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ORIGINAL ARTICLE

Combined plasma levels of IL-10 and testosterone, but not soluble HLA-G5, predict the risk of death in COVID-19 patients

Giada Amodio[1] | Paolo Capogrosso[2,3] | Marina Pontillo[4] | Michela Tassara[4] | Luca Boeri[2,5] | Cristina Carenzi[2] | Daniele Cignoli[2,6] | Anna Maria Ferrara[2] | Giuseppe A. Ramirez[6,7] | Cristina Tresoldi[8] | Massimo Locatelli[4] | Luca Santoleri[9] | Antonella Castagna[6,10] | Alberto Zangrillo[6,11] | Francesco De Cobelli[6,12] | Moreno Tresoldi[13] | Giovanni Landoni[6,11] | Patrizia Rovere-Querini[6,14] | Fabio Ciceri[6,15] | Francesco Montorsi[2,6] | Andrea Salonia[2,6] | Silvia Gregori[1]

[1]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), IRCCS Ospedale San Raffaele, Milan, Italy

[2]Division of Experimental Oncology/Unit of Urology, URI, IRCCS Ospedale San Raffaele, Milan, Italy

[3]Department of Urology and Andrology, Ospedale di Circolo and Macchi Foundation, Varese, Italy

[4]Laboratory Medicine Service, IRCCS Ospedale San Raffaele, Milan, Italy

[5]IRCCS Foundation Ca' Granda, Maggiore Policlinico Hospital, Department of Urology, University of Milan, Milan, Italy

[6]University Vita-Salute San Raffaele, Milan, Italy

[7]Immunology, Rheumatology, Allergology and Rare Diseases Unit, IRCCS Ospedale San Raffaele, Milan, Italy

[8]Molecular Hematology Unit, IRCCS Ospedale San Raffaele, Milan, Italy

[9]Immunohematology and Transfusion Medicine, IRRCS Ospedale San Raffaele, Milan, Italy

[10]Department of Infectious Diseases, IRCCS Ospedale San Raffaele, Milan, Italy

[11]Anesthesia and Intensive Care Department, IRCCS Ospedale San Raffaele, Milan, Italy

[12]Department of Radiology, IRCCS Ospedale San Raffaele, Milan, Italy

[13]General Medicine and Advanced Care Unit, IRCCS Ospedale San Raffaele, Milan, Italy

[14]Internal Medicine, Diabetes, and Endocrinology Unit, IRCCS San Raffaele Scientific Institute, Milan, Italy

[15]Hematology and Bone Marrow Transplant Unit, IRCCS Ospedale San Raffaele, Milan, Italy

Correspondence
Silvia Gregori and Andrea Salonia, Via Olgettina 60, 20132 Milano, Italia.
Email: gregori.silvia@hsr.it and salonia.andrea@hsr.it

Abstract

Background: The identification of biomarkers correlated with coronavirus disease 2019 (COVID-19) outcomes is a relevant need for clinical management. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is characterized by elevated interleukin (IL)-6, IL-10, HLA-G, and impaired testosterone production.

Objectives: We aimed at defining the combined impact of sex hormones, interleukin-10, and HLA-G on COVID-19 pathophysiology and their relationship in male patients.

Materials and methods: We measured by chemiluminescence immunoassay, electrochemiluminescent assays, and enzyme-linked immunosorbent assay circulating total

testosterone, 17β-estradiol (E_2), IL-10, and -HLAG5 as well as SARS-CoV-2 S1/S2 Immunoglobulin G from 292 healthy controls and 111 COVID-19 patients with different disease severity at hospital admission, and in 53 COVID-19 patients at 7-month follow-up.

Results and discussion: We found significantly higher levels of IL-10, HLA-G, and E_2 in COVID-19 patients compared to healthy controls and an inverse correlation between IL-10 and testosterone, with IL-10, progressively increasing and testosterone progressively decreasing with disease severity. This correlation was lost at the 7-month follow-up. The risk of death in COVID-19 patients with low testosterone increased in the presence of high IL-10. A negative correlation between SARS-CoV-2 Immunoglobulin G and HLA-G or IL-10 at hospitalization was observed. At the 7-month follow-up, IL-10 and testosterone normalized, and HLA-G decreased.

