exploratory correspondence analysis was conducted in R Core Team (R_Core_Team, 2020). Principal component analysis (PCA) was performed with FactoMineR (Lê et al., 2008). Optimal cutoff values were estimated according to Youden Index (J) using the pROC package (Robin et al., 2011).

3. Results

3.1. COVID-19 patients have high-affinity IgG and low-affinity IgA anti-RBD antibodies

Seventy-seven patients with COVID-19 were included in this study, including 25 individuals with moderate disease, 27 individuals with severe disease and 25 individuals with critical disease. The group of patients with critical COVID-19 included older individuals ($P = 0.0493$) and a higher proportion of male patients, compared with the group of patients with moderate COVID-19. In addition, critical patients had

increased peripheral blood neutrophils ($P = 0.0009$), increased levels of the acute phase proteins C-reactive protein ($P = 0.024$) and procalcitonin ($P = 0.0001$), and increased levels of the fibrinolysis-associated D-dimer ($P = 0.0001$), compared to moderate patients. They also had higher SOFA scores than moderate ($P < 0.0001$) and severe patients ($P < 0.0001$) (Table 1). These parameters were also increased in non-surviving patients, compared to surviving patients ($P < 0.05$) (supplementary Table 1).

We first measured anti-RBD antibodies and their affinities in the COVID-19 patients. The amount of anti-RBD IgG antibodies was significantly increased in moderate ($P = 0.0006$), severe ($P < 0.0001$) and critical patients ($P = 0.0055$), compared to a group of 25 asymptomatic individuals that were household contacts of the patients, but there were no differences in the IgG levels between the three patient groups (Fig. 1A). However, most of the contacts had detectable levels of these antibodies. In patients with critical COVID-19, the anti-RBD IgG antibody levels remained constant for up to 10 days (Fig. 1B). Most of the IgG antibodies had high affinity, as assessed by the urea method (Fig. 1D, E). No differences were observed in the anti-RBD IgG levels or in their affinity between surviving and non-surviving patients (Fig. 1C, F). The amount of anti-RBD IgA antibodies was significantly increased in moderate ($P = 0.0002$), severe ($P < 0.0001$) and critical patients ($P < 0.0001$), compared to the contacts, and most of the contacts had no detectable levels of these antibodies (Fig. 2A). In patients with critical COVID-19, the anti-RBD IgA antibody levels remained constant for up to 10 days (Fig. 2B). In contrast to the IgG antibodies, almost all of the IgA antibodies had a low affinity for RBD (Fig. 2D), although this affinity increased with time in the critical patients (days 2 vs 10, $P = 0.0151$; days 4 vs 10, $P = 0.0044$) (Fig. 2E). The levels of IgA antibodies were significantly increased in non-surviving patients, compared to surviving patients ($P = 0.0198$; median levels in non-surviving patients = 2.136; median levels in surviving patients = 1.550) (Fig. 2C), although the IgA affinity was similar in both groups ($P = 0.7937$) (Fig. 2F).

3.2. Severe and critical COVID-19 patients have a mixed profile of pro- and anti-inflammatory cytokines, with increased intestinal permeability and secondary infections

Cytokines have been previously evaluated as possible biomarkers in COVID-19. Here we measured a panel of pro-inflammatory and anti-inflammatory cytokines, cytokines associated with anti-viral responses and cytokines associated with NK cell and CD8 T cell responses, and found that the levels of IL-6, IL-10 and sFas were increased in patients with critical disease, compared with patients with moderate disease ($P < 0.0001$, $P < 0.0001$ and $P = 0.0031$, respectively) and with contacts ($P < 0.0001$ for the three cytokines) (Fig. 3A, 3C and 3E, left panels). The levels of these three cytokines were also increased in patients with severe disease, compared with the contacts ($P = 0.0018$, $P < 0.0001$ and $P = 0.0005$, respectively) (Fig. 3A, 3C, and 3E, left panels), and the levels of sFas were higher in patients with moderate disease, compared with the contacts ($P = 0.0351$) (Fig. 3E, left panel). IL-8 was increased in patients with critical COVID-19, compared to the contacts ($P = 0.0062$) (Fig. 3B, left panel), while IFN-β was increased in patients with critical disease, compared to patients with severe disease ($P = 0.0199$) (Fig. 3D, left panel). Granulysin and IP-10 were increased in critical ($P < 0.0001$ and $P = 0.0026$, respectively) and in severe patients ($P = 0.0197$ and $P = 0.0470$, respectively), compared to the contacts, and IP-10 was also increased in moderate patients, compared to the contacts ($P = 0.0494$) (Fig. 3F and 3G, left panels). Thus, in this cohort, sFas and IP-10 were the only cytokines that were significantly increased in all COVID-19 patients (moderate, severe or critical), in comparison with the contacts. IL-6, IL-8, IL-10, IFN-β, sFas and IP-10 were also significantly increased in non-surviving patients ($P < 0.0001$, $P = 0.0009$, $P < 0.0001$, $P = 0.0032$, $P = 0.0001$ and $P = 0.0332$, respectively) (Fig. 3A to 3G, middle panels).

The levels of sFas were increased in male patients (n = 49, 14,296 ± 2012 pg/mL), compared to female patients (n = 28, 9,991 ± 2559 pg/

Table 1
Demographic and clinical data of COVID-19 patients.

	Reference value	Moderate [a] (n = 25)	Severe [b] (n = 27)	Critical [c] (n = 25)	P value
Age (years)	NA	51.8 ± 12.0	54.9 ± 10.9	59.8 ± 12.8	0.0493 [ac]
Gender (females/ males)	NA	13/12	9/18	6/19	NA
Time between symptom onset and collection of the first blood sample (days)	NA	9.5 ± 3.9	10.0 ± 4.6	5.2 ± 3.7	0.0272 [ac] 0.0237 [bc]
Survivors	NA	100 %	77.8 %	12.0 %	NA
Secondary infection (bacterial or yeast)	NA	44.4 %	74.1 %	100 %	NA
Gastrointestinal symptoms (abdominal pain, nausea, vomiting and/ or diarrhea)	NA	11.1 %	29.6 %	26.9 %	NA
Sequential Organ Failure Assessment (SOFA) score	NA	2.1 ± 1.0	3.0 ± 1.2	9.2 ± 2.3	<0.0001 [ac] <0.0001 [bc]
Respiratory rate (/min)	12–20	23.4 ± 5.5	25.4 ± 5.2	22.0 ± 2.3	0.034 [bc]
pO_2 (mmHg)	80–100	72.6 ± 27.6	68.6 ± 17.6	59.4 ± 20.8	ns
Peripheral oxygen saturation (%)	97–98	82.5 ± 5.8	78.7 ± 8.0	77.4 ± 5.2	0.0024 [ab] <0.0001 [ac]
PaO_2/FiO_2 (mmHg)	400–500	231.4 ± 104.5	137.8 ± 62.9	190.9 ± 79.6	0.0004 [ab] 0.037 [bc]
Temperature (°C)	36.1–37.0	36.6 ± 0.4	36.9 ± 0.8	36.5 ± 0.5	ns
Heart rate (/min)	60–100	85.6 ± 15.0	90.7 ± 8.8	97.9 ± 8.2	<0.0001 [ac] 0.017 [bc]
Leukocytes (x $10^3/\mu L$)	4.5–10	8.5 ± 4.5	10.8 ± 4.2	11.6 ± 4.2	0.048 [ab] 0.011 [ac]
Lymphocytes (x $10^3/\mu L$)	1–3	1.3 ± 0.7	0.8 ± 0.4	1.0 ± 0.8	0.034 [ac]
Neutrophils (x $10^3/\mu L$)	3–7	6.3 ± 4.6	9.8 ± 4.0	10.1 ± 4.0	0.0014 [ab] 0.0009 [ac]
Monocytes (x $10^3/\mu L$)	0.3–0.8	0.5 ± 0.3	0.5 ± 0.3	0.4 ± 0.3	ns
Neutrophil-to-lymphocyte ratio	0.78–3.53	9.8 ± 22.6	16.1 ± 14.0	16.0 ± 12.0	0.0006 [ab] 0.0002 [ac]
Creatinine (mg/ dL)	0.66–1.09	4.0 ± 15.2	0.9 ± 0.4	0.9 ± 0.4	ns
Urea (mg/dL)	17–43	39.0 ± 31.3	40.3 ± 17.4	54.7 ± 36.8	0.012 [ac]
L-lactate (mmol/ L)	1–1.5	2.2 ± 0.9	2.2 ± 0.9	2.9 ± 1.3	ns
Lactate dehydrogenase (U/L)	140–271	257.3 ± 104.7	401.9 ± 303.6	405.8 ± 155.2	0.0062 [ab] 0.0006 [ac]
C-reactive protein (mg/ dL)	0–8	120.4 ± 151.6	172.9 ± 137.1	204.7 ± 120.5	0.024 [ac]
Procalcitonin (ng/dL)	Bacterial infection: <0.10, very unlikely <0.25, unlikely <50,	0.18 ± 0.33	0.34 ± 0.53	0.86 ± 2.31	0.0001 [ac]

