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1) Baker, Monya. "1,500 scientists lift the lid on reproducibility." Nature, no. 533 (May 26, 2016): 452-54. doi:10.1038/533452a.

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RESEARCH ARTICLE

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The antibody response to the glycan α-Gal correlates with COVID-19 disease symptoms

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has affected millions of people worldwide. Characterization of the immunological mechanisms involved in disease symptomatology and protective response is important to progress in disease control and prevention. Humans evolved by losing the capacity to synthesize the glycan Galα1-3Galβ1-(3)4GlcNAc-R (α-Gal), which resulted in the development of a protective response against pathogenic viruses and other microorganisms containing this modification on membrane proteins mediated by anti-α-Gal immunoglobulin M (IgM)/IgG antibodies produced in response to bacterial microbiota. In addition to anti-α-Gal antibody-mediated pathogen opsonization, this glycan induces various immune mechanisms that have shown protection in animal models against infectious diseases without inflammatory responses. In this study, we hypothesized that the immune response to α-Gal may contribute to the control of COVID-19. To address this hypothesis, we characterized the antibody response to α-Gal in patients at different stages of COVID-19 and in comparison with healthy control individuals. The results showed that while the inflammatory response and the anti-SARS-CoV-2 (Spike) IgG antibody titers increased, reduction in anti-α-Gal IgE, IgM, and IgG antibody titers and alteration of anti-α-Gal antibody isotype composition correlated with COVID-19 severity. The results suggested that the inhibition of the α-Gal-induced immune response may translate into more aggressive viremia and severe disease inflammatory symptoms. These results support the proposal of developing interventions such as probiotics based on commensal bacteria with α-Gal epitopes to modify the microbiota and increase α-Gal-induced protective immune response and reduce severity of COVID-19.

KEYWORDS
antibody, coronavirus, COVID-19, immunology, microbiota, α-Gal

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1 | INTRODUCTION

The coronavirus disease 19 (COVID-19), a pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has rapidly evolved from an epidemic outbreak to a disease affecting the global population. SARS-CoV-2 infects human host cells by binding to the angiotensin-converting enzyme 2 (ACE2) receptor.[1] It has been established that COVID-19 mainly affects the respiratory tract, but as a systemic disease, it affects multiple processes including the gastrointestinal, cardiovascular, neurological, hematopoietic, and immune systems.[2] Several days after the onset of symptoms, the SARS-CoV-2 infection becomes more systemic and affects various organs with inflammatory responses and lymphocytopenia.[2] Lymphocytopenia is likely caused by the direct lethal effect of SARS-CoV-2 on lymphocytes with the ACE2 receptor[3] and the release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1) and IL-6 that induce apoptosis in lymphocytes.[4] The "cytokine storm syndrome (CSS)" has been associated with COVID-19 through the activation of the nuclear factor-kB (NF-kB) innate immune pathway resulting in the upregulation of pro-inflammatory cytokines.[5] Lymphocytopenia in patients with COVID-19 along with the rise in neutrophils has been associated with worse disease prognosis. Consequently, patients with respiratory distress syndrome in intensive care unit (ICU) show lower lymphocyte counts and higher mortality when compared to other COVID-19 patients.[6,7] Additionally, COVID-19 patients suffer dysbacteriosis in the gut and lung microbiota due to enrichment of opportunistic pathogens and depletion of beneficial commensals, which recommends the development of interventions such as probiotics to reduce the severity of COVID-19 through modification of the microbiota composition.[1,8,9]

Humans evolved by losing the capacity to synthesize the glycan Galα1-3Galβ1-(3)4GlcNAc-R (α-Gal), which resulted in the development of a protective response of anti-α-Gal IgM/IgG antibodies against pathogenic viruses (e.g., HIV), bacteria (e.g., *Mycobacterium*) and parasites (e.g., *Plasmodium*) containing this modification on membrane proteins.[10–14] The natural IgM/IgG antibodies against α-Gal are produced in response to bacteria with this modification in the microbiota.[10] In addition to anti-α-Gal antibody-mediated pathogen opsonization, this glycan induces various immune mechanisms such as B-cell maturation, macrophage response, activation of the complement system, upregulation of pro-inflammatory cytokines through the Toll-like receptor 2 (TLR2)/NF-kB innate immune pathway, and TLR-mediated induction of the anti-inflammatory nuclear factor-erythroid 2-related factor 2 signalling pathway.[14–16] In conjunction, the immune response to α-Gal in animal models has shown protection against infectious diseases without inflammatory responses.[10,12–14,17]

Based on these results, we have hypothesized that the immune response to α-Gal may play a role in the person-to-person variability in COVID-19 disease symptoms with a putative protective capacity.[18] First, if the virus contains α-Gal, it would be possible to limit the zoonotic transmission of SARS-CoV-2 by antibody-mediated opsonization.[18] Secondly, boosting α-Gal-mediated protective immune and anti-inflammatory responses may contribute to the control of COVID-19 while increasing protection to pathogens with α-Gal on their surface that negatively affect the individual response to SARS-CoV-2.[14,18]

To address this hypothesis, herein we characterized the antibody response to α-Gal in patients at different stages of COVID-19 and in comparison with healthy control individuals. The results showed that while the inflammatory response and the anti-SARS-CoV-2 (Spike) IgG antibody titers increased, reduction in anti-α-Gal antibody titers and alteration of anti-α-Gal antibody isotype composition correlated with COVID-19 severity. These results suggested that the inhibition of the α-Gal-induced immune response translates into more aggressive viremia and severe disease symptoms.

2 | MATERIALS AND METHODS

2.1 | COVID-19 patients and healthy control individuals

A retrospective case-control study was conducted in patients suffering from COVID-19 admitted to the University General Hospital of Ciudad Real (HGUCR), Spain from March 1 to April 15, 2020. The infection by SARS-CoV-2 was confirmed in all patients included in the study by the real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay from Abbott Laboratories (Abbott RealTime SARS-COV-2 assay, Abbott Park, IL) from upper respiratory tract samples after hospital admission. Clinical features, as well as laboratory determinations, were obtained from patients' medical records. The patients were grouped into (a) hospital discharge ($n = 27$), (b) hospitalized ($n = 29$) and (c) ICU ($n = 25$) (Table 1). Patients were hospitalized for developing a moderate-severe clinical condition with radiologically demonstrated pneumonia and failure in blood oxygen saturation. Patients with acute respiratory failure who needed mechanical ventilation support were admitted to a hospital ICU. Patients were discharged from the hospital due to the clinical and radiological improvement of pneumonia caused by the SARS-CoV-2, along with the normalization of analytical parameters indicative of inflammation, such as C-reactive protein (CRP), D-dimer, and blood cell count (Table 1). Samples from asymptomatic COVID-19 cases with positive anti-SARS-CoV-2 IgG antibody titers but negative by RT-PCR ($n = 10$) were collected on May 22-29, 2020 and included in the analysis. Samples from healthy control individuals (individuals without a record of tick bites and allergic reactions; $n = 37$, sex ratio F/M = 1.1, 41.4 ± 11.1 years old) were collected before the COVID-19 pandemic in April 2019. The use of samples and individual's data was approved by the Ethical and Scientific Committee (University Hospital of Ciudad Real, C-352, and SESCAM C-73).