Conclusion: Our findings indicate that combined evaluation of IL-10 and testosterone predicts the risk of death in men with COVID-19 and support the hypothesis that IL-10 fails to suppress excessive inflammation by promoting viral spreading.

KEYWORDS
COVID-19, HLA-G, IL-10, male, SARS-CoV-2, testosterone

1 | INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[1,2] SARS-CoV-2 infection is characterized by the elevated release of inflammatory cytokines and chemokines, defined as a "cytokine storm", leading to acute respiratory distress syndrome and death in numerous cases.[3] The main pro-inflammatory cytokines increased during SARS-CoV-2 infection include interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF), which are responsible for initiating immune responses.[4] Moreover, the anti-inflammatory molecule IL-10, which is secreted during viral infection to control inflammation,[5] is also increased in the plasma of COVID-19 patients, and its levels are associated with disease severity.[6–9] The early and dramatic IL-10 elevation upon SARS-CoV-2 infection plays a detrimental role in COVID-19 severity. Indeed, it has been proposed that elevated IL-10 at the early phase of infection can either fail to suppress inflammation or act as a pro-inflammatory and immunostimulatory molecule, as previously reported in other contexts.[10,11]

Human leukocyte antigen (HLA)-G is a non-classical MHC class I molecule that can inhibit innate and adaptive immune responses.[12,13] Beyond its expression at the fetal-maternal interface,[14] HLA-G is involved in several pathological contexts including viral infections, where it favors immune escape mechanisms leading to viral spreading.[15–18] HLA-G can be found as four membrane-bound (HLA-G1 to -G4) and three soluble (HLA-G5 to -G7) isoforms.[19] High membrane-bound HLA-G expression in an early phase of SARS-CoV-2 infection[20] and significantly higher levels of soluble HLA-G in patients with COVID-19[21] have been reported. HLA-G expression is influenced by several immunomodulatory molecules and IL-10 is one of the most studied cytokines known to increase the expression of HLA-G and its receptors.[22,23] It has been also demonstrated that HLA-G-mediated signaling promotes IL-6 transcription in myeloid cells.[24] Moreover, systemic delivery of IL-6 up-regulates IL-10 and IL-1RA plasma levels in vivo.[25] This evidence suggests potential crosstalk among IL-10, IL-6, and HLA-G. Finally, being associated with fertility, the expression of HLA-G is influenced by sex hormones, being progesterone an inducer of its expression.[26–28] Conversely, serum levels of testosterone inversely associate with HLA-G.[29]

Men affected by COVID-19 have delayed viral clearance and a greater risk of severe illness and death than women.[30–33] The major causes are attributable to genetically-imprinted differences in the immune responses to pathogens (e.g., a different expression of pattern recognition receptors), and to sex hormones,[34,35] which directly regulate the expression of several receptors on leukocytes.[34] It has been demonstrated that testosterone significantly up-regulates, while estrogen downregulates, angiotensin-converting enzyme 2 expressions in males and females, indicating an intrinsic role of steroids in explaining sex differences in COVID-19.[36,37] Recently, it has been reported that low testosterone levels are associated with COVID-19 severity already at hospital admission,[38–40] being the lowest total testosterone (tT) levels in men with more severe clinical outcomes (e.g., intensive care unit [ICU] admission or death).[38]

Sex hormones have been shown to regulate anti-inflammatory responses or to sustain tolerance: testosterone and IL-6 induce IL-10[25,27]; likewise, progesterone and 17b-estradiol (E_2), during pregnancy, promote HLA-G.[26–28] Based on the hypothesis that IL-10 and

HLA-G and their relationship with IL-6 and sex hormone have a relevant impact throughout the COVID-19 course we aimed to (a) investigate the levels of circulating IL-10, soluble HLA-G5, tT, and E_2 in a cohort of male patients with COVID-19 at hospital admission compared to a cohort of healthy men, and at 7-month follow-up; and, (b) test the association between IL-10, HLA-G5, tT, and E_2, alone or combination, and disease severity, risk of death, and SARS-CoV-2 infection.

