

HIGHLIGHTED ARTICLE

Severe COVID-19 is marked by dysregulated serum levels of carboxypeptidase A3 and serotonin

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Abstract

The immune response plays a critical role in the pathophysiology of SARS-CoV-2 infection ranging from protection to tissue damage and all occur in the development of acute respiratory distress syndrome (ARDS). ARDS patients display elevated levels of inflammatory cytokines and innate immune cells, and T and B cell lymphocytes have been implicated in this dysregulated immune response. Mast cells are abundant resident cells of the respiratory tract and are able to release different inflammatory mediators rapidly following stimulation. Recently, mast cells have been associated with tissue damage during viral infections, but their role in SARS-CoV-2 infection remains unclear. In this study, we examined the profile of mast cell activation markers in the serum of COVID-19 patients. We noticed that SARS-CoV-2-infected patients showed increased carboxypeptidase A3 (CPA3) and decreased serotonin levels in their serum when compared with symptomatic SARS-CoV-2-negative patients. CPA3 levels correlated with C-reactive protein, the number of circulating neutrophils, and quick SOFA. CPA3 in

Abbreviations: ACE2, angiotensin-converting enzyme II; ARDS, acute respiratory distress syndrome; AUC, area under the curve; COVID-19, coronavirus disease 2019; CPA3, carboxypeptidase A3; CRP, C-reactive protein; MC, mast cells; qSOFA, quick Sepsis-related Organ Failure Assessment; ROC, receiver operating characteristic; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

serum was a good biomarker for identifying severe COVID-19 patients, whereas serotonin was a good predictor of SARS-CoV-2 infection. In summary, our results show that serum CPA3 and serotonin levels are relevant biomarkers during SARS-CoV-2 infection. This suggests that mast cells and basophils are relevant players in the inflammatory response in COVID-19 and may represent targets for therapeutic intervention.

KEYWORDS

carboxypeptidase A3, COVID-19, Mast cell, SARS-CoV-2, serotonin

1 | INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by the SARS-CoV-2 virus, which was identified for the first time in Wuhan, China in late 2019. Since then, the virus has spread across the globe infecting more than 100 million people and causing the death of more than 2.1 million infected individuals.¹

SARS-CoV-2 infects cells of the mucosa that express the angiotensin-converting enzyme II (ACE2), which is particularly abundant in type II alveolar cells of the lung and in other tissues.² The COVID-19 infection has an incubation period of 3–7 days, generating moderate symptoms, such as head and muscle aches, sore throat, nasal congestion, dry cough, fatigue, and fever. However, 5–10% of infected patients develop acute respiratory distress syndrome (ARDS), a serious complication that causes respiratory failure leading to high mortality.³

A relevant factor for the development of ARDS in patients with COVID-19 is the exacerbated immune response triggered by the infection, reflected as a cytokine storm in which different cytokines such as: IL-1 β , IL-2, IL-4, IL-6, IL-10, IFN- γ , and TNF- α are elevated.⁴ Besides, COVID-19 patients are known to show profound alterations in cell populations associated with the immune response against viruses, such as monocytes, macrophages, neutrophils, NK cells, B lymphocytes, CD8+ T lymphocytes, and memory and regulatory CD4+ lymphocytes.⁵ However, it is unclear how SARS-CoV-2 affects mast cells (MC).

MC are tissue resident leukocytes derived from hematopoietic precursors, distributed throughout the body and abundantly found along the respiratory tract.⁶ These cells are characterized by having many cytoplasmic granules that contain different chemical mediators released after activation. Among the molecules abundant in MC granules are tryptase, carboxypeptidase, chymase, serotonin, histamine and TNF- α . MC also produce other types of inflammatory mediators including prostaglandins, leukotrienes and reactive nitrogen species. In addition, activated MCs secrete different *de novo* synthesized cytokines and chemokines.⁷