2.2 | Serum and saliva samples

Serum samples were collected for confirmed COVID-19 patients and healthy control individuals. Nursing personnel at the HGUCR extracted blood samples. Blood samples were drawn in a vacutainer

TABLE 1 Clinical parameters and laboratory tests of COVID-19 symptomatic cohort

Parameters	Hospital discharge	Hospitalized	ICU	*f* ratio	*p*
Age (year)	61.0 ± 18.0	73.7 ± 12.6	57.2 ± 14.6	9.196	<.001
Female/male sex ratio	0.9	0.8	0.7	0.039	.962
Neutrophils (10^3 cells/μl)	7.0 ± 4.0	7.7 ± 4.4	14.2 ± 9.6	12.116	<.001
Neutrophils (%)	68.9 ± 14.1	76.8 ± 10.8	85.1 ± 10.9	13.771	<.001
Lymphocytes (10^3 cells/μl)	1.5 ± 0.5	1.2 ± 0.6	1.1 ± 0.7	4.223	.018
Lymphocytes (%)	19.0 ± 10.1	13.9 ± 8.4	8.4 ± 7.4	11.521	<.001
Neutrophil–lymphocyte count ratio (NLR)	5.4 ± 4.2	10.1 ± 10.0	18.8 ± 14.8	14.231	<.001
D-dimer (ng/ml)	712 ± 623	1514 ± 1528	6528 ± 9436	7.066	.002
C-reactive protein (CRP) (mg/dl)	1.0 ± 1.4	4.4 ± 5.7	10.4 ± 9.7	17.558	<.001

Note: The patients were grouped into hospital discharge ($n = 27$), hospitalized ($n = 29$) and ICU ($n = 25$). The results (average ± *SD*) were compared between different groups by one-way ANOVA test.

Abbreviations: ANOVA, analysis of variance; COVID-19, coronavirus disease 2019; ICU, intensive care unit.

tube without anticoagulant. The tube remained at rest for 15–30 min at room temperature (RT) for clotting. Subsequently, the tube was centrifuged at 1500*g* for 10 min at RT to remove the clot and obtain the serum sample. Serum samples were heat-inactivated for 30 min at 56°C and conserved at -20°C until used for analysis.[19] Saliva samples from asymptomatic COVID-19 cases were collected and stored at -20°C until used for analysis.

2.3 | Determination of antibody titers against SARS-CoV-2

Antibody titers specific for the recognition of virus infection based on IgG against SARS-CoV-2 Spike (EI 2606-9601G) and Nucleocapsid (EI 2606-9601-2G) proteins and IgA (EI 2606-9601A) were determined by ELISA (Euroimmun) following the manufacturer′s indications.[19,20] Briefly, 100 μl of the calibrator, positive and negative controls and serum samples at 1:100 dilution was added to the 96-microwell plate coated with SARS-CoV-2 proteins and incubated for 1 h at 37°C. After washing three times with 300 μl/well of wash buffer, 100 μl/well of enzyme conjugate (peroxidase-labelled anti-human IgG or IgA) were added and incubated for 30 min at RT. Then, after 3 washes with 300 μl/well of wash buffer, 100 μl/well of chromogen substrate solution were added and incubated for 15 min (EI 2606-9601-2 G) or 30 min (EI 2606-9601 G; EI 2606-9601 A) at RT. Finally, the colorimetric reaction was stopped with 100 μl/well of stop solution and the absorbance was measured in a spectrophotometer (Thermo Fisher Scientific) at O.D. of 450 nm. Results were evaluated semi-quantitatively by calculating the ratio between O.D. of the sample and the O.D of the calibrator, those under 0.8 considered as negative and those over 1.1 as positive.

2.4 | Determination of antibody titers against α-Gal

High absorption capacity polystyrene microtiter plates were coated with 50 ng of BSA coated with α-Gal (BSA-α-Gal, thereafter named α-Gal; Dextra) per well in carbonate-bicarbonate buffer (Sigma-Aldrich) and used for ELISA. After an overnight incubation at 4°C, coated plates were washed once with 100 μl/well PBS with 0.05% Tween 20 (PBST; Sigma-Aldrich), blocked with 100 μl/well of 1% human serum albumin (HAS) in PBST (Sigma-Aldrich) for 1 h at RT and then washed four times with 100 μl/well of PBST. Human serum and saliva samples were diluted 1:100 and 1:2, respectively, in PBST with 1% HAS and 100 μl/well were added into the wells of the antigen-coated plates and incubated for 1 h at 37°C. Plates were washed four times with PBST and 100 μl/well of goat anti-human immunoglobulins-peroxidase IgG (FC specific; Sigma-Aldrich), IgM (μ-chain specific; Sigma-Aldrich), IgE (ε-chain specific; Sigma-Aldrich), and IgA (heavy chain specific; Bio-Rad) secondary antibodies diluted 1:1000, v/v in blocking solution were added and incubated for 1 h at RT. Plates were washed four times with 100 μl/well of PBST and 100 μl/well of 3,3,´5,5-tetramethylbenzidine TMB (Promega) were added and incubated for 20 min at RT. Finally, the reaction was stopped with 50 μl/well of 2 N H_2SO_4 and the O.D. was measured in a spectrophotometer at 450 nm. The average of two technical replicates per sample was used for analysis after background (coated wells incubated with PBS and secondary antibodies) subtraction.

Reference values for serum immunoglobulin levels[21] were considered in the analysis of the profile of anti-α-Gal antibody isotypes. Although this methodology has been previously validated,[22,23] the anti-α-Gal immunoglobulin E (IgE) antibody titers were determined in sera from selected individuals ($n = 8$) with α-Gal syndrome, anaphylaxis, and skin local reactions to tick bites and healthy individuals without record of tick bites and allergic reactions using the ImmunoCAP Phadia 250 automated platform (Thermo Fisher Scientific) with the commercial ImmunoCap α-Gal bovine Thyroglobulin kit according to the manufacturer's instructions.[22] These values were used to draw a trendline ($R^2 = 0.83$) to calculate the corresponding kU/l values for the ELISA O.D. at 450 nm values using the formula IgE titers (kU/l) = 100 × EXP ([O.D.450 nm − 2.9]/0.2). Positive anti-α-Gal IgE levels were considered at a cut-off value of 0.35 kU/l.[23]

2.5 | Determination of IL-1 and IL-4 serum levels

Serum levels of IL-1 and IL-4 were determined by ELISA (Invitrogen) following the manufacturer's instructions. Briefly, 96-microwell plates coated in duplicate with anti-human IL-1β or IL-4 were washed twice with 400 μl/well of wash buffer and 100 μl of human IL-1β or IL-4 standard (20.00 pg/ml) at serial dilutions (1:2, 1:4, 1:8, 1:16, 1:32), 100 μl/well of sera at 1:2 dilution, and 100 μl/well of sample diluent as negative control. Then, 50 μl/well of biotin-conjugate were added to all wells. After incubation for 2 h at RT and three washes with 400 μl/well of wash buffer, 100 μl/well of streptavidin-HRP were added to all wells. After incubation for 1 h at RT and 3 washes with 400 μl/well of wash buffer, 100 μl/well of 3,3′,5,5′-tetramethylbenzidine or TMB substrate solution were added to all wells. As soon as the Standard 1 well reached an O.D. of 0.9 at 620 nm, the colorimetric reaction was stopped with 100 μl/well of stop solution and the absorbance was measured in a spectrophotometer (Thermo Fisher Scientific) at O.D. of 450 nm. Human IL-1β or IL-4 concentration (pg/ml) in each sample was calculated from the obtained standard curve.

2.6 | Statistical analysis

The ELISA O.D. at 450 nm values were compared between different groups by one-way analysis of variance (ANOVA) test ($p = .05$; https://www.socscistatistics.com/tests/ANOVA/default2.aspx). Clinical parameters and laboratory tests of COVID-19 symptomatic cohort (Table 1) were compared between different groups by one-way ANOVA test ($p = .05$). Pairwise comparisons between groups were conducted by Student's t test ($p = .05$). A Spearman Rho (r_s) correlation analysis ($p = .05$; https://www.socscistatistics.com/tests/spearman/default2.aspx) was conducted between anti-SARS-CoV-2 Spike IgG titers and COVID-19 disease severity (2 = asymptomatic, 3 = hospital discharge, 4 = hospitalized, 5 = ICU), anti-α-Gal IgA, IgE, IgM and IgG antibody titers and disease severity (1 = healthy, 2 = asymptomatic, 3 = hospital discharge, 4 = hospitalized, 5 = ICU), and for anti-α-Gal IgA and IgG antibody titers between serum and saliva samples.