2 | MATERIALS AND METHODS

2.1 | Study subjects

Clinical data from 111 men with COVID-19 first admitted at the San Raffaele University Hospital, Milan, Italy, with SARS-CoV-2 diagnosed based on quantitative reverse transcription-polymerase chain reaction between February and May 2020 were comprehensively collected. Blood withdrawal was performed at the time of hospitalization before steroid or antiviral treatment was started. A full blood count laboratory test was performed for all the samples received. Samples were collected upon informed consent in accordance with the Helsinki Declaration and with local ethical committee approvals: Covid-BioB, ClinicalTrials.gov NCT04318366; Ethical Committee approval number 34/int/2020. The Charlson Comorbidity Index (CCI) used to score health-significant comorbidities was coded using the International Classification of Diseases, 10th revision.[41] Body mass index (BMI) was obtained for each patient. Moreover, a validated composite risk score based on the characteristics at the time of first hospital admission was calculated for every patient (e.g., Critical-ill COVID-19 score)[42]; the score provides an estimate of the risk of developing critical illness for a patient with COVID-19, taking into account the following parameters: chest radiography, age, hemoptysis, dyspnoea, unconsciousness, number of comorbidities, cancer history, neutrophil/lymphocytes ratio, lactate dehydrogenase, and direct bilirubin.

Clinical data from a subgroup of 53 of the same COVID-19 patients initially enrolled, were collected 7-months after the first hospital admission. During hospitalization, 23% of these patients were treated with corticosteroids that were withdrawn when patients were discharged from the hospital. Patients were treatment free at the follow-up blood withdrawal.

Healthy controls (HC) were voluntary blood male donors (aged > 18 years) with a negative serological test for SARS-CoV-2 S1/S2 IgM and SARS-CoV-2 S1/S2 IgG, who arrived between June and July 2020 at the Blood Donor Centre of the San Raffaele University Hospital, Milan, Italy. According to the internal research protocol (ethics committee approval number 91/int/2020), HC underwent the same comprehensive clinical and biochemical assessment of the infected counterpart.

2.2 | Biochemical measurements

For all subjects included in the study, baseline venous blood samples were drawn at hospital admission and kept at 4°C until processing. Serum and plasma aliquots were separated by centrifugation and stored at −80°C until assay. Sex hormone levels were measured using assays used in clinical practice for medical reports. Specifically, tT and E_2 were measured by a direct chemiluminescence immunoassay using commercially available kits. tT: LIAISON Testosterone (Cat. 310410); E_2: LIAISON Estradiol II Gen (Cat. 310680); all from DiaSorin SpA, Saluggia, Italy.

IL-6 was measured by electrochemiluminescent assays (Elecsys IL-6; COBAS ROCHE), and IL-10 was evaluated by enzyme-linked immunosorbent assay (ELISA) using the Human IL-10 High Sensitivity ELISA kit (Cat. BMS215HS, ThermoFisher Scientific, Vienna, Austria) following manufacturer's instruction. The limit of detection for IL-10 was 0.39 pg/ml.

Soluble HLA-G5 was determined by ELISA, as previously described.[43] Briefly, 96 well plates (Nunc-Immuno Plate PolySorp, Thermo Scientific, Denmark) were coated with the mAb 5A6G7 (Exbio, Czech Republic) and HLA-G5 was detected with biotinylated W6/32 mAb (Exbio, Czech Republic). Supernatants from HeLa HLA-G5-transfected cells (kindly provided by Dr. Rizzo, Università di Ferrara) purified by affinity chromatography by using the W6/32 mAb were used for the generation of standard calibration curves. The limit of detection was 15 ng/ml.