(*continued on next page*)

Table 1 (*continued*)

	Reference value	Moderate [a] (n = 25)	Severe [b] (n = 27)	Critical [c] (n = 25)	*P* value
	probable >50, highly probable				
D-dimer (μg/L)	0–550	908 ± 1483	2079 ± 4290	4609 ± 9487	0.0001 [ac]
Ferritin (ng/mL)	20–204	748.7 ± 572.2	1985 ± 2408	1381 ± 1459	0.025 [ab]

NA, Not applicable. ns, not significant. Values represent mean ± SD.
For critical patients, the reported values were measured on ICU admission.

mL, $P = 0.0302$), while the levels of IL-6, IL-8, IL-10, IFN-β, granulysin and IP-10 were not significantly different between male and female patients. The serum concentrations of these cytokines did not show a significant correlation with the SOFA score or the WHO COVID-19 clinical progression scale (Supplementary Fig. 1). ROC curves indicate that IL-6 and IL-10 have the highest accuracy to discriminate both between critical patients and contacts, and between surviving and non-surviving patients, followed by sFas and granulysin (Fig. 3A to 3G, right panels). IL-6 has a sensitivity of 73.1 % and a specificity of 78.6 % to discriminate between surviving and non-surviving patients (optimal cutoff value, 37.2 pg/mL), while IL-10 has a sensitivity of 80.8 % and a specificity of 85.7 % (optimal cutoff value, 12.3 pg/mL), sFas has a sensitivity of 82.7 % and a specificity of 63 % (optimal cutoff value, 11714.5 pg/mL) and granulysin has a sensitivity of 76.9 % and a specificity of 51.9 % (optimal cutoff value, 9673.5 pg/mL). The serum concentrations of IL-1β, IFN-λ1, IL-12p70, IFN-α2, IFN-λ2/3, GM-CSF, TNF-α, IFN-γ, IL-2, IL-4, IL-17A, sFasL, granzyme A, granzyme B and perforin were not significantly different between the groups (Supplementary Fig. 2).

Most of the COVID-19 patients included in this study had secondary infections (Table 1), with *Acinetobacter baumannii, Enterococcus faecalis, Escherichia coli* and *Pseudomonas aeruginosa* as the most frequently isolated microorganisms. We next measured the serum concentrations of