3 | RESULTS

3.1 | Inflammatory biomarkers are associated with severity in COVID-19 patients

In the blood cell analysis, the ICU patients showed a higher lymphocytopenia, percentage and neutrophil counts when compared to hospital discharge and hospitalized individuals ($p < .001$; Figure 1A and Table 1). The cellular and biochemical indicators of systemic inflammation, neutrophil-lymphocyte count ratio (NLR), C-reactive protein (CRP), and D-dimer levels were higher in ICU patients when compared to other patients ($p < .002$; Figure 1A and Table 1).

All patients had pneumonia associated with COVID-19. Hospitalized patients in the plant were treated with oxygen therapy with a fraction of inspired oxygen (FiO2) above 50% and with the objective of reaching an O_2 saturation above 95%. When, despite the oxygen therapy, patients presented an O_2 saturation below 90%, a sustainable rate of breaths per minute (bpm) > 30 and an increase in respiratory work and assisted mechanical ventilation was required, and consequently they were admitted to ICU. Although more severe symptoms have been associated with elderly patients, herein older patients were recorded in the hospitalized and not the ICU group ($p < .001$; Table 1). The healthy and asymptomatic individuals did not show symptoms of inflammation. These results corroborated a higher inflammation rate in the most critical COVID-19 patients independently of the age factor.

3.2 | Immune response to SARS-CoV-2 increased with severity in COVID-19 patients

All COVID-19 symptomatic patients showed both IgA and IgG antibody titers against SARS-CoV-2 (Figure 1B). In asymptomatic cases, only IgG antibody titers were determined, and all tested positive (Figure 1B). However, only the IgG titers against the SARS-CoV-2 Spike protein significantly increased in accordance with disease symptoms ($p = .02$; Figure 1B) with a positive correlation ($r_s > 0$; $p = 0$; Figure 1B). These results showed that COVID-19 patients were immunocompetent despite the inflammatory response.

3.3 | Immune response to α-Gal varied in COVID-19 patients

The serum IgA, IgE, IgM and IgG antibody response to α-Gal was characterized in healthy individuals and COVID-19 patients at different disease stages (Figures 2 and 3a). The calculated anti-α-Gal IgE levels were below (8.3E − 5 to 3.4E − 02 kU/l) the cut-off value of 0.35 kU/l used for the diagnosis of the α-Gal syndrome. A negative

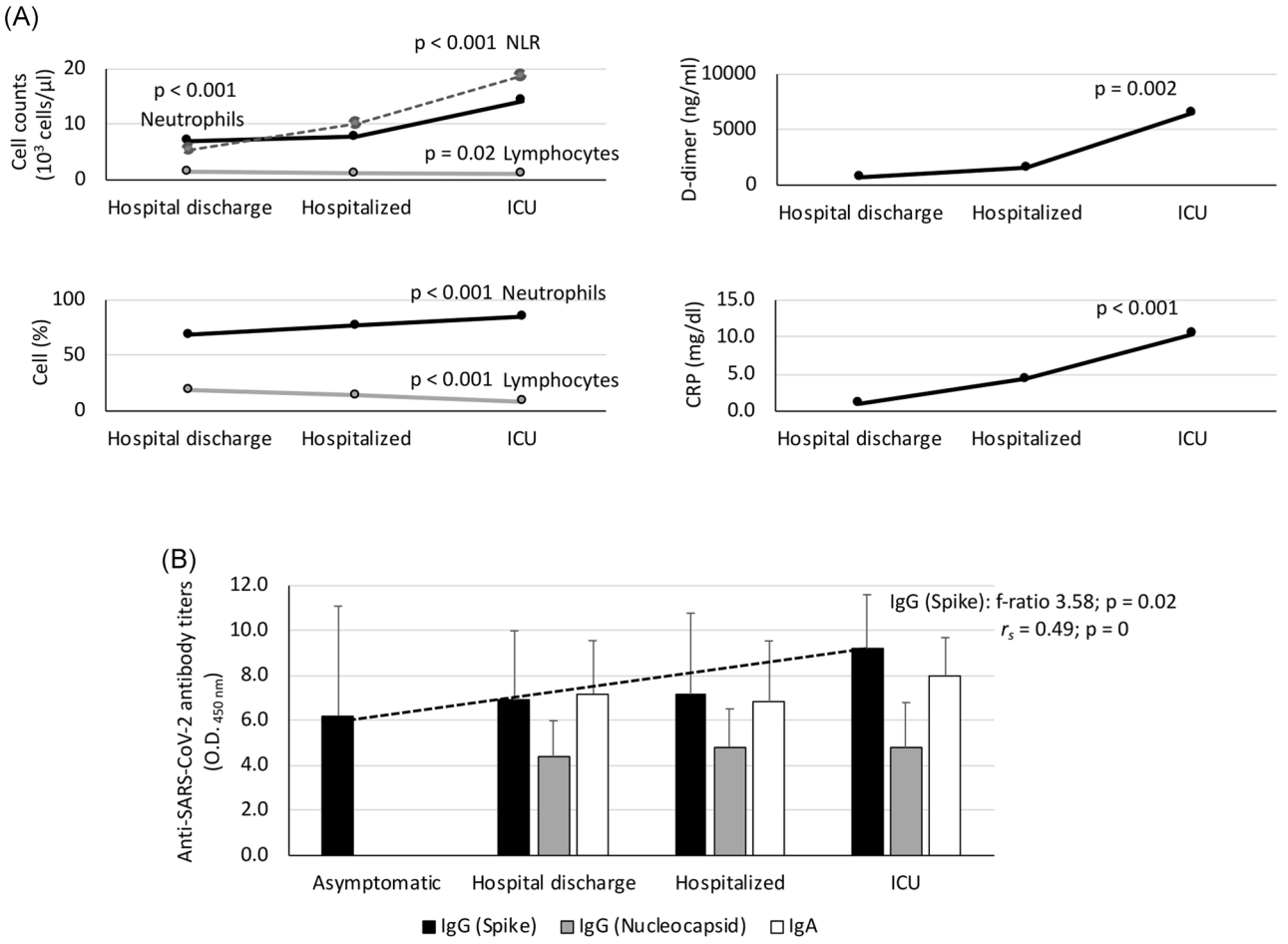

FIGURE 1 Laboratory tests in COVID-19 patients. A, Cellular and biochemical indicators of systemic inflammation included neutrophils (cell counts and percent), lymphocytes (cell counts and percent), NLR, D-dimer and CRP levels (Table 1). B, Serum anti-SARS-CoV-2 IgA, IgG (spike) and IgG (nucleocapsid) antibody levels were determined by ELISA. The patients were grouped as asymptomatic (n = 10), hospital discharge (n = 27), hospitalized (n = 29) and ICU (n = 25). The results were compared between different groups by one-way ANOVA test (p < .05). A Spearman rho (r_s) correlation analysis (p < .05) was conducted between anti-Spike IgG antibody titers and disease severity (2 = asymptomatic, 3 = hospital discharge, 4 = hospitalized, 5 = ICU). ANOVA, analysis of variance; COVID-19, coronavirus disease 2019; CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; ICU, intensive care unit; IgA, immunoglobulin A; NLR, neutrophil-lymphocyte count ratio; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

correlation was observed for IgE, IgM, and IgG between anti-α-Gal antibody titers and disease severity (r_s < 0; p = 0; Figure 3A). The anti-α-Gal IgA antibody titers did not vary between the different groups (p = .21136; Figure 3A) nor correlate with disease severity (r_s = 0.02; p = .91; Figure 3A). For anti-α-Gal IgM and IgG antibodies, the titers decreased from healthy to ICU individuals (p < .00001; Figures 2 and 3a). However, in asymptomatic cases, the anti-α-Gal IgE titers were higher than in healthy individuals and symptomatic COVID-19 patients (p < .000001; Figure 3A). In COVID-19 patients, the IgE but not IgM and IgG antibody titers were higher in hospitalized patients than in hospital discharge and ICU cases (p < .05; Figure 2).