MC are well known for their role in mediating allergic reactions, but recent evidence indicates an important role in the innate immune response to different pathogens including viruses, bacteria, protozoa, fungi and nematodes.⁷ The ability of MC to participate in viral infections is mediated by different Pattern Recognition Receptors,

such as TLR-3, -7, RIG-I, MDA-5, etc., which are essential in the innate antiviral response.⁸ Recently, it has been shown that MCs are associated with tissue damage induced by an excessive inflammatory response during viral infections.⁹ For instance, in influenza virus infections, the observed lung tissue damage is due to an excessive inflammatory response characterized by the overproduction of cytokines and chemokines or “cytokine storm.”¹⁰ In mouse models of infection with the H5N1 influenza virus, treatment of mice with ketotifen, an inhibitor of MC activation, reduces damage to lung tissue. Furthermore, the combination of ketotifen with oseltamivir, an antiviral drug, significantly increased mice survival.¹¹ On the other hand, it has been observed that mice deficient in MC show less lung damage when infected with influenza A virus, compared with wild-type mice. Interestingly, this effect was associated with a decreased production of TNF- α , CCL2, CCL3, CCL4, CXCL2, and CXCL10, suggesting a crucial role of MC in the “cytokine storm” triggered by influenza infection.¹² Considering that MC activation plays a crucial role during the damage induced by viral infections and that a cytokine storm is a crucial feature during SARS-CoV-2 infection, this study was conducted to evaluate MC activation markers in the serum of patients with COVID-19 and to determine if they are associated with disease severity.

2 | MATERIAL AND METHODS

2.1 | Patients and control

Patients with COVID-19 and a control group were enrolled at Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, between March and April 2020. All participants in this study had COVID-19 suggestive symptoms, but only those showing a positive PCR test for SARS-CoV-2 were considered infected, whereas PCR test-negative patients with respiratory symptoms were selected as control. Based on clinical records, samples from asthmatic, HIV, cancer, autoimmune, or pregnant patients were not included in this study. Serum samples were taken on the first day of admission. Demographic and clinical parameters were collected, and laboratory indicators were tested with conventional methods in COVID-19 patients (Supplemental Table 1). Disease severity was classified as mild/moderate when patients showed fever, signs of airway disease, with or without a tomographic image indicating pneumonia. Severe

COVID-19 patients showed either respiratory failure, an increased respiratory rate (>30 bpm), decreased oxygen saturation at rest ($<92\%$), or decreased $\text{PaO}_2/\text{FiO}_2$ (<300 mmHg). This study was approved by the Research and Ethics Committee of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (Ref. 3341). All protocols were in accordance with the Declaration of Helsinki. All participants of this study provided informed written consent to participate in this study.

2.2 | Assessment of mast cell activation markers

Patient serum was analyzed by the ELISA, using commercial kits for histamine (Cat. RE59221; IBL International, Hamburg, Germany), human carboxypeptidase A3 (Cat. OKCD01671; Aviva Systems Biology, San Diego, CA, USA), serotonin (Cat. ab133053; Abcam, Cambridge, UK), and heparin (Cat. abx 258893; Abbeva, Houston, TX, USA), according to the manufacturer's instructions.

Nitric oxide was evaluated using a colorimetric method that measures the levels of the breakdown product NO_2^- (Griess Reagent System, Cat. G2930; Promega, Madison, WI, USA).

2.3 | IL-6 quantification

IL-6 was evaluated with the Milliplex Map Human Cytokine/Chemokine Bead Panel-Premixed 29 Plex (Cat. HCYTMAG-60K-PX29; Millipore, USA) following manufacturer's instructions.

2.4 | Images and statistical analysis

To identify potential organ or tissue sources of CPA3, protein and mRNA expression were analyzed in Human Protein Atlas (www.proteinatlas.org/ENSG00000163751-CPA3/tissue) and mRNA expression was obtained from HPA RNA-sEquation (www.ncbi.nlm.nih.gov/gene/1359#gene-expression).

All statistical analyses were performed with SigmaPlot software version 14.0 (Systat Software, San Jose, CA, USA). Data normality was assessed by Kolmogorov-Smirnov with Lilliefors correction. Data are shown as mean or median \pm range, as appropriate. For comparisons between 2 groups, Student's *t*-test or Mann-Whitney rank sum test with Yates correction were used. For comparisons of 3 groups, one-way ANOVA with Student-Newman-Keuls post-hoc or Kruskal-Wallis test followed by a Dunn's post-hoc test were used. Results were considered significant at a *P* value < 0.05 . For correlations of 2 variables, Spearman Rank Order Correlation was used. The *r* values for each of the correlations were plotted in a bubble chart generated with Microsoft Excel 2019 (<https://office.microsoft.com/excel>). Receiver operating characteristic (ROC) curves were generated to find the accuracy of biomarkers to distinguish infected individuals and the severity of COVID-19.