LIAISON SARS-CoV-2 S1/S2 IgG serological tests (DiaSorin SpA, Saluggia, Italy) were used to assess SARS-CoV-2 IgG in every participant.

2.3 | Statistical methods

The distribution of data was tested with the Shapiro–Wilk test. Data are presented as medians (interquartile range) or frequencies (proportions). We used one-way ANOVA on ranks (Kruskal–Wallis test associated with Dunn's multiple comparison test) or Chi-Square test to compare hormonal levels and other demographics, clinical, and laboratory characteristics between COVID-19 patients and HC. The same analyses were used to compare clinical characteristics and hormonal values among patients with different severity in terms of clinical outcomes. Differences in IL-10 and HLA-G5 levels among groups were evaluated by Kruskal–Wallis test associated with Dunn's multiple comparison test. For statistical purposes, to all donors in whom HLA-G5 was below the detection limit of the ELISA, we assigned levels of 15 ng/ml, representing the assay detection limit. Linear regression analyses were performed to define associations between IL-10, HLA-G5, E_2, tT, and SARS-CoV-2 IgG. To test the hypothesis that IL-10, HLA-G5, and E_2 could be associated with lower tT levels, we performed logistic regression models predicting serum tT. Lastly, the associa-

tion between tT levels and death outcomes was tested with logistic regression models adjusted for baseline clinical factors and for markers of systemic inflammation (e.g., IL-6) which could have influenced the hormonal values. We hypothesized that the association between disease severity and the risk of death could vary according to the various tT levels, therefore an interaction term between IL-10 and tT levels was included in the logistic regression model to test this hypothesis.

Statistical analyses were performed using Stata 14.0 (StataCorp, College Station, TX, USA) or GraphPad Prism 9.0 (GraphPad Software, Inc. La Jolla, CA, USA). All tests were two-sided, and p-values less than 0.05 were considered significant. All p-values of the analyses were reported in the figures and/or (Tables S1 and S2).

3 | RESULTS

3.1 | Clinical and laboratory characteristics of study subjects

The entire cohort of 403 participants was subdivided according to SARS-CoV-2 infection status in 111 patients with COVID-19 and 292 HC (Table 1). COVID-19 patients were stratified as patients in good clinical conditions and discharged home from the emergency department (asymptomatic, Asy $n = 10$); patients admitted in the internal medicine unit until discharge home (symptomatic, Sym $n = 63$); patients invasively ventilated in the ICU ($n = 29$) and subsequently discharged home; and patients either transferred to ICU or in the internal medicine unit who eventually died (dead, n = 9) (Table 2). Demographic and clinical parameters including BMI, CCI, arterial hypertension, C-reactive protein, and laboratory parameters are presented in Tables 1 and 2. The HC group was significantly ($p < 0.0001$) younger than COVID-19 patients. Moreover, analysis of the age in COVID-19 patients revealed a homogeneous distribution with a significant ($p = 0.0458$) difference only between asymptomatic and dead patients (Tables 1 and 2, Figure S1, and Table S1). The CCI score was higher in COVID-19 patients compared to HC, but no differences were observed among COVID-19 cohorts. As expected, a significant difference in CCI corrected for the age (CCI-age)[41] was observed between dead and asymptomatic patients ($p = 0.0420$, Table S1). However, CCI age resulted significantly different only between ICU and dead patients ($p = 0.0309$, Table S1) and not between symptomatic and dead patients, despite ICU and symptomatic cohorts being homogenous in terms of age (Table 2 and Table S1). In line with previous studies,[44] the absolute number of white blood cells (WBC), neutrophils, and levels of IL-6 were significantly higher in patients with COVID-19 compared to HC ($p < 0.0001$), while lymphocytes count, and hemoglobin levels were decreased ($p < 0.0001$ for both parameters). Finally, IL-6 and E_2 plasma levels were significantly higher and tT significantly lower in the severe outcome groups (ICU and dead patients, Table 2). Since tT levels observed in severe COVID-19 patients were lower compared to those previously reported in age-matched healthy subjects,[45] we can exclude a possible tT level association with age.