The profile of anti-α-Gal antibody isotypes was qualitatively compared between groups including reference values for serum immunoglobulin levels (Figure 3B). The results evidenced that anti-α-Gal IgE and IgM antibodies are more abundant than reference values even in healthy individuals. However, the most abundant anti-α-Gal antibodies varied from IgM/IgG in healthy individuals to IgE (asymptomatic), IgG (hospital discharge), none (hospitalized) and IgA (ICU) in COVID-19 cases (Figure 3B). These results suggested a role for anti-α-Gal IgA, which increased in relative representation in ICU patients by four-fold (vs. healthy individuals), six-fold (vs. asymptomatic cases) and two-fold (vs. hospital discharge and hospitalized cases).

Despite differences in absolute values due to dilutions of the samples used for ELISA (1:100 for serum vs. 1:2 for saliva), as expected, anti-α-Gal IgA but not IgG antibody titers were higher in saliva than in serum samples (p = .0002; Figure 4A) but without a significant correlation (p > .05). The saliva anti-α-Gal IgA antibody titers were similar between asymptomatic COVID-19 cases and healthy individuals (p = .3049; Figure 4B).

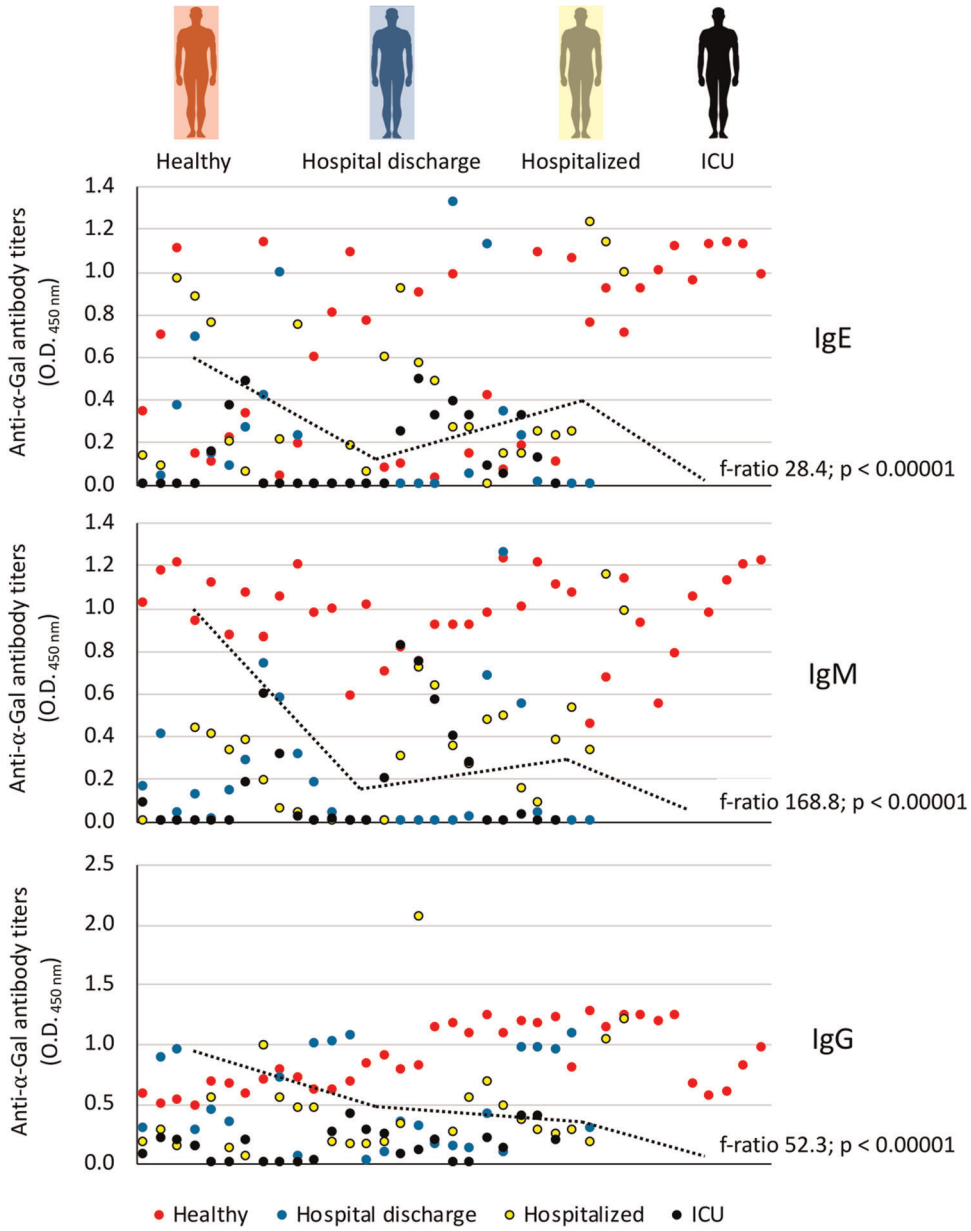

FIGURE 2 Anti-α-Gal antibody response in COVID-19 symptomatic patients and healthy controls. The IgE, IgM, and IgG anti-α-Gal antibody titers were determined by ELISA. Individuals were grouped as healthy controls ($n = 37$), hospital discharge ($n = 27$), hospitalized ($n = 29$) and ICU ($n = 25$). Values for individuals from all groups are shown in different colors and distributed across the entire *X*-axis. The results were compared between different groups by one-way ANOVA test ($p < .05$). ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; ICU, intensive care unit; IgE, immunoglobulin E; α-Gal, Galα1-3Galβ1-(3)4GlcNAc-R

The analysis of the cytokine response was focused on anti-inflammatory IL-4 and pro-inflammatory IL-1 serum levels. For both IL-4 and IL-1, serum levels did not vary significantly with disease symptoms and were similar in COVID-19 patients than in healthy individuals ($p > .4$). In hospitalized patients only, three and two different cases showed cytokine levels above 20 pg/ml (IL-4) and 0.2 pg/ml (IL-1), respectively. However, these results may be affected by different interventions and treatments to which patients were subjected that alter cytokine serum levels.

4 | DISCUSSION

Systemic inflammation is associated with changes in the quantity and composition of circulating blood cells and has been identified as the primary basic mechanism resulting in disability and increased mortality in COVID-19.[24] As previously reported,[25,26] in the blood cell analysis of cellular and biochemical indicators of systemic inflammation, ICU patients showed a higher lymphocytopenia independent of the age factor associated with more severe COVID-19 symptoms.[27]