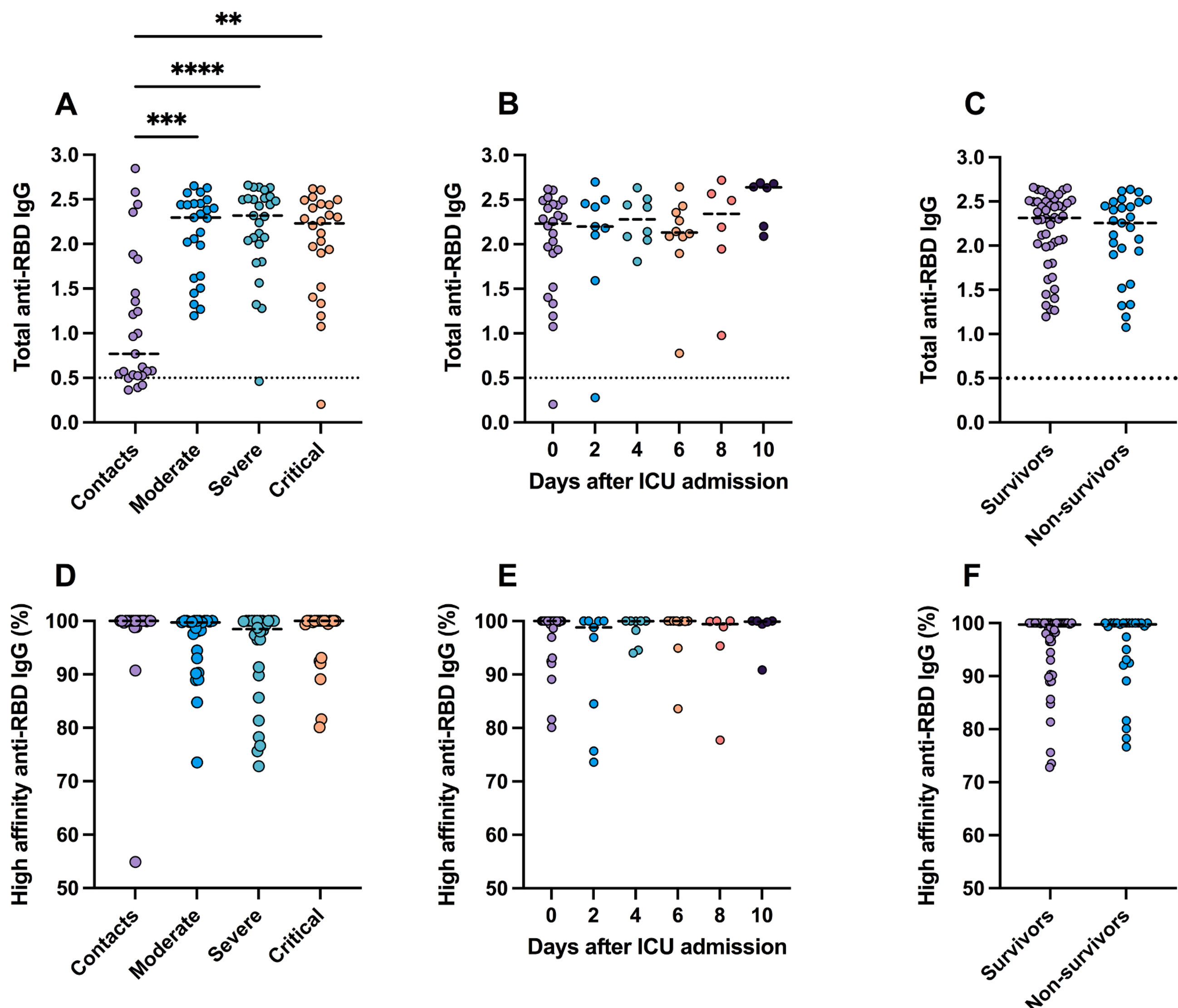

Fig. 1. COVID-19 patients have high-affinity anti-RBD IgG antibodies. (A) anti-RBD IgG antibodies were measured in the sera of asymptomatic individuals that were household contacts of COVID-19 patients (n = 25), and in the sera of patients with moderate (n = 25), severe (n = 27) and critical (n = 25, upon ICU admission) COVID-19. **(B)** In patients with critical COVID-19, anti-RBD IgG antibodies were measured upon ICU admission (day 0), and 2, 4, 6, 8 and 10 days later. **(C)** COVID-19 patients were also classified as survivors (n = 49) and non-survivors (n = 28). **(D to F)** The percentage of high-affinity anti-RBD IgG antibodies was determined in each serum sample with the urea 7 M method. The test cutoff value is indicated with dotted lines. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0001$.

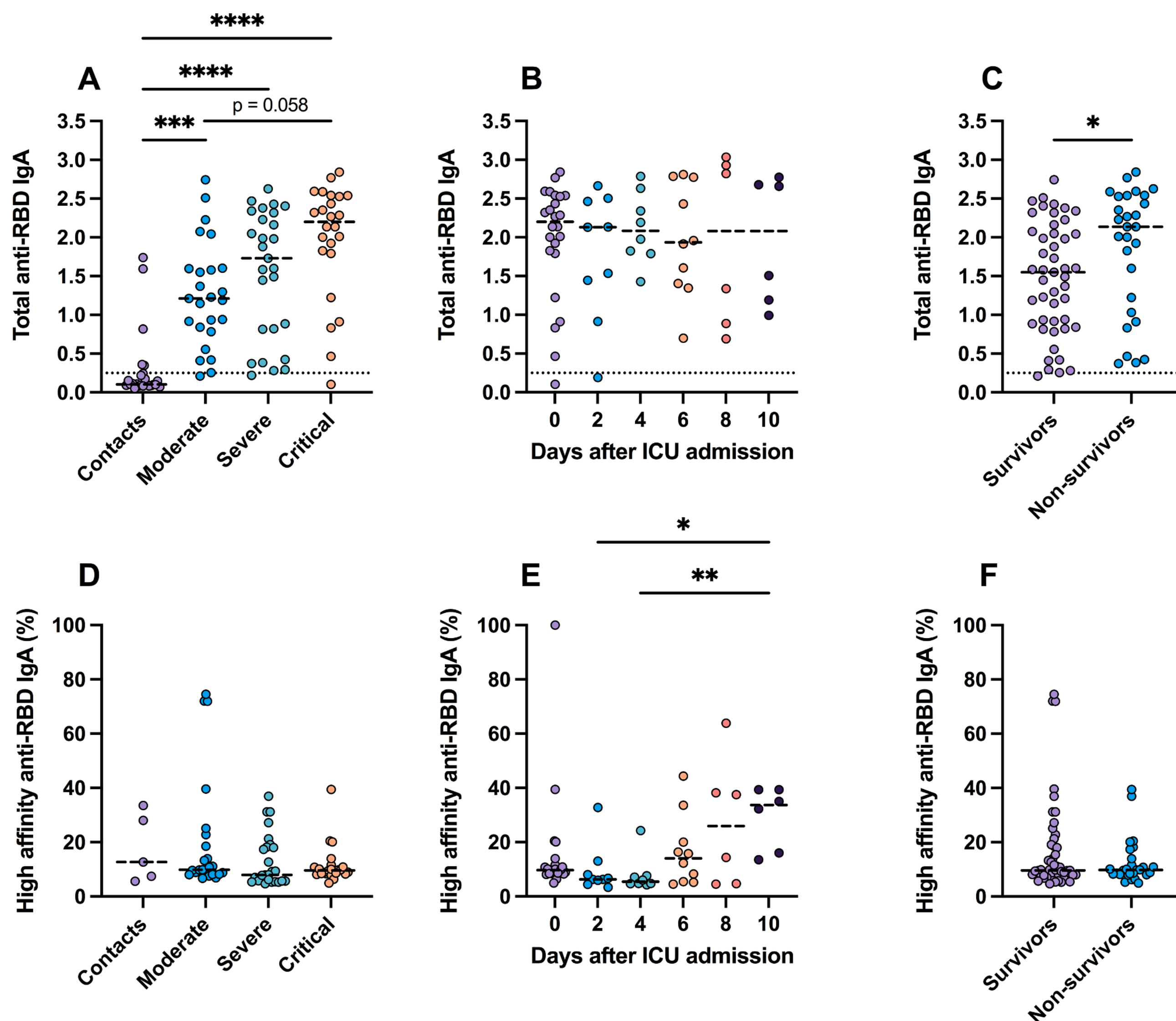

Fig. 2. Low-affinity anti-RBD IgA antibodies are increased in non-surviving COVID-19 patients. (A) anti-RBD IgA antibodies were measured in the sera of asymptomatic individuals that were household contacts of COVID-19 patients (n = 25), and in the sera of patients with moderate (n = 25), severe (n = 27) and critical (n = 25, upon ICU admission) COVID-19. **(B)** In patients with critical COVID-19, anti-RBD IgA antibodies were measured upon ICU admission (day 0), and 2, 4, 6, 8 and 10 days later. **(C)** COVID-19 patients were also classified as survivors (n = 49) and non-survivors (n = 28). **(D to F)** The percentage of high-affinity anti-RBD IgA antibodies was determined in each serum sample with the urea 7 M method. The test cutoff value is indicated with dotted lines. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0001$.

the intestinal permeability markers D-lactate, I-FABP and zonulin, in addition to IL-22, in these patients. The concentration of D-lactate, a metabolite that is produced by bacterial fermentation (Smith et al., 1986), was increased in the serum of severe patients, compared to the contacts ($P = 0.0028$) (Fig. 4A, first panel). In critical COVID-19 patients, the serum concentrations of D-lactate increased over time ($P = 0.0141$) (Fig. 4A, second panel). The levels of D-lactate were not significantly different in surviving and non-surviving patients (Fig. 4A, third panel), but they were increased in patients with secondary infections ($P = 0.0340$) (Fig. 4A, fourth panel). The serum levels of IL-22, a cytokine associated with intestinal barrier integrity (Duffin et al., 2016), were not significantly different in moderate, severe and critical patients, in surviving and non-surviving patients, or in patients with or without secondary infections (Fig. 4B). However, the levels of this cytokine decreased over time in critical patients ($P = 0.0001$) (Fig. 4B, second panel).