3.2 | IL-10 and HLA-G5 plasma levels significantly increased with disease severity at hospitalization

In the hypothesis that pro-tolerogenic molecules could influence viral spreading and disease severity during SARS-CoV-2 infection, we evaluated IL-10 and soluble HLA-G5 in plasma samples from all participants. As previously reported,[7,8,10] IL-10 was significantly higher ($p < 0.0001$) in COVID-19 patients compared to HC (Table 1). In line with the previous observation, the IL-10 plasma levels significantly increased in symptomatic ($p < 0.0001$), ICU ($p < 0.0001$), and deceased ($p = 0.0002$) COVID-19 patients compared to HC (Figure 1A, Table 2, and Table S2). Similarly, the concentration of soluble HLA-G5 was significantly higher ($p < 0.0001$) in COVID-19 patients compared to HC (Table 1), and the highest HLA-G5 plasma levels were detected in symptomatic and ICU patients compared to HC ($p < 0.0001$ and $p = 0.0443$, respectively). HLA-G5 levels in asymptomatic and deceased patients were comparable to that observed in HC (Figure 1B, Table 2, and Table S2).

3.3 | IL-10 and HLA-G5 inversely correlate with tT in COVID-19 patients

IL-10 and HLA-G5 expression can be regulated by sex hormones including tT and E_2.[26–28] Accordingly with previous data,[38] the highest tT levels were detected in HC with a progressive and statistically significant decrease in asymptomatic ($p = 0.0334$), symptomatic ($p < 0.0001$), ICU ($p < 0.0001$), and deceased COVID-19 patients ($p < 0.0001$) (Table 1, Figure 1C, Table 2, and Table S1). Circulating E_2 levels were significantly higher in COVID-19 patients compared to HC ($p < 0.0001$) with deceased patients having the highest E_2 amounts (Table 1, Figure 1D, Table 2, and Table S2).

Linear regression analyses revealed that IL-10 ($p = 0.002$), HLA-G5 ($p < 0.0001$), and E_2 ($p = 0.01$) are independent predictors of low tT levels, since the increased IL-10, HLA-G5, and E_2 plasma levels are associated with tT decrease (Table S3). Linear regression analyses performed to assess the correlation between IL-10 or HLA-G5 with E_2, showed that IL-10 positively correlated with E_2 levels ($p = 0.0029$), while HLA-G5 did not ($p = 0.0726$; not shown). Thus, we can conclude that IL-10, HLA-G5, and E_2 levels are inversely associated with circulating tT, and IL-10 positively correlated with circulating E_2 in men with COVID-19 at hospital admission.

3.4 | IL-10 and testosterone levels, but not HLA-G5, predict the risk of death in men with COVID-19

High IL-10 and low tT levels were detected in severe COVID-19 patients, and linear regression analysis revealed the independent association between IL-10, HLA-G5, E_2, and tT. To define whether one or more of these parameters can predict the risk of death in men with COVID-19 we performed multivariable regression

TABLE 1 Demographic, clinical, and laboratory parameters of subjects included in the study at admission