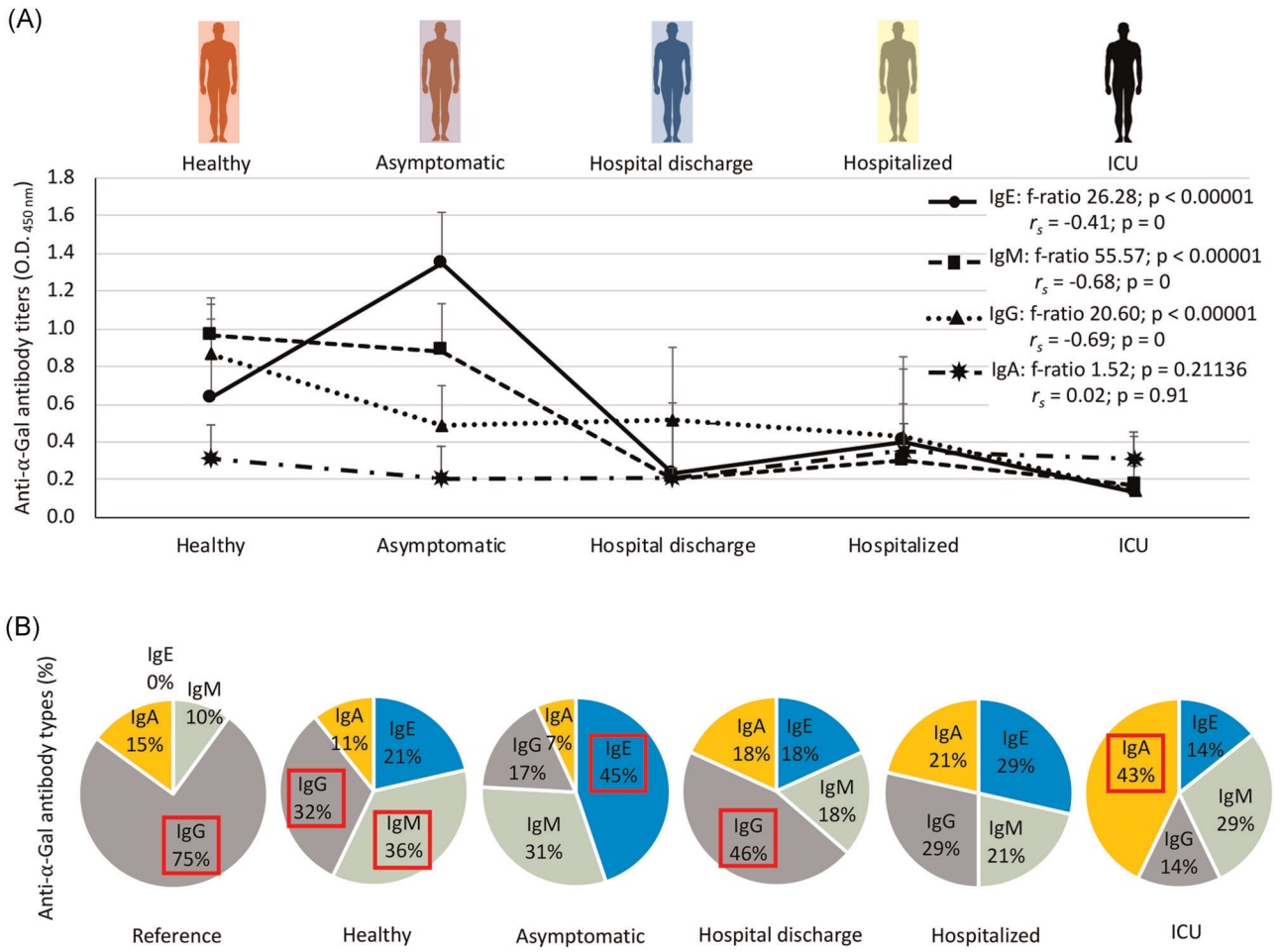

FIGURE 3 Serum anti-α-Gal antibody response in COVID-19 asymptomatic and symptomatic cases and healthy controls. A, The IgA, IgE, IgM and IgG anti-α-Gal antibody titers were determined by ELISA. Individuals were grouped as healthy controls ($n = 37$), asymptomatic ($n = 10$), hospital discharge ($n = 27$), hospitalized ($n = 29$) and ICU ($n = 25$). The results were compared between different groups by one-way ANOVA test ($p < .05$). A Spearman rho (r_s) correlation analysis ($p < .05$) was conducted between anti-α-Gal IgA, IgE, IgM and IgG antibody titers and disease severity (1 = healthy, 2 = asymptomatic, 3 = hospital discharge, 4 = hospitalized, 5 = ICU). B, Profile of anti-α-Gal antibody isotype (shown as percentage of antibody titers) for each group. Reference values for serum immunoglobulin levels were included. Antibody isotypes with highest representation on each group are highlighted in red. ANOVA, analysis of variance; COVID-19, coronavirus disease 2019; ELISA, enzyme-linked immunosorbent assay; ICU, intensive care unit; IgA, immunoglobulin A; α-Gal, Galα1-3Galβ1-(3)4GlcNAc-R

The results of our study showed a negative correlation between anti-α-Gal antibody titers and COVID-19 disease severity. However, these results raised the question of whether the observed reduction in the anti-α-Gal antibody response at the population level is a consequence or a cause of COVID-19 symptomatology. In our study, COVID-19 patients were immunocompetent with a positive correlation between anti-SARS-CoV-2 Spike antibody titers and disease severity.[28] Therefore, our results suggested that the decrease in the anti-α-Gal antibody response occurred by mechanisms different from humoral immunosuppression and as a consequence of SARS-CoV-2 infection.

In addition to the observed negative correlation between anti-α-Gal IgE, IgM, and IgG antibody titers and COVID-19 disease severity, our results showed differences in the profile of anti-α-Gal antibody isotypes in COVID-19 cases that may be associated with different disease stages (Figure 5). These results suggested that higher anti-α-Gal IgE levels in asymptomatic cases may reflect an allergic response mediated by this glycan, which reflects the trade-off associated with the immune response to α-Gal that benefits humans by providing immunity to pathogen infection while increasing the risk of developing allergic reactions to this molecule.[12,13,17] In healthy individuals as in hospital discharge cases, the higher representation of anti-α-Gal IgM and/or IgG antibodies may be associated with a protective response to COVID-19. However, in hospitalized patients, the representation of anti-α-Gal antibody isotypes did not vary, which could reflect the absence of protection. Finally, the higher representation of anti-α-Gal IgA antibodies in ICU patients may be associated with the inflammatory response observed in these cases. In accordance with these results, it was recently shown in endogenous α-Gal-negative turkeys that treatment with probiotic

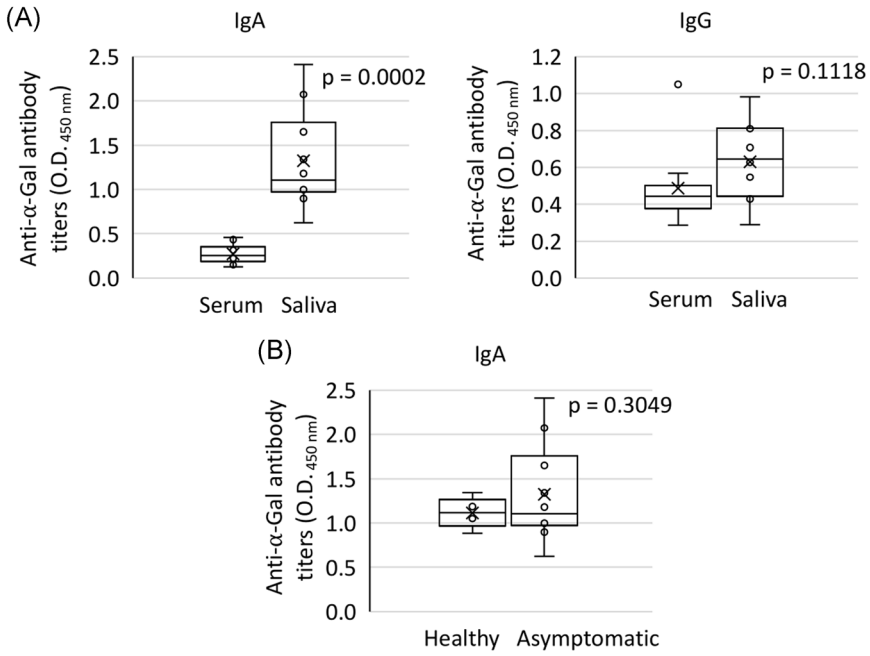

FIGURE 4 Salivary anti-α-Gal antibody response in COVID-19 asymptomatic cases and healthy controls. A, The anti-α-Gal IgA and IgG antibody titers were determined by ELISA and compared in asymptomatic cases between serum and saliva samples by Student's *t*-test ($p < .05$; $n = 10$). B, The anti-α-Gal IgA antibody titers in saliva were determined by ELISA and compared between asymptomatic COVID-19 cases and healthy individuals by Student's *t*-test ($p < .05$; $n = 10$). COVID-19, coronavirus disease 2019; ELISA, enzyme-linked immunosorbent assay; ICU, intensive care unit; IgA, immunoglobulin A; α-Gal, Galα1-3Galβ1-(3)4GlcNAc-R

bacteria with high α-Gal content results in protection against aspergillosis through reduction by still unknown mechanisms in the pro-inflammatory anti-α-Gal IgA response in the lungs.[29]