The levels of I-FABP, a protein that is found in enterocytes and is released during necrosis (Derikx et al., 2010), were also not significantly different in moderate, severe and critical patients, in surviving and non-surviving patients, or in patients with or without secondary infections (Fig. 4C), but the levels of this protein increased with time in some critical patients (Fig. 4C, second panel). The levels of zonulin, which are associated with increased intestinal permeability (Tripathi et al., 2009), were increased in severe patients, compared with the contacts ($P = 0.0290$), and in patients with secondary infections, compared to patients without these infections ($P = 0.0290$) (Fig. 4D). COVID-19 patients with secondary infections had increased levels of IL-6, IL-8, IL-10, IFN- β and sFas, compared to COVID-19 patients without these infections ($P = 0.0074$, $P = 0.0027$, $P = 0.0005$, $P = 0.0171$ and $P = 0.0126$, respectively), but the levels of anti-RBD IgA antibodies, granulysin and IP-10 were not significantly different between these groups (Fig. 5, A-H). Patients with secondary infections had higher SOFA scores than patients without these infections ($P < 0.0001$) (Fig. 5I), and this increased disease severity was associated with higher mortality: 95 % of the patients without secondary infections survived, while only 52 % of the patients with secondary infections survived.

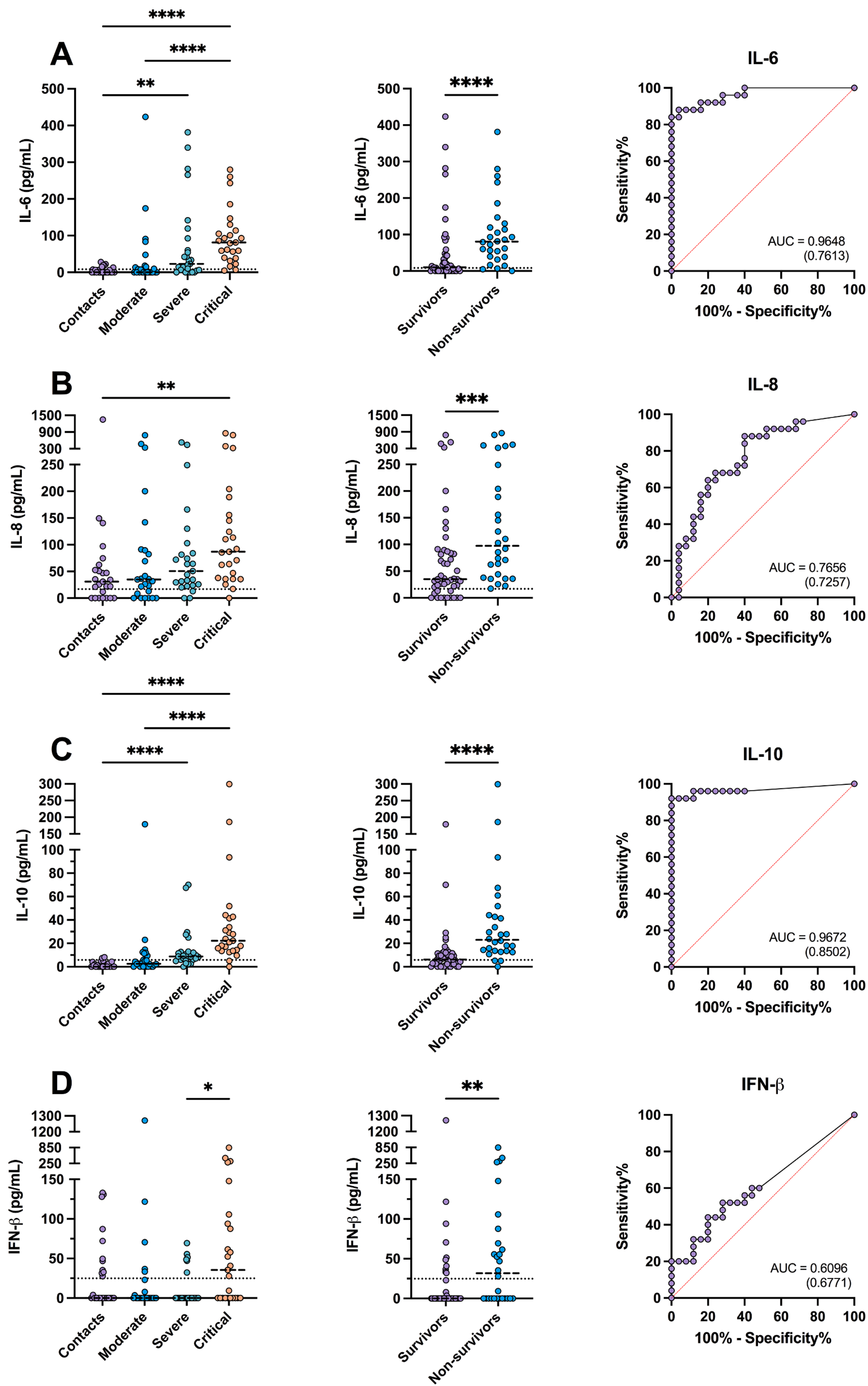
A
B
C
D
IL-6 (pg/mL)
IL-8 (pg/mL)
IL-10 (pg/mL)
IFN-β (pg/mL)
Contacts
Moderate
Severe
Critical
Survivors
Non-survivors
IL-6
IL-8
IL-10
IFN-β
Sensitivity%
100% - Specificity%
AUC = 0.9648
(0.7613)
AUC = 0.7656
(0.7257)
AUC = 0.9672
(0.8502)
AUC = 0.6096
(0.6771)

Fig. 3. Severe and critical COVID-19 patients have a mixed serum profile of pro-inflammatory, anti-inflammatory and anti-viral cytokines. The serum concentrations of IL-6 **(A)**, IL-8 **(B)**, IL-10 **(C)**, IFN-β **(D)**, sFas **(E)**, granulysin **(F)** and IP-10 **(G)** were measured in asymptomatic individuals that were household contacts of COVID-19 patients (n = 25), and in COVID-19 patients (n = 77), classified according to disease severity (left panels) or survival (middle panels). The assay lower detection limits are indicated with dotted lines. Receiver operating characteristic (ROC) curves (right panels) were used to analyze the accuracy of each cytokine to discriminate between critical COVID-19 patients and contacts. The corresponding areas under the curve (AUC) are shown in each ROC curve. The numbers in parenthesis indicate the AUC of ROC curves that analyze the accuracy of each cytokine to discriminate between surviving and non-surviving COVID-19 patients. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0001$. ns, not significant.