Variables	HC (*N* = 292; 72%)	COVID-19 (*N* = 111; 28%)	*p*-Value
Age	46.0 (35.0, 53.0)	59.0 (48.7, 66.0)	<**0.0001**
Ethnicity			
White-European	284 (97.2)	97 (87.4)	N/A
Latin-American	7 (2.4)	9 (8.1)	N/A
African	0 (0.0)	4 (3.6)	N/A
Asian-Far East Asian	1 (0.4)	1 (0.9)	N/A
BMI	25.2 (23.52, 27.58)	27.81 (25.13, 30.42)	<**0.0001**
Comorbidities			
CCI	0.0 (0.0, 0.0)	0.0 (0.0, 1.0)	**0.0094**
CCI-age	0.0 (0.0, 1.0)	2.0 (0.0, 3.0)	<**0.0001**
CCI (score)			
0	292 (100)	63 (53.3)	<**0.0001**
1	0.0 (0.0)	26 (23.6)	<**0.0001**
≥ 2	0.0 (0.0)	22 (0.2)	<**0.0001**
Arterial hypertension	32 (11)	41 (37.3)	<**0.0001**
CRP (mg/L)	38 (13%)	39 (35%)	**0.001**
Laboratory parameters			
WBC (count/ul)	5.8 (4.9, 6.5)	7.2 (5.6, 9.9)	<**0.0001**
Neutrophils (count/ul)	3.1 (2.5, 3.7)	5.6 (4.1, 7.7)	<**0.0001**
Lymphocytes (count/ul)	1.8 (1.5, 2.1)	1.0 (0.7, 1.4)	<**0.0001**
Hemoglobin (g/dl)	15.1 (14.4, 15.6)	13.55 (12.1, 15)	<**0.0001**
IL-6 (pg/ml)	2.5 (2.5, 4.0)	47.2 (18.35, 106.5)	<**0.0001**
IL-10 (pg/ml)	3.0 (1.5, 3.0)	6.0 (3.0, 12.6)	<**0.0001**
HLA-G5 (ng/ml)	178.0 (100.5, 275.7)	315.5 (174.4, 410.0)	<**0.0001**
tT (nmol/L)	10.6 (8.1, 13.9)	2.4 (0.9, 4.2)	<**0.0001**
E_2 (pg/ml)	22.9 (18.9, 43.1)	34.5 (18.2, 43.1)	<**0.0001**

Continuous variables are presented as medians (interquartile range); categorical variables are presented as frequency (%). For each variable, the two-tailed Mann-Whitney test and Chi Square test were used for testing the difference between groups. Statistically significant p-values are indicated in bold.
Abbreviations: BMI, Body Mass Index; CCI, Charlson Comorbidity Index; CRP, C-reactive protein; E2, 17β-estradiol; HC, healthy controls; tT, total testosterone; WBC, white blood cells.

analyses. Results showed that IL-10, but not HLA-G5, levels at hospital admission are associated with the risk of death ($p = 0.01$); indeed, the higher the IL-10 level, the higher is the risk of death (Table 3 and Table S4). The interaction test assessing the hypothesis that circulating IL-10 could differently impact the relationship between tT levels and the risk of death, previously reported,[38] revealed that the risk of death in men with COVID-19 with low tT increased in the presence of high IL-10 (Figure 2). Thus, IL-10 and tT levels at hospital admission represent critical biomarkers of risk of death in COVID-19 patients.

To confirm the hypothesis of a link between IL-10 and tT, we collected plasma samples from a subgroup of 53 of the same COVID-19 patients initially enrolled, 7 months after the first hospital admission (Table 4). On average, IL-10 levels were comparable between hospital admission and follow-up (4.9 and 5.6 pg/ml, respectively). Since high variability in IL-10 levels among patients at hospital admission was observed (Figure 3A), we segregated patients according to IL-10 levels at admission in IL-10^{high} and IL-10^{low}. The threshold value of 5 pg/ml was determined as > 95% of the confidence interval of IL-10 levels detected in HC at admission ($n = 292$). Of 25 patients with IL-10^{high}, 18 (72%) men depicted IL-10 levels significantly reduced at follow-up compared to hospital admission ($p = 0.0088$), reaching the levels detected in HC (Figure 3B). Conversely, the majority (20 out of 28; 71.4%) of IL-10^{low} patients, at follow-up showed a significant increase of IL-10 compared to hospital admission ($p < 0.0001$) (Figure 3B). In the same cohort of patients at follow-up, tT levels mostly increased (50 out of 53; 94.3%) compared to hospital admission ($p < 0.0001$) (Table 4 and Figure 3C). As previously reported,[38] patients at 7-month follow-up did not reach the levels of tT observed in HC. Linear regression analyses between IL-10 and tT at 7-month follow-up revealed that the inverse correlation observed at hospital admission was lost ($p = 0.9996$) (Figure 3D), indicating that IL-10 and tT are