In Spain, differences have been observed in the number of reported cases per 100 000 people by age and sex, with more females at age 20–59 and males at age 60–89 with a higher mortality in males.[30] In this study, differences in age, but not sex, were observed in symptomatic COVID-19 cases (Table 1). However, the youngest cases corresponded to ICU patients and healthy control individuals, thus reducing the possible effect of age and sex on the observed

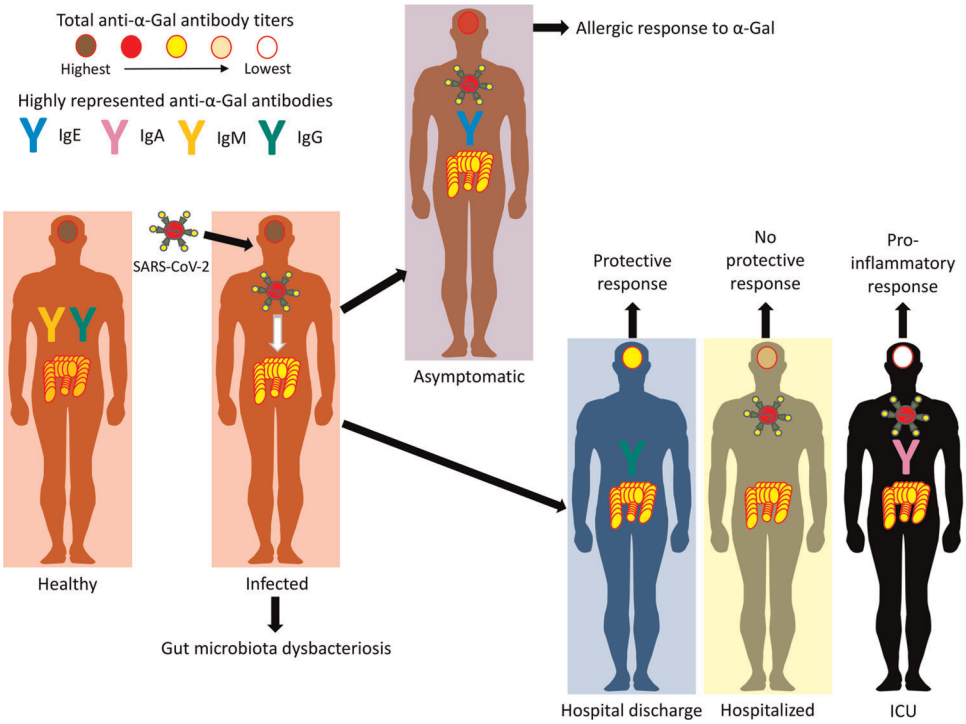

FIGURE 5 A negative correlation between anti-α-Gal antibody titers and COVID-19 disease severity and differences in the profile of anti-α-Gal antibody isotypes may be associated with different disease stages. Our hypothesis is that the dysbacteriosis observed in COVID-19 patients translates into a reduction in total anti-α-Gal antibody titers and alteration of anti-α-Gal antibody isotype composition due to the reduction in the microbiota of α-Gal-containing commensal bacteria. COVID-19, coronavirus disease 2019; IgA, immunoglobulin A; α-Gal, Galα1-3Galβ1-(3)4GlcNAc-R; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

disease symptoms. Furthermore, currently a clear correlation has not been found between age, sex, and the antibody response to α-Gal.[31–33]

The protective response of anti-α-Gal IgM/IgG antibodies against pathogenic organisms containing this modification on membrane proteins has been well documented.[10–14,17,23] In contrast, IgE antibody response against α-Gal has been associated with the allergy to mammalian meat or α-Gal syndrome and other diseases such as atopy, coronary artery disease and atherosclerosis.[33,35–37] In this study and based on anti-α-Gal IgE levels, all individuals were negative for α-Gal syndrome.[38]

The anti-α-Gal IgM/IgG antibodies can protect from infection by opsonizing pathogens with α-Gal on their surface.[10,12,13] In preliminary analyses, it was suggested that blood type O individuals are less susceptible to COVID-19 than other blood type groups,[39,40] a finding that was recently confirmed by genetic analyses.[41] ABO blood groups contain highly fucosylated antigens,[42,43] a property shared with the glycans present in SARS-CoV-2.[44] The glycans on envelope glycoproteins are linked to host-cell glycosylation machinery and thus the viral surface envelope is dominantly covered by host-derived glycans.[17,44] For example, glycosylation in spike asparagine (N343) is highly fucosylated with 98% of detected glycans bearing fucose residues.[44] Accordingly, the monoclonal antibody S309 that neutralizes SARS-CoV-2 binds core fucose moieties in N343 and *N*-acetylglucosamine (GlcNAc), a structural glycan found in both SARS-CoV-2[44] and ABO blood groups.[42,43] Human cells do not express α-Gal, but SARS-CoV-2 can carry structurally similar blood group B antigen when it replicates in cells expressing the blood group enzyme.[45,46] Therefore, the blood type B antigen can be then targeted by pre-existing and cross-reactive anti-α-Gal and anti-B antibodies produced by blood type B-negative individuals,[45,46] thus preventing infection by blocking the virus cell attachment and entry. Other immune-mediated mechanisms may be also activated in response to α-Gal,[14–16] which can be activated by SARS-CoV-2 expressing blood type B antigen on their envelope. Although not addressed in this study, these findings prompted us to consider that blood type O individuals could produce antibodies against A and B antigens that in addition to IgM/IgG antibodies against α-Gal, which cross-react with the structurally similar blood B antigen,[46] could be involved in a polyvalent recognition of the SARS-CoV-2 Spike that may be implicated in the human protection to COVID-19.

In our study, we did not collect information on ABO blood type in COVID-19 patients. However, in a related study with a similar group of patients ($n = 73$; 9, 40 and 24 hospital discharge, hospitalized and ICU patients, respectively), the results did not show significant differences in ABO blood group distribution. Nevertheless, the results suggest that the ABO blood factor should be considered when evaluating the antibody response to α-Gal. Blood type O individuals, who produce anti-A and anti-B antibodies, can be protected against SARS-CoV-2 particles carrying blood antigens A or B. However, blood type A and B individuals, who produce either anti-A or anti-B antibodies, would be protected only against SARS-CoV-2 particles carrying antigen A or B, respectively. Therefore, both blood type A and B individuals will be highly susceptible to SARS-CoV-2 particles coming from blood type O individuals because these viral particles do not carry either blood antigen A or B on the envelope.[45] Assuming equal replicative fitness for viruses replicating in cells expressing any of the ABO blood group enzymes, an epidemiological dynamics would be created in which, after a large proportion of the population being exposed and infected by SARS-CoV-2, the frequency of individuals with blood types B and A would be equally represented among COVID-19 patients. In contrast, blood type O individuals would be underrepresented, relative to the frequency of these blood groups in the general population. These predictions were corroborated in a large study ($n = 750\,000$) even after adjusting for age, sex, body mass index, race, ethnicity, and co-morbidities.[47] Therefore, the blood type's effects are not explained by other risk factors including age, sex, race, ethnicity, hypertension, diabetes mellitus, obesity, and cardiovascular and respiratory diseases,[40] which support the immunological considerations discussed above.