3 | RESULTS AND DISCUSSION

Several MC-derived biomarkers are used to diagnose and predict outcomes in allergic and infectious diseases. Among them, histamine, heparin, carboxypeptidase A3 (CPA3), serotonin, and nitric oxide have been studied.^{13,14} To address whether these markers are affected during COVID-19, serum samples were collected from patients admitted at a tertiary care center in Mexico City. Demographic and clinical characteristics of patients are shown in Supplemental Table 1. We noticed that COVID-19 patients had increased serum levels of CPA3 in serum when compared with the control group (Fig. 1A). Interestingly, serotonin showed decreased levels in sera from SARS-CoV-2-infected patients compared with those not infected (Fig. 1B). Serum levels of histamine, heparin, and nitric oxide were not affected in COVID-19 patients (Supplemental Fig. 1).

Previous reports indicate that different immune markers are differentially expressed during severe COVID-19.¹⁵ Therefore, we investigated whether MC-derived biomarkers were altered depending on COVID-19 severity. We observed that patients with severe disease showed increased levels of CPA3 when compared with those with mild/moderate disease, or individuals in the control group (Fig. 1C). Serotonin levels were decreased in severe COVID-19 (Fig. 1D). No other MC-associated biomarker evaluated showed significant differences in patients with severe COVID-19, compared with patients with mild/moderate symptoms or controls (Supplemental Fig. 2).

Because CPA3 and serotonin levels were altered in severe COVID-19 patients, we next assessed their correlation with clinical and laboratory parameters. A matrix with Spearman's *r* coefficient values and representing correlation showed that CPA3 had a stronger correlation with an increased number of clinical and laboratory parameters in comparison with serotonin (Fig. 2A). Furthermore, CPA3 showed a significant positive correlation with inflammation-associated markers indicated by the level of circulating neutrophils ($r = 0.291$, $P = 0.0447$) (Fig. 2B) and C-reactive protein ($r = 0.390$, $P = 0.00703$) (Fig. 2D). Remarkably, CPA3 also associated with the disease severity score quick Sepsis-related Organ Failure Assessment (qSOFA), ($r = 0.335$, $P = 0.00862$) (Fig. 2C).

Finally, to evaluate the potential clinical utility of serotonin and CPA3, ROC curves were performed to distinguish SARS-CoV-2 infection. Serotonin showed an acceptable AUC (area under the curve) values that allow to distinguish between SARS-CoV-2 infected from non-infected individuals (AUC 0.77) (Fig. 2E). When ROC curves were analyzed to differentiate between mild/moderate and severe COVID-19 patients, CPA3 (AUC 0.77) was more reliable than serotonin (AUC 0.58) or IL-6 (AUC 0.56) to detect patients with severe disease. This was close to the predictive values seen for C-reactive protein (AUC 0.93) (Fig. 2F). As a whole, these results suggest a relationship between MC activation, as reflected by CPA3 levels in serum, and severe COVID-19.

To the best of our knowledge, this is the first evidence of alteration in serum CPA3 levels during COVID-19. The source of this CPA3 in serum is unknown; however, CPA3 is abundantly expressed in different