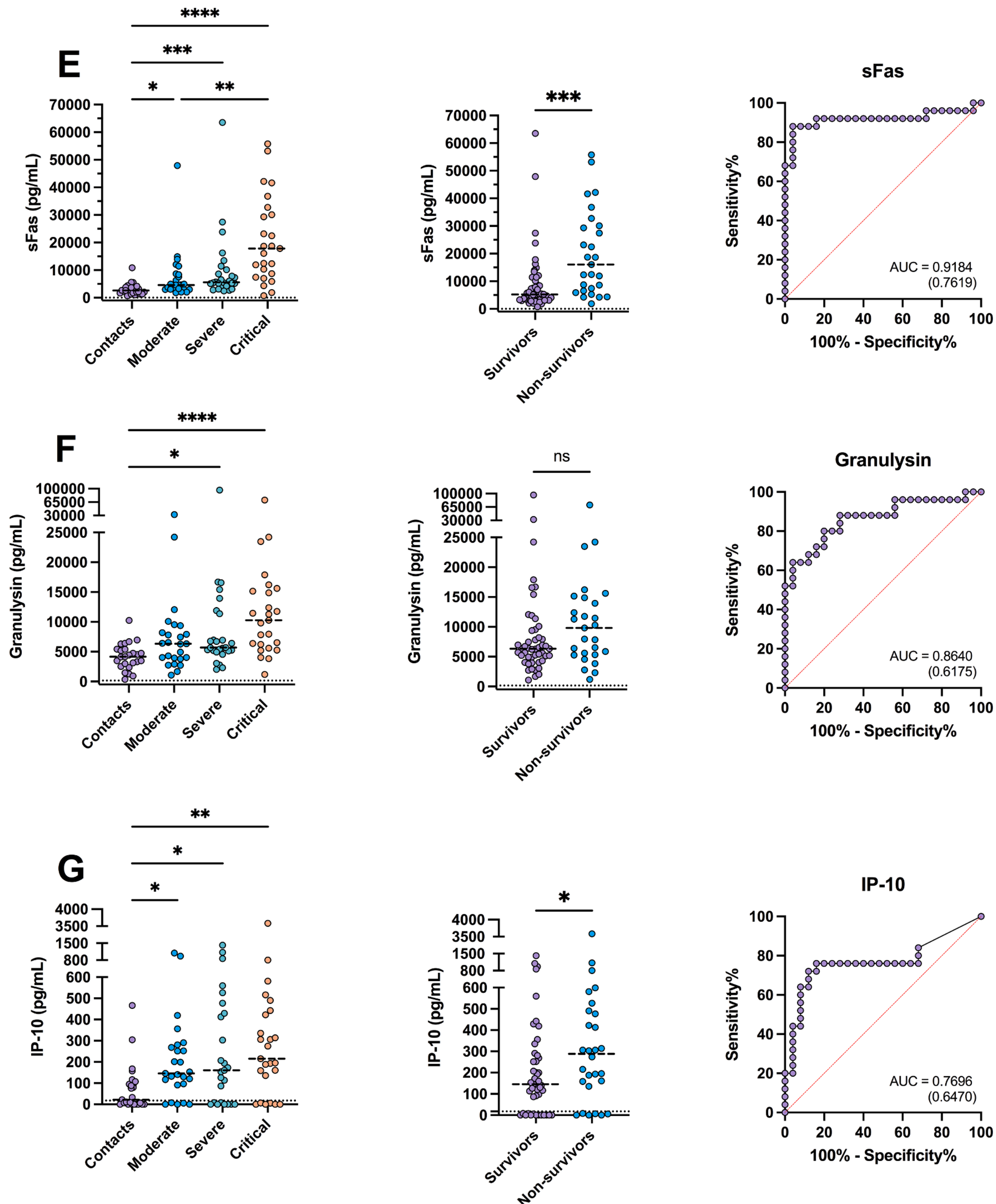

Fig. 3. (*continued*).

ROC curves indicate that D-lactate, IL-22, I-FABP and zonulin had a lower accuracy to discriminate both between critical COVID-19 patients and contacts, and between surviving and non-surviving patients, compared to IL-6, IL-10, sFas and granulysin. In addition, ROC curves indicate that the levels of anti-RBD IgA antibodies had a high accuracy to discriminate between critical COVID-19 patients and contacts, but a lower accuracy to discriminate between surviving and non-surviving patients, compared to IL-6, IL-10 and sFas (Supplementary Fig. 3). The serum concentrations of the intestinal permeability markers did not show significant correlations with the analyzed cytokines, with anti-RBD IgA antibodies, with clinical markers of inflammation, or with the SOFA score (Supplementary Fig. 1). However, principal component

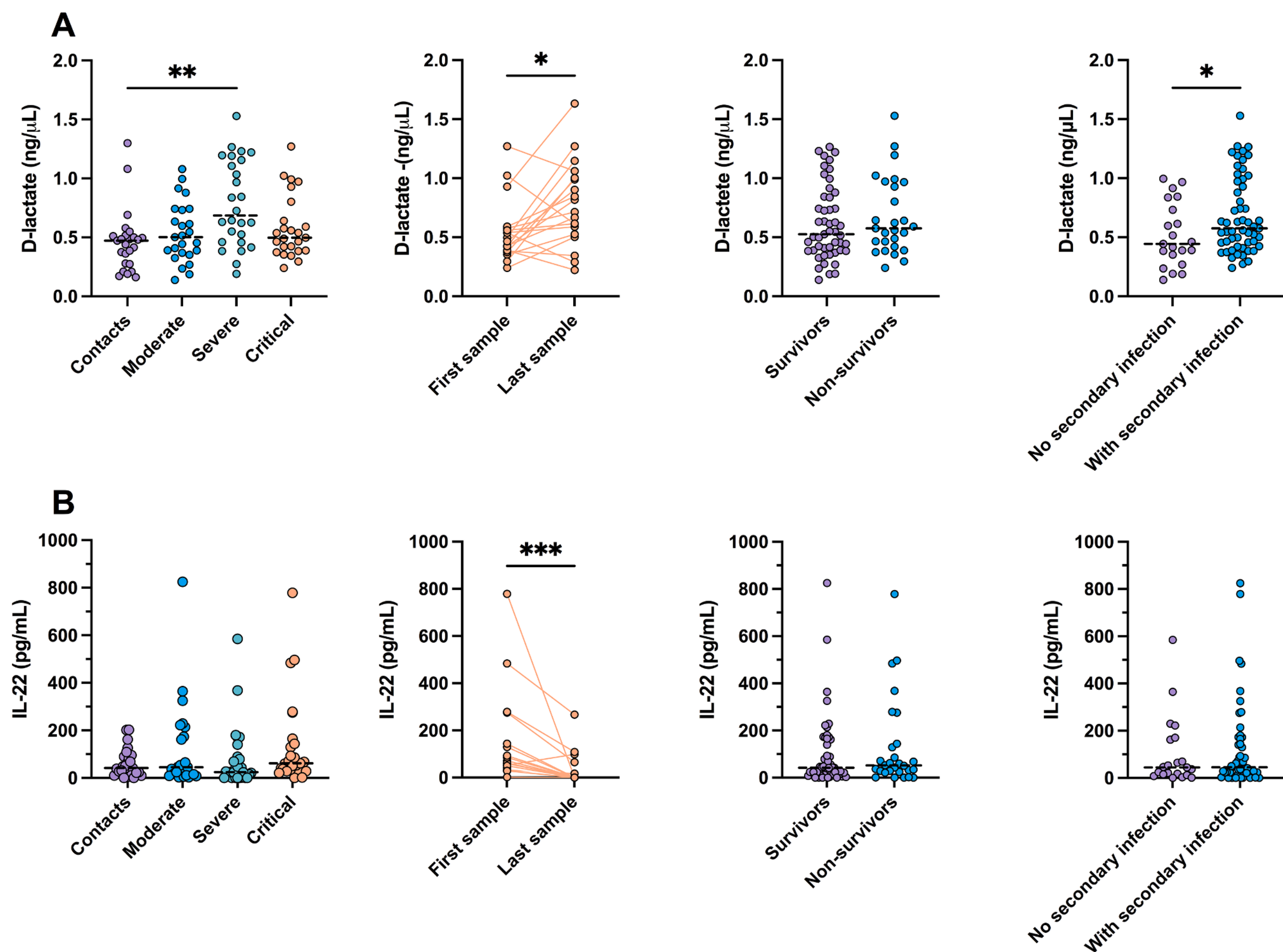

Fig. 4. Severe COVID-19 patients have increased intestinal permeability, and markers of increased intestinal permeability are elevated in COVID-19 patients with secondary infections. The serum concentrations of D-lactate **(A)**, IL-22 **(B)**, I-FABP **(C)** and zonulin **(D)** were measured in asymptomatic individuals that were household contacts of COVID-19 patients (n = 25), and in COVID-19 patients (n = 77), which were classified according to disease severity (first panels). In patients with critical COVID-19 (n = 25), these markers were measured upon ICU admission (first sample) and in the last available sample for each individual (second panels). COVID-19 patients were also classified according to survival (third panels) and to the presence of secondary infections (fourth panels). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

analyses of these intestinal permeability markers, in combination with IL-6, IL-8, IL-10, IFN- β, sFas, granulysin and IP-10, and with the clinical parameters in Table 1, discriminate critical patients (Fig. 6A), non-surviving patients (Fig. 6B) and patients with secondary infection (Fig. 6C), which highlights the overlap of these clinical conditions and their association with the measured parameters.