Based on the fact that natural antibodies against α-Gal are produced in response to bacteria with this modification in the microbiota,[10] our hypothesis is that the dysbacteriosis observed in COVID-19 patients[34] translates into a reduction in total anti-α-Gal antibody titers and alteration of anti-α-Gal antibody isotype composition due to the reduction in the microbiota of α-Gal-containing commensal bacteria and other still uncharacterized mechanisms (Figure 5). Alternatively, and hypothetically, individuals with higher α-Gal content in the microbiota may be less susceptible to COVID-19. Additionally, the pulmonary microbiota can be affected with the presence of gut bacteria in the lungs.[9]

In conclusion, according to these results and previous findings in retrovirus,[48,49] the inhibition of the α-Gal-induced immune response may translate into more aggressive viremia and severe disease inflammatory symptoms.[50] These results further encourage addressing the proposal of developing interventions such as probiotics based on commensal bacteria with α-Gal epitopes to modify the microbiota and increase the α-Gal-induced protective immune response and reduce the severity of COVID-19.[8,18] Furthermore, as recently proposed, the production of coronavirus or virus-like particles (VLPs) in non-catarrhine mammalian cells may be used to produce vaccines with α-Gal-containing antigens to induce anti-α-Gal protective response and increase vaccine efficacy for the control of COVID-19.[51–53]

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

José Miguel Urra, Christian Gortázar, and José de la Fuente designed the study; Elisa Ferreras-Colino, Marinela Contreras, Carmen M. Cabrera, Isabel G. Fernández de Mera, José Miguel Urra, Christian Gortázar, and José de la Fuente performed the experiments; José de la Fuente, José Miguel Urra, Elisa Ferreras-Colino, Marinela Contreras, Carmen M. Cabrera, Isabel G. Fernández de Mera, and Alejandro Cabezas-Cruz analyzed data; José Miguel Urra, Christian Gortázar, A.M., Margarita Villar, and José de la Fuente supervised the project. All authors wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Accepted Article

Jeffrey Wilson ORCID iD: 0000-0002-5975-1760

Running title: alpha-Gal and COVID-19

Reply to: The antibody response to the glycan α-Gal correlates with COVID-19 symptoms

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To the Editor:

We read with interest two recent papers which discussed possible connections between the immune response to galactose-α-1,3-galactose (α-Gal) and COVID-19(1, 2). First described nearly a hundred years ago by Landsteiner and Miller as a "B-like" blood group substance of non-primate mammals (but not higher primates), work over the last 40 years has made it clear that α-Gal is also expressed on some species of bacteria and multi-cellular parasites, and that all immunocompetent humans produce large quantities of IgM, IgG and IgA antibodies (Ab) specific for the oligosaccharide (3, 4). More recent research indicates that a subset of the population can also produce IgE specific for α-Gal. IgE sensitization to α-Gal has largely been attributed to tick bites and is an important cause of allergic reactions to mammalian meat and dairy(5, 6).

On this backdrop Urra et al. reported that patients with COVID-19 had altered levels of anti-α-Gal IgG, IgM, IgA and IgE Ab as compared to a control cohort(1). Specifically, they found that levels of α-Gal-specific IgG, IgM and IgE (but not IgA), were lower in patients hospitalized in an ICU with severe COVID-19 as compared to healthy uninfected controls. They also reported that

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relative amounts of different anti-α-Gal antibody isotypes varied in relation to disease severity. Interestingly, they noted that IgE represented 14-45% of the overall repertoire of anti-α-Gal antibody levels, with the highest amount of specific IgE observed in asymptomatic COVID-19 patients. The authors speculate that dysbacteriosis could have caused the reduced antibody response to α-Gal, which in turn translated to greater SARS-CoV-2 viral loads and systemic inflammation. This hypothesis leads to the idea that restoring anti-α-Gal antibodies could be protective against COVID-19. This paper raises some intriguing points, but we think additional commentary is merited.

In our recent investigation of COVID-19, which utilized a quantitative ImmunoCAP-based approach, we did not observe differences in levels of anti-α-Gal IgG when comparing patients with severe COVID-19 to a reference cohort of healthy uninfected controls(7). The explanation for the discrepancy between our findings and Urra et al. is not clear. One possibility is that inflammatory mediators which are present during acute severe COVID-19 could be interfering with one or both of our assays. However, we would highlight a recent report that used a glycan array and did not find lower anti-α-Gal IgG levels in COVID-19 patients compared to controls(8). To look at this question in a different light, here we have extended that analysis by monitoring anti-α-Gal IgG levels among five patients with severe COVID-19 in which longitudinal data were available. The data indicates that levels were relatively stable across time, including at a follow-up time point where the patients had convalesced and recovered from their infection (Fig A). We also measured IgG to tetanus toxoid as a reference control antigen (to which most individuals are vaccinated) and found little fluctuation in antibody levels across the time points (Fig B). It is also important to note that the anti-α-Gal antibody levels reported by Urra and colleagues were based on semi-quantitative ELISA assays, where the read-out is in OD450 units. Because there is not an internal calibrator curve, the antibody levels cannot be expressed in a quantitative fashion. As a consequence, there are major limitations in comparing isotype specific antibody levels with each

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other. This is particularly true when considering IgE, which usually represents only a minor fraction of the antibody repertoire. Using the quantitative ImmunoCAP assay, here we show that levels of anti-α-Gal IgE were log orders of magnitude lower than specific IgG in severe COVID-19 patients (Fig A).

The recent article by Chen discussed the possibility that α-Gal could be used as a means of enhancing immune responses to COVID-19 vaccinations(2). The concept, which takes advantage of pre-existing humoral immunity to α-Gal, is interesting and biologically plausible. Data cited in that report from non-human studies was also encouraging that α-Gal can indeed boost vaccine-related immune responses. Nonetheless, we were struck that there was no mention of the fact that IgE to α-Gal could be an important confounder to this strategy. IgE is a critical mediator of allergic reactions and IgE specific for α-Gal has been linked with cases of anaphylaxis that were caused by gelatin-containing vaccines (in which the gelatin was a source of α-Gal)(9, 10). Because α-Gal occurs in some areas of the world with frequencies approaching or exceeding 20% there are good reasons to think that inclusion of α-Gal could lead to issues with vaccine safety(5, 11). We don't doubt that α-Gal could have potential immune-enhancing benefits for certain personalized immunotherapies (eg, cancer vaccines), but have reservations that it is a rational choice for designing vaccines that would be implemented widely on a population basis(12).

Conflicts of interest:

TPM has a patent on an IgE assay to α-Gal and has received assay support from Thermo Fisher Scientific. JW has received consultant fees and assay support from Thermo Fisher Scientific. The other authors have no conflicts of interest to declare.

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Woodfolk, Lyndsey Muehling, Pat Pramoonjago and colleagues affiliated with the University of Virginia COVID-19 Biorepository for access to COVID-19 samples.