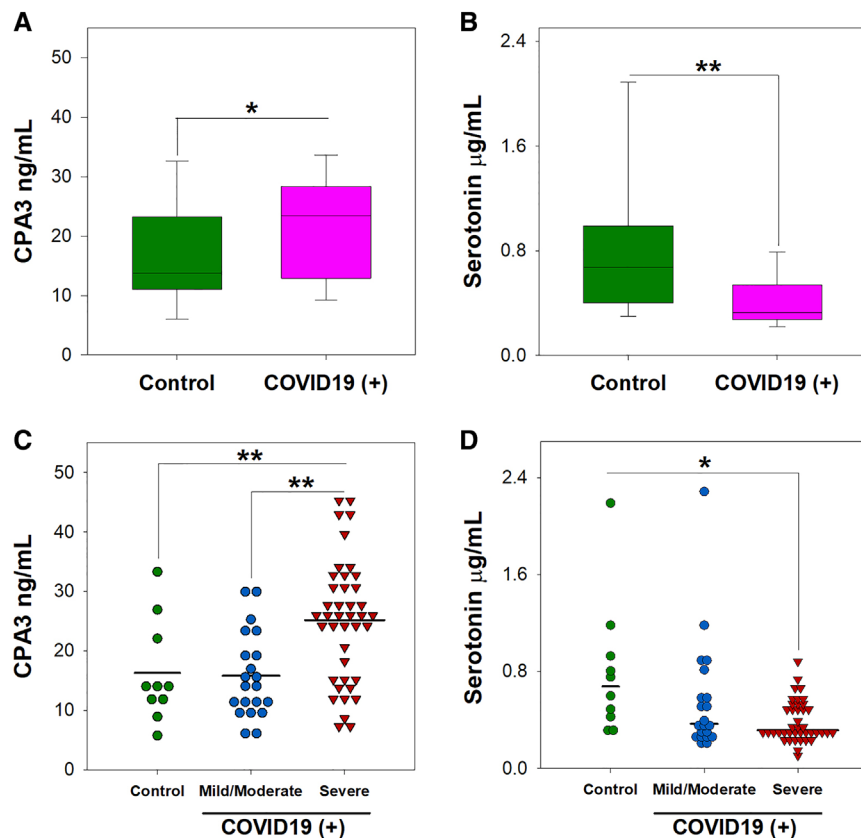


FIGURE 1 Serotonin and carboxypeptidase A3 are altered in COVID-19 patients.

The serum concentration of (A and C) carboxypeptidase A3 (CPA3), and (B and D) serotonin were measured upon patient admission by ELISA. Data from 21 patients with mild/moderate disease, 41 patients with severe COVID-19, and 10 control individuals is shown. Data are presented as mean or median \pm range, as appropriate. * $P < 0.05$; ** $P < 0.01$. Student's *t* test (A) Mann-Whitney test (B), one-way ANOVA test (C), and Kruskal-Wallis test (D)

organs, including human lungs,¹⁶ with MC being the main cell source, and releases this enzyme after degranulation¹⁷ (Supplemental Fig. 3). Remarkably, MC granules contain other abundant proteases, such as chymase and tryptase, which are relevant in clinical pathologies such as hemorrhagic dengue¹⁸ and mastocytosis.¹⁹ Further work is needed to ascertain whether other MC proteases are modified during COVID-19. Interestingly, a second potential cellular source of CPA3 are basophils, which are found in blood and migrate to inflamed tissues.²⁰ CPA3 is an enzyme that cleaves C-terminal amino acid residues from proteins and is an abundant protein in MC granules (16 μg CPA3 per 10^6 MC). This enzyme is released after cell degranulation and is associated with allergic pathologies of the respiratory tract.²¹ Identified substrates for CPA3 include neurotensin, kinetensin, neuromedin N, angiotensin I, and endothelin-1, which is associated with pulmonary fibrosis,²² a sequela observed in COVID-19 patients.²³ Interestingly, we noticed that CPA3 correlated with clinical parameters associated with systemic inflammation during COVID-19. Remarkably, CPA3 correlated with circulating neutrophils and CPR, which are associated with an exacerbated inflammatory response during COVID-19¹⁵; however, CPA3 potential as a disease severity biomarker needs to be further analyzed in larger prospective cohorts. Previous studies have noticed the importance of MC activation for the recruitment of neutrophils to sites of infection.²⁴ Furthermore, this increase in tissue neutrophil is proposed to be one mechanism of tissue damage and organ failure during COVID-19.²⁵ Our results are in agreement with a recent histologic study, where an increased number of MC in the lungs of COVID-19 patients was observed.²⁶ These results suggest an important role of

MC and basophils in SARS-CoV-2 infection, but further work is needed to understand the mechanisms involved.