4. Discussion

In order to identify biomarkers for the severity and the lethality of COVID-19, we first evaluated the levels and affinities of anti-RBD IgG and IgA antibodies in COVID-19 patients. In accordance with previous reports (Chen et al., 2021, Zervou et al., 2021), we found increased levels of anti-RBD IgG and IgA antibodies in moderate, severe and critical COVID-19 patients, compared to asymptomatic individuals that were household contacts of the patients. In addition, we found that the IgG antibodies had high affinity for RBD, while the IgA antibodies had low affinity for RBD. The affinity of the IgG antibodies remained constant for up to 10 days, while the affinity of the IgA antibodies increased significantly during these 10 days in the critical patients. Tang *et al.* reported an increase in the affinity of polyclonal antibodies (plasma samples) from surviving COVID-19 patients for the SARS-CoV-2 prefusion spike protein, and this affinity maturation was detectable within days. In contrast, non-surviving patients had minimal or no affinity maturation during the same period (Tang et al., 2021). Our results suggest that the affinity maturation observed by Tang *et al.* could be attributed to IgA antibodies.

We also noted that most of the contacts included in our study had detectable levels of anti-RBD IgG antibodies, which could indicate a current asymptomatic infection with SARS-CoV-2. These individuals shared a house with symptomatic COVID-19 patients for several days (the average times between the symptom onset and the collection of the first blood sample in each patient group are given in Table 1), which would be enough time to induce antibody production in response to infection (Siracusano et al., 2021). However, most of the contacts had no detectable levels of anti-RBD IgA antibodies. *Siracusano et al.* report that SARS-CoV2-infected asymptomatic individuals have both anti-S1 IgG and IgA antibodies (Siracusano et al., 2021), so the presence of anti-RBD IgG antibodies without anti-RBD IgA antibodies may indicate a previous exposure of the contacts to SARS-CoV-2, rather than a current infection with the virus.

We report that the levels of anti-RBD IgA antibodies, but not of anti-RBD IgG antibodies, were significantly increased in non-surviving COVID-19 patients, compared to surviving patients. Tang *et al.* also observed higher levels of anti-spike protein IgA antibodies in non-surviving patients, compared to surviving patients (Tang et al., 2021).

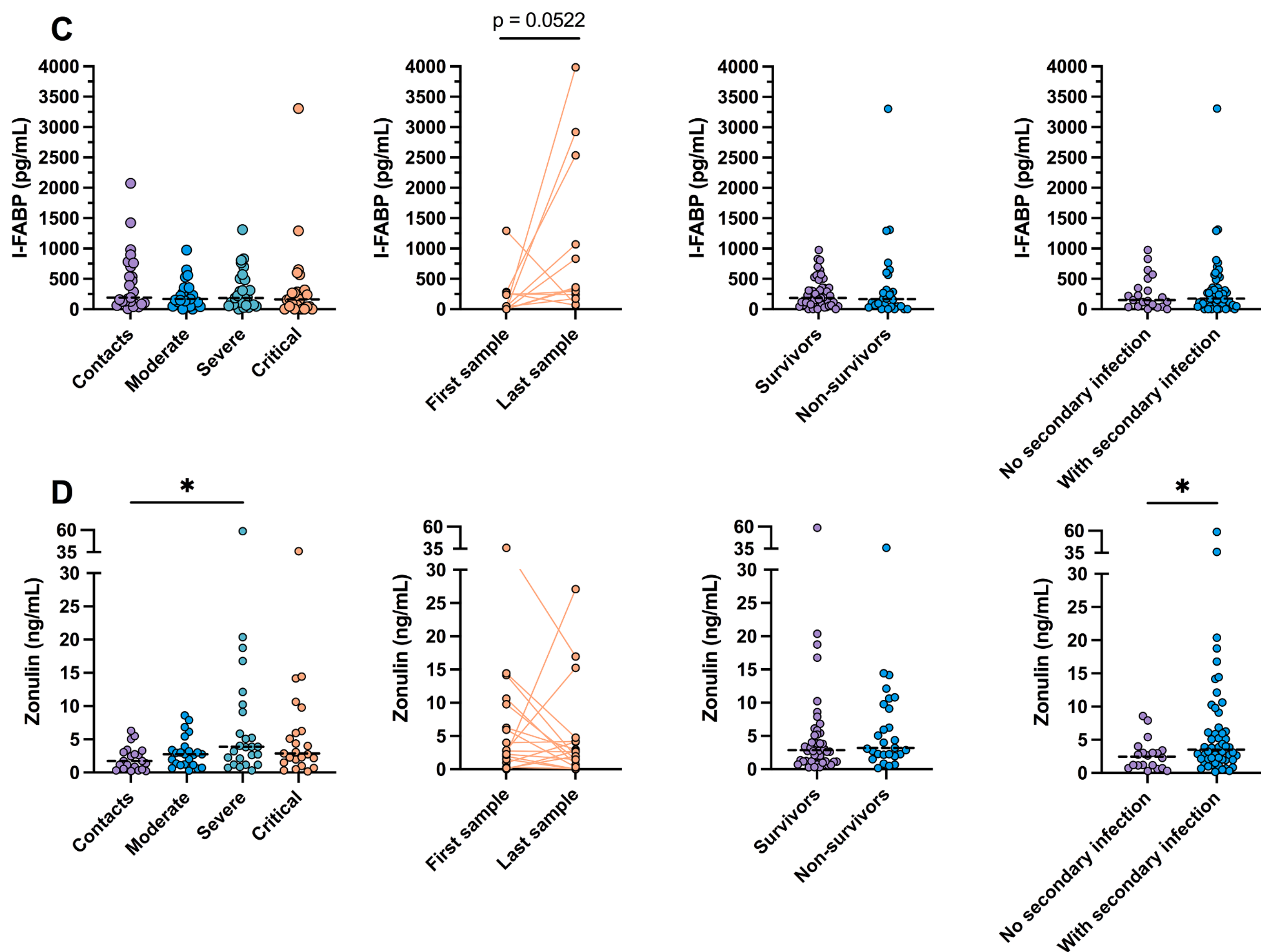

Fig. 4. (*continued*).

IgA immune complexes can bind to the Fc receptor FcαRI on neutrophils, eosinophils, monocytes, macrophages and platelets (van Gool and van Egmond, 2020), and induce the production of pro-inflammatory cytokines and chemokines (Breedveld and van Egmond, 2019). In addition, FcαRI signaling induces phagocytosis, degranulation, reactive oxygen species production, neutrophil extracellular trap formation and antigen presentation (Breedveld and van Egmond, 2019). Our results and those of Tang *et al.* suggest that, in non-surviving COVID-19 patients, IgA antibodies may amplify the inflammatory response through FcαRI, further complicating the deregulated immune response that is observed in these patients.