TABLE 2 Demographic, clinical, and laboratory parameters of coronavirus disease 2019 (COVID-19) patients at admission, as divided according to outcome status

Variables	Asy ($N = 10$; 9.01%)	Sym ($N = 63$; 56.76%)	ICU ($N = 29$; 26.13%)	Dead ($N = 9$; 8.11%)	*p*-Value
Age	51.0 (45.7, 64.0)	58.5 (48.0, 65.2)	58.0 (50.0, 64.5)	72.0 (58.0, 74.0)	**0.0406**
Ethnicity					
White-European	6 (60.0)	56 (88.9)	27 (93.1)	8 (88.9)	N/A
Latin-American	3 (30.0)	3 (4.8)	2 (6.9)	1 (11.1)	N/A
African	1 (10.0)	3 (4.8)	0 (0.0)	0 (0.0)	N/A
Asian-Far East Asian	0 (0.0)	1 (1.6)	0 (0.0)	0 (0.0)	N/A
BMI	27.0 (22.9, 31.9)	28.1 (25.7, 36.7)	27.7 (25.1, 31.0)	28.4 (24.2, 29.4)	0.9785
Comorbidities					
CCI	0.5 (0.0, 1.2)	0.0 (0.0, 1.0)	0.0 (0.0, 1.0)	1.0 (0.0, 2.0)	0.0802
CCI-age	1.0 (0.0, 3.5)	2.0 (1.0, 3.0)	2.0 (1.0, 2.5)	3.0 (3.0, 4.5)	**0.0261**
CCI (score)					
0	5 (50.0)	34 (54.0)	21 (72.3)	3 (34.0)	0.1515
1	3 (30.0)	15 (23.8)	6 (20.7)	2 (22.0)	0.9455
≥2	2 (20.0)	14 (22.2)	2 (7.0)	4 (44.0)	0.0817
Arterial hypertension	2 (20.0)	23 (37.0)	11 (38.0)	5 (56.0)	0.4671
CRP (mg/L)	16.4 (2.1, 121.7)	64.6 (29.6, 133.1)	135.0 (40.9, 225.3)	160.0 (33.0, 270.6)	**0.0209**
Laboratory parameters					
Critical-Ill COVID-19	68.9 (60.1, 96.9)	94.1 (72.4, 111.1)	107.1 (83.7, 122.8)	115.5 (96.5, 142.5)	**0.0184**
IL-6 (pg/ml)	5.4 (3.3, 33.8)	31.3 (21.1, 74.8)	80.3 (17.3, 308.3)	108.0 (46.9, 165.0)	**0.0011**
IL-10 (pg/ml)	4.5 (1.6, 6.0)	6.0 (3.8, 9.0)	8.2 (2.2, 20.0)	19.1 (5.7, 62.8)	**0.0202**
HLA-G5 (ng/ml)	246.4 (159.6, 463.7)	330.1 (230.7, 450.9)	304.3 (153.4, 397.9)	276.9 (122.3, 332.8)	0.2284
tT (nmol/L)	4.3 (0.4, 11.7)	2.9 (2.0, 5.6)	0.94 (0.5, 1.5)	0.4 (0.3, 2.9)	**<0.0001**
E_2 (pg/ml)	28.3 (18.4, 38.1)	34.9 (17.2, 43.2)	31.0 (18.3, 43.7)	40.4 (32.8, 63.9)	**<0.0001**
SARS-CoV-2 IgG (AU/ml)	17.7 (3.8, 82.7)	16.8 (5.5, 66.7)	94.4 (7.8, 128.5)	10.1 (3.8, 102.5)	**0.0784**

Continuous variables are presented as medians (interquartile range