The second marker that was modified during SARS-CoV-2 infection was serotonin. Traditionally serotonin is considered as an important neurotransmitter regulating several neuronal activities in the central nervous system. However, recent evidence indicates that systemic serotonin distributed by the blood plays a more complex function in the organism. Blood serotonin is produced by different cell lineages, including MC, but is mainly produced by enterochromaffin cells of the intestine. Once in the blood, serotonin levels are primarily regulated by platelets that capture and store in dense granules that are secreted after cell activation.²⁷ Previous studies have shown that infections can decrease, increase, or maintain serotonin levels in blood.²⁸⁻³⁰ For instance, in dengue infection, decreased levels of serum serotonin were associated with disease severity. Moreover, decreased serotonin levels correlate with thrombocytopenia, a clinical feature of severe dengue, associating decreased serum serotonin with decreased numbers of platelets.^{28,30} However, we did not observe a difference in platelet count in COVID-19 patients (Supplemental Table 1), implying other mechanisms could be involved in this decrease. A recent report noticed that platelet numbers are not affected in COVID-19, but their gene expression profile is different when compared with those from healthy individuals.³¹ Because platelets usually introduce serotonin from blood through a transporter (SERT or SLC6A4),³² an overexpression of this protein could explain the decrease of serotonin in blood. However, Manne et al.³¹ showed that SERT is not modified in platelets from COVID-19 patients.