The severe and critical COVID-19 patients included in this study had increased levels of IL-6, IL-10, sFas, granulysin and IP-10, and non-surviving patients had increased levels of IL-6, IL-8, IL-10, IFN-β, sFas and IP-10. IL-6 has been identified as a biomarker for disease progression and fatality in COVID-19 (Santa Cruz et al., 2021), while IL-8, IL-10 and IP-10 have been evaluated as predictors of disease severity (Elemam et al., 2022, Guo et al., 2021). IP-10 (CXCL10) limits endothelial repair (Lupieri et al., 2020), which may promote thrombosis in critical and severe COVID-19 patients (Chen et al., 2020). Granulysin is present in the cytolytic granules of NK cells and cytotoxic CD8 T cells; it has lytic and pro-inflammatory activities (Krensky and Clayberger, 2009), and its plasma levels are higher in COVID-19 patients than in healthy controls (Li et al., 2020b). sFas blocks the interaction of membrane-bound Fas with its ligand, and so it prevents apoptosis. sFas increases during sepsis, particularly in non-surviving patients (Doughty et al., 2002, Lorente et al., 2020), and Lorente *et al.* reported that it is also increased in non-surviving COVID-19 patients, compared to surviving patients (Lorente et al., 2021), which is in accordance with our observations. The prevention of Fas-mediated neutrophil apoptosis by sFas may explain the neutrophilia that is observed in COVID-19 patients, and it may also contribute to the perpetuation of the inflammatory response that leads to organ failure. We noted that the levels of sFas were increased in male patients, compared to female patients, and this was the only cytokine whose concentrations differed according to gender. Male gender is associated with a higher risk for infection and for severe COVID-19 (Chaturvedi et al., 2022), and higher sFas levels in males may contribute to these increased risks. We performed ROC curves analysis, which indicated that IL-6 had the highest accuracy to discriminate between surviving and non-surviving COVID-19 patients, followed by IL-10, sFas and granulysin. These four molecules had high sensitivity and specificity to discriminate between these two groups of individuals, indicating that they could be used for the early identification of patients with increased risk of a fatal outcome.

Systemic inflammation has been associated with an increase in intestinal permeability and in microbial translocation, which could be a source of secondary infections (Mittal and Coopersmith, 2014). Here we report that the levels of anti-RBD IgA antibodies were significantly increased in non-surviving COVID-19 patients, compared to surviving patients, and a possible explanation for this observation is that the increase in IgA antibodies is a response to increased microbial translocation from the intestine. The levels of anti-RBD IgA antibodies tended to be higher in COVID-19 patients with secondary infections, compared to patients without these infections, but without reaching statistical significance. However, the serum concentrations of D-lactate, a metabolite that is produced through fermentation by several bacteria (Derikx

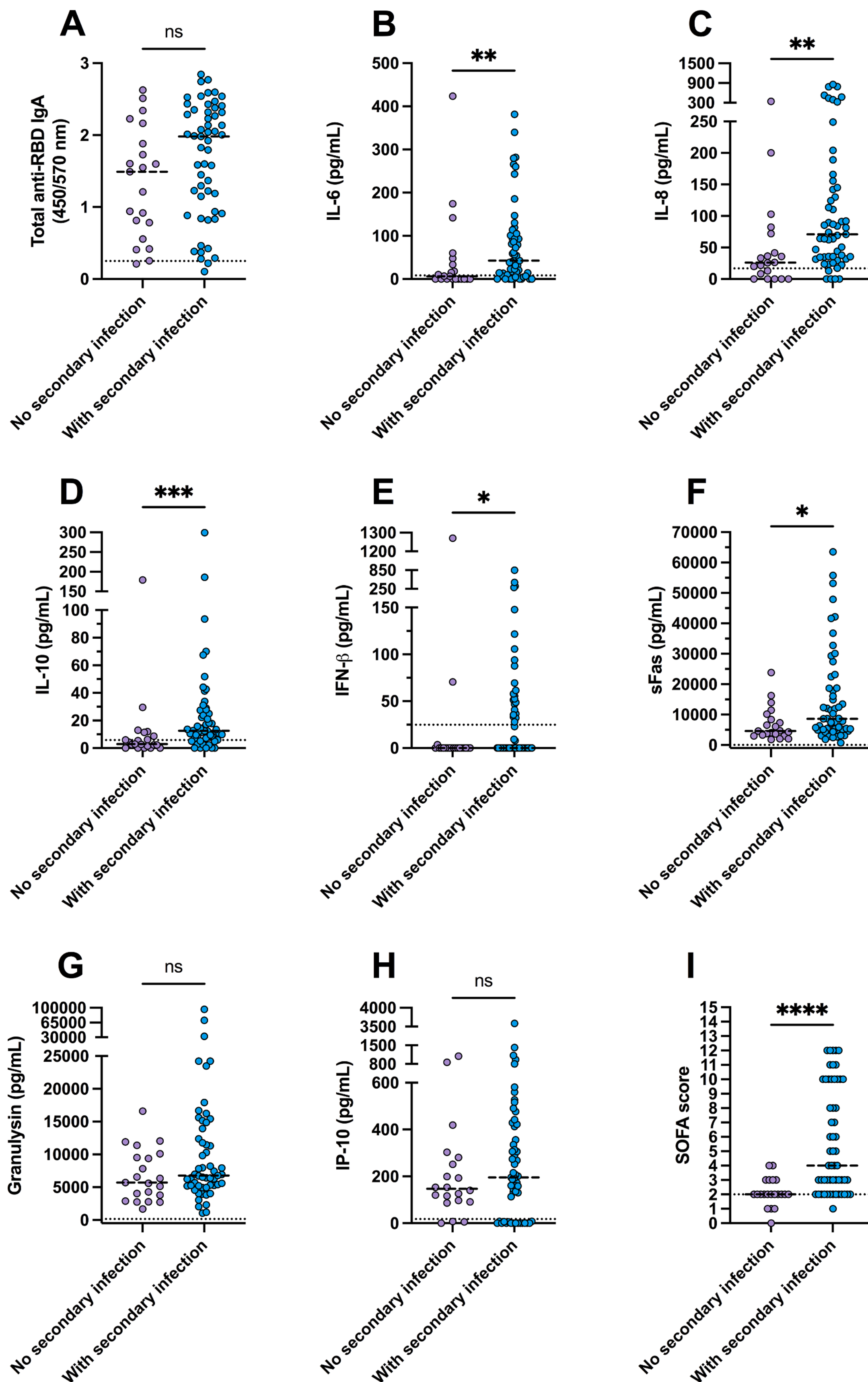

Fig. 5. COVID-19 patients with secondary infections have increased levels of IL-6, IL-8, IL-10, IFN-β and sFas, and an increased disease severity. The serum levels of anti-RBD IgA antibodies **(A)**, IL-6 **(B)**, IL-8 **(C)**, IL-10 **(D)**, IFN-β **(E)**, sFas **(F)**, granulysin **(G)** and IP-10 **(H)** were measured in COVID-19 patients (n = 77), which were classified according to the presence of secondary infections. The Sequential Organ Failure Assessment (SOFA) score was also determined in these patients **(I)**. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ****, $P < 0.0001$. ns, not significant.