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FIGURE LEGEND

Fig. Quantitative assessment of antibodies in five patients admitted to the ICU with severe COVID-19 using ImmunoCAP. A) IgG levels to α-Gal assessed at day of admission (D0; median 10 days post-symptom onset), day 7 of admission (D7; median 17 days post-symptom onset) and at a recovery follow-up clinic (median 74 days post symptom onset). IgE levels to α-Gal were measured at the recovery timepoint, expressed in µg/mL using the same units/axis as for

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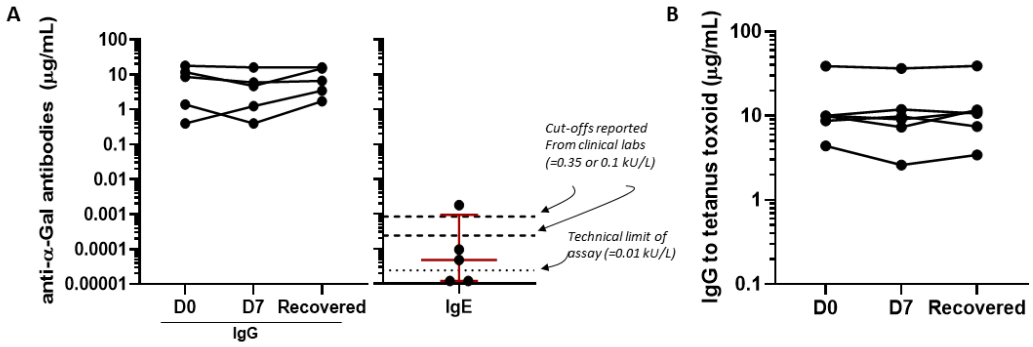
IgG. Two samples in which no IgE was detected were plotted as 0.5x the technical limit of the assay. Both the IgG and IgE assays used α-Gal-HSA as the assay solid-phase, as previously described(7). B) IgG to tetanus toxoid was measured by ImmunoCAP using the commercial assay (Thermo-Fisher/Phadia).



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LETTER TO THE EDITOR

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Reply to: The antibody response to the glycan α-Gal correlates with COVID-19 symptoms

To the Editor:

We read with interest two recent papers which discussed possible connections between the immune response to galactose-α-1,3-galactose (α-Gal) and coronavirus disease 2019 (COVID-19).[1,2] First described nearly a hundred years ago by Landsteiner and Miller as a "B-like" blood group substance of non-primate mammals (but not higher primates), work over the last 40 years has made it clear that α-Gal is also expressed on some species of bacteria and multi-cellular parasites, and that all immunocompetent humans produce large quantities of immunoglobulin M (IgM), IgG and IgA antibodies (Ab) specific for the oligosaccharide.[3,4] More recent research indicates that a subset of the population can also produce IgE specific for α-Gal. IgE sensitization to α-Gal has largely been attributed to tick bites and is an important cause of allergic reactions to mammalian meat and dairy.[5,6]

On this backdrop, Urra et al. reported that patients with COVID-19 had altered levels of anti-α-Gal IgG, IgM, IgA, and IgE Ab as compared to a control cohort.[1] Specifically, they found that levels of α-Gal-specific IgG, IgM, and IgE (but not IgA), were lower in patients hospitalized in an intensive care unit (ICU) with severe COVID-19 as compared to healthy uninfected controls. They also reported that relative amounts of different anti-α-Gal antibody isotypes varied in relation to disease severity. Interestingly, they noted that IgE represented 14%–45% of the overall repertoire of anti-α-Gal antibody levels, with the highest amount of specific IgE observed in asymptomatic COVID-19 patients. The authors speculate that dysbacteriosis could have caused the reduced antibody response to α-Gal, which in turn translated to greater severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral loads and systemic inflammation. This hypothesis leads to the idea that restoring anti-α-Gal antibodies could be protective against COVID-19. This paper raises some intriguing points, but we think additional commentary is merited.

In our recent investigation of COVID-19, which utilized a quantitative ImmunoCAP-based approach, we did not observe differences in levels of anti-α-Gal IgG when comparing patients with severe COVID-19 to a reference cohort of healthy uninfected controls.[7] The explanation for the discrepancy between our findings and Urra et al. is not clear. One possibility is that inflammatory mediators which are present during acute severe COVID-19 could be interfering with one or both of our assays. However, we would highlight a recent report that used a glycan array and did not find lower anti-α-Gal IgG levels in COVID-19 patients compared to controls.[8] To look at this question in a different light, here we have extended our previous analysis by monitoring anti-α-Gal IgG levels among five patients with severe COVID-19 in which longitudinal data were available. The data indicate that levels were relatively stable across time, including at a follow-up timepoint where the patients had convalesced and recovered from their infection (Figure 1A). We also measured IgG to tetanus toxoid as a reference control antigen (to which most individuals are vaccinated) and found little fluctuation in antibody levels across the time points (Figure 1B). It is also important to note that the anti-α-Gal antibody levels reported by Urra and

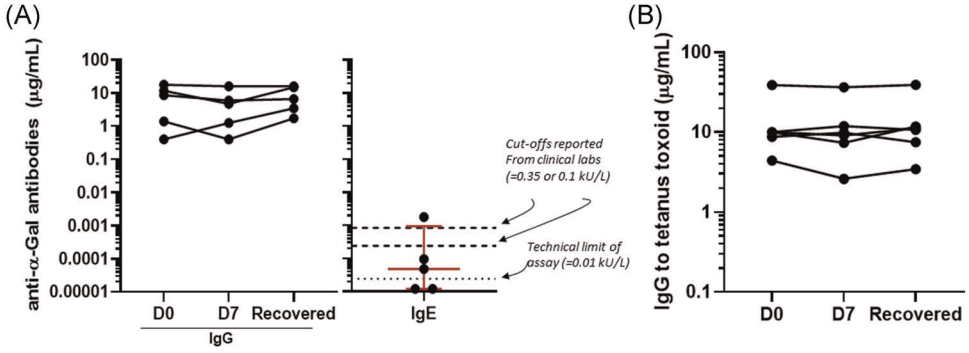

FIGURE 1 Quantitative assessment of antibodies in five patients admitted to the intensive care unit (ICU) with severe COVID-19 using ImmunoCAP. (A) Immunoglobulin G (IgG) levels to galactose-α-1,3-galactose (α-Gal) assessed at day of admission (D0; median 10 days post-symptom onset), Day 7 of admission (D7; median 17 days post-symptom onset) and at a recovery follow-up clinic (median 74 days post-symptom onset). IgE levels to α-Gal were measured at the recovery timepoint, expressed in μg/ml using the same units/axis as for IgG. Two samples in which no IgE was detected were plotted as ×0.5 the technical limit of the assay. Both the IgG and IgE assays used α-Gal-HSA as the assay solid-phase, as previously described.[7] (B) IgG to tetanus toxoid was measured by ImmunoCAP using the commercial assay (Thermo-Fisher/Phadia)

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colleagues were based on semi-quantitative enzyme-linked immunosorbent assays, where the read-out is in OD_{450} units. As there is not an internal calibrator curve, the antibody levels cannot be expressed in a quantitative fashion. As a consequence, there are major limitations in comparing isotype-specific antibody levels with each other. This is particularly true when considering IgE, which usually represents only a minor fraction of the antibody repertoire. Using the quantitative ImmunoCAP assay, here we show that levels of anti-α-Gal IgE were log orders of magnitude lower than specific IgG in the five severe COVID-19 patients (Figure 1A).

The recent article by Chen discussed the possibility that α-Gal could be used as a means of enhancing immune responses to COVID-19 vaccinations.[2] The concept, which takes advantage of pre-existing humoral immunity to α-Gal, is interesting and biologically plausible. Data cited in that report from nonhuman studies was also encouraging that α-Gal can indeed boost vaccine-related immune responses. Nonetheless, we were struck that there was no mention of the fact that IgE to α-Gal could be an important confounder to this strategy. IgE is a critical mediator of allergic reactions and IgE specific for α-Gal has been linked with cases of anaphylaxis that were caused by gelatin-containing vaccines (in which the gelatin was a source of α-Gal).[9,10] As α-Gal occurs in some areas of the world with frequencies approaching or exceeding 20%, there are good reasons to think that inclusion of α-Gal could lead to issues with vaccine safety.[5,11] We don't doubt that α-Gal could have potential immune-enhancing benefits for certain personalized immunotherapies (e.g., cancer vaccines), but have reservations that it is a rational choice for designing vaccines that would be implemented widely on a population basis.[12]

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