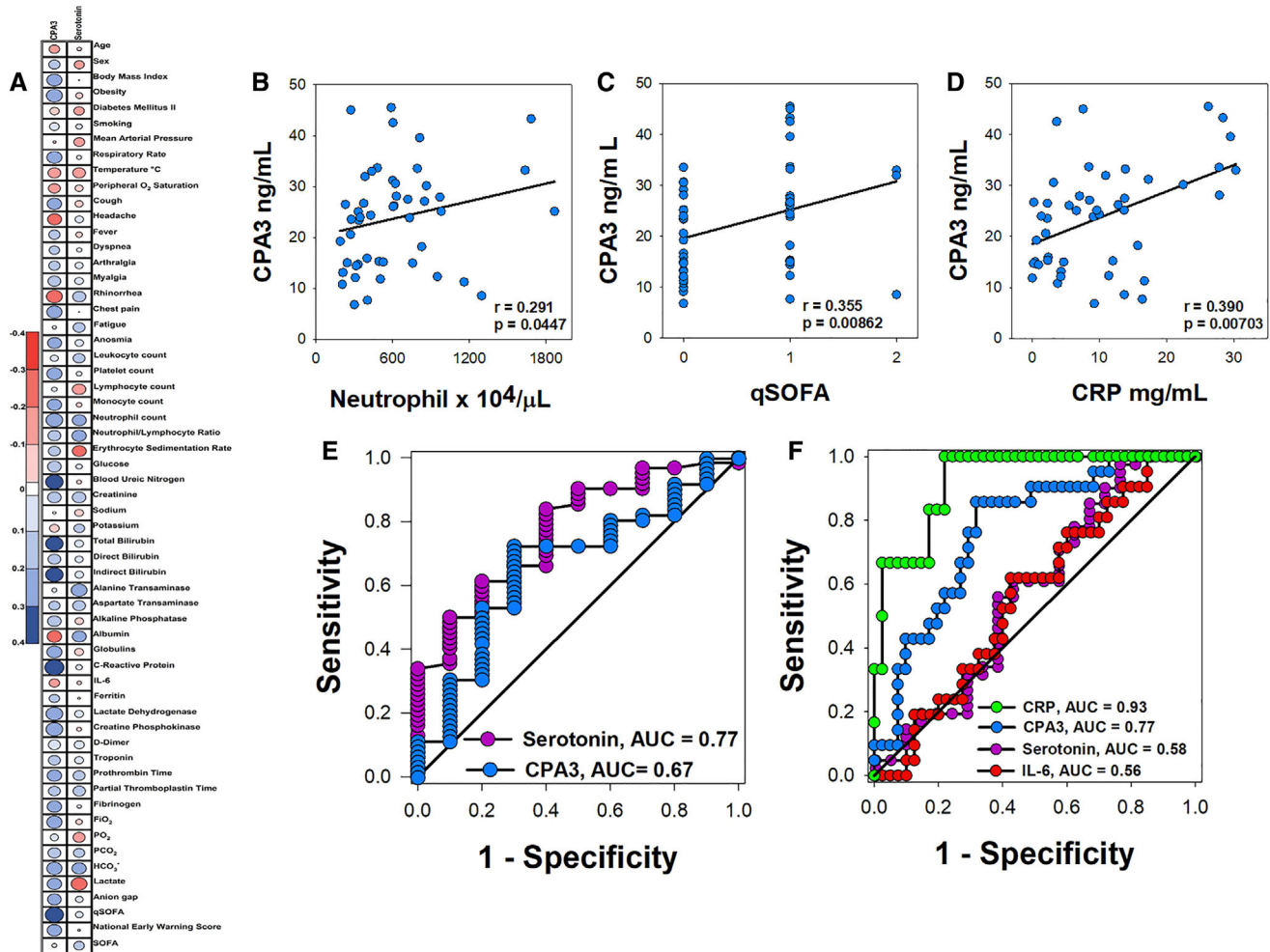


FIGURE 2 Serum levels of carboxypeptidase A3 correlates with clinical parameters of disease severity in COVID-19 patients. (A) Correlation matrix representing correlation carboxypeptidase A3 or serotonin serum levels with clinical and laboratory parameters used to determine COVID-19 severity. Spearman's coefficient value r is used as correlation descriptor, and the size of each circle symbolizes correlation strength (color scale of red and blue indicates negative or positive correlation, respectively). Correlation between serum concentration of carboxypeptidase A3 and (B) blood neutrophils, (C) qSOFA, (D) C-reactive protein. Value of Spearman's correlation (r) and significant P values ($P < 0.05$) are shown. (E) Receiver-operator characteristics (ROC) curves of carboxypeptidase A3 (CPA3) and serotonin serum levels for the prediction of SARS-CoV-2 infection. (F) ROC curve of C-reactive protein (CRP), carboxypeptidase A3 (CPA3), serotonin, and IL-6 in the prediction of COVID-19 severity

Another explanation could be related to an alteration in enterochromaffin cells functions by SARS-CoV-2, a phenomenon that is observed in other viral infections,³³ or to increased serotonin degradation by monoamine oxidase,²⁷ which is overexpressed by platelets in COVID-19 patients.³¹ How this serotonin decrease can affect the pathophysiology of SARS-CoV-2 infection is unknown; however, among the diverse functions of blood serotonin, one involves modulation of the immune response. Several cells of the immune system express serotonin receptors, including those that participate in innate and adaptive immune response.²⁷ Activation of serotonin receptor 5-H₂TA diminishes inflammation induced by TNF- α .³⁴ Furthermore, serotonin decreases IL-6 and TNF- α production by macrophages and lymphocytes,³⁵ suggesting the importance of serotonin in regulating exacerbated inflammation. Interestingly, recent reports have shown

increased levels of serotonin in the serum of COVID-19 patients in relation to healthy subjects.^{36,37} This discrepancy with our results can be explained by the experimental design, where we compared serotonin levels from SARS-CoV-2-infected patients with noninfected patients, but with symptoms of other uncharacterized respiratory disease. Furthermore, in our study, serum serotonin levels in samples from COVID-19 patients are increased (median mild/moderate 366.36 ng/ml, severe 313.12 ng/ml) when compared with serotonin reference level in serum (283 ng/ml).³⁸

In conclusion, our results demonstrate that serum levels of CPA3 and serotonin are affected during SARS-CoV-2 infection and can be considered as potential biomarkers during COVID-19. We suggest that MC and basophils play an important role in this disease and that these cells are potential therapeutic targets.

AUTHORSHIP

R.S.-C., Y.G.M.-P., and G.M.R.-L. contributed equally to this work, designed, performed experiments, analyzed data, and drafted the manuscript. S.R.-R., V.A.S.-H., R.C.-D., A.P.-F., J.J.T.-R., D.G.-M. assisted in processing and preservation of patient samples, collected patient data, generated, and organized clinical database. M.C.-N., V.D.A.-J., S.M.P.-T., A.D.C.-B., S.E.-P., J.L.M.-M., and R.C.-S. analyzed, interpreted data, and drafted the manuscript. J.L.M.-M. and R.C.-S. designed and supervised the study, and obtained funding. All authors critically revised and approved the final version of this manuscript.

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DISCLOSURES

The authors declare no conflicts of interest. The data supporting the conclusions of this article will be made available by the authors upon reasonable request.

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SUPPORTING INFORMATION

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

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