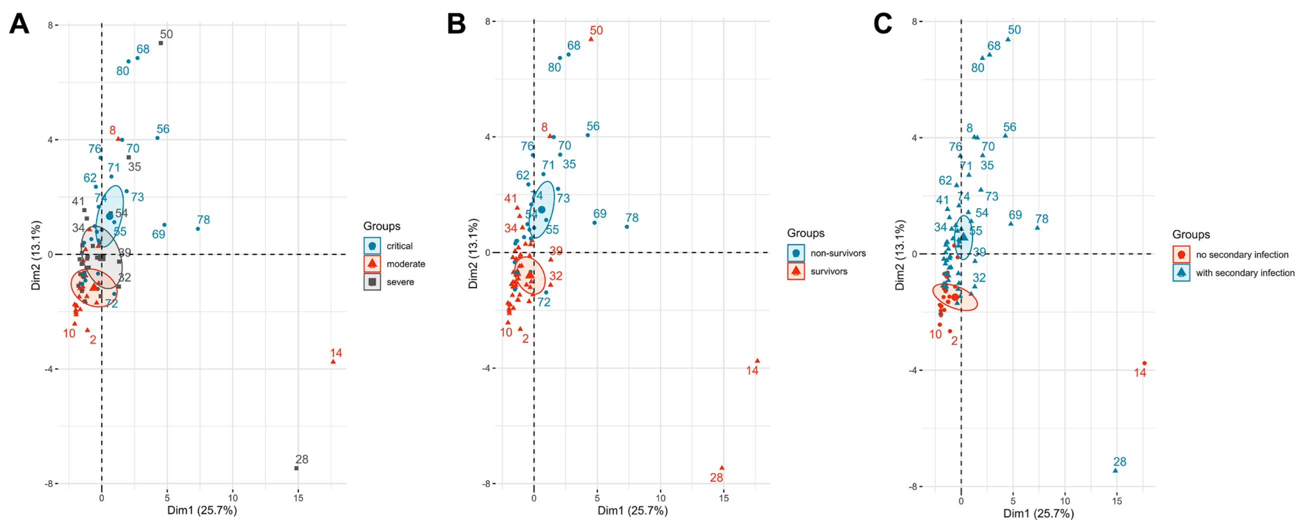

Fig. 6. Principal component analyses of cytokines, intestinal permeability markers and clinical parameters in COVID-19 patients. Principal component analysis (PCA) score plots of COVID-19 patients (n = 77) indicating their disease severity **(A),** survival **(B)** and presence of secondary infections **(C).** The numbers in parenthesis indicate the explained percentage of the total variance for each dimension. The analysis was performed with the cytokines in Fig. 3, the intestinal permeability markers in Fig. 4 and the clinical parameters in Table 1.

et al., 2010), were increased in COVID-19 patients with secondary infections, which suggests that these patients had increased bacterial translocation from the intestine to the blood. The levels of D-lactate were increased in severe COVID-19 patients, and they increased over time in critical COVID-19 patients, suggesting that the intestinal barrier deteriorated progressively in these patients. In support of this suggestion, we also observed a decrease over time in the serum concentrations of IL-22 [a cytokine associated with intestinal barrier integrity (Duffin et al., 2016, Longman et al., 2014)] in these critical patients.

The severe COVID-19 patients included in this study had increased levels of zonulin. Zonulin disassembles the tight junctions in the intestinal epithelium, allowing the paracellular influx of microorganisms and dietary antigens (Sturgeon and Fasano, 2016). Our data suggest that this mechanism occurs in patients with severe COVID-19. In addition, we observed that the levels of zonulin were increased in patients with secondary infections, compared with patients without these infections, indicating that microbial translocation from the intestine could be a relevant source of these infections. Giron *et al.* report increased levels of zonulin, LPS-binding protein (which reflects exposure to LPS) and β-glucan (a fungal component) in severe COVID-19 patients, compared with mild COVID-19 patients and with healthy individuals (Giron et al., 2021). Oliva *et al.* report increased levels of zonulin and LPS in severe COVID-19 patients, compared with healthy individuals (Oliva et al., 2021a). They also report increased levels of LPS-binding protein, but not of I-FABP (a marker of enterocyte necrosis), in COVID-19 patients with pneumonia, compared with healthy individuals, and in COVID-19 patients that required ICU admission (Oliva et al., 2021b).

These studies, together with our results, provide evidence that severe COVID-19 is associated with increased tight junction permeability and with the translocation of microbial products into the blood, and they also support the investigation of zonulin antagonists as possible treatments for severe COVID-19, as proposed by Llorens *et al.* (Llorens et al., 2021). Our study strongly supports the association of severe COVID-19 with increased microbial translocation, because it is the first one that analyzes intestinal permeability markers according to the presence or absence of secondary infections in these patients. The translocation of microbial products from the intestine to the circulation would amplify the systemic inflammatory response. Accordingly, we observed that COVID-19 patients with secondary infections had increased levels of IL-6, IL-8, IL-10, IFN- β and sFas, and that they also had increased disease severity and mortality than patients without secondary infections. The simultaneous analysis of these cytokines, in combination with the intestinal permeability markers and with clinical parameters that we report here indicates that, in COVID-19, there is a significant overlap between patients with secondary infection, critical patients and non-surviving patients.

In conclusion, we report that non-surviving COVID-19 patients had higher levels of low-affinity anti-RBD IgA antibodies than surviving patients. We identified sFas and granulysin, in addition to IL-6 and IL-10, as possible biomarkers with high sensitivity and specificity to discriminate between surviving and non-surviving COVID-19 patients. Finally, we report that D-lactate and zonulin are increased in patients with severe COVID-19 and in COVID-19 patients with secondary infections, suggesting that increased intestinal permeability (caused by tight junction disassembly and not by enterocyte necrosis) is a source of secondary infections in these patients. Since COVID-19 patients with secondary infections had a higher risk of death than patients without these infections, as was also reported by Shafran *et al.* (Shafran et al., 2021), our results suggest that intestinal permeability markers could increase the sensitivity and specificity of the serum cytokines for the early identification of patients with a high risk of a fatal outcome.

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CRediT authorship contribution statement

Alejandro Hernández-Solis: Conceptualization, Funding acquisition, Investigation, Supervision. **Azmavet M. Güemes-González:** Data curation, Formal analysis, Investigation, Visualization. **Ximena Ruiz-Gómez:** Formal analysis, Investigation. **Pablo Álvarez-Maldonado:** Investigation. **Jessica Castañeda-Casimiro:** Investigation, Validation. **Argelia Flores-López:** Investigation. **Martha Alicia Ramírez-Guerra:** Investigation. **Omar Muñoz-Miranda:** Investigation. **Ruth L. Madera-Sandoval:** Formal analysis, Methodology, Visualization. **Lourdes A. Arriaga-Pizano:** Formal analysis. **Alejandro Nieto-Patlán:**

Investigation. **Sergio Estrada-Parra:** Formal analysis, Supervision. **Sonia Mayra Pérez-Tapia:** Formal analysis, Resources. **Jeanet Serafín-López:** Formal analysis, Project administration. **Rommel Chacón-Salinas:** Formal analysis, Funding acquisition, Writing – review & editing. **Alejandro Escobar-Gutiérrez:** Formal analysis, Writing – review & editing. **Rodolfo Soria-Castro:** Investigation. **Bibiana Patricia Ruiz-Sánchez:** Conceptualization, Formal analysis, Project administration, Writing – original draft. **Isabel Wong-Baeza:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.imbio.2022.152288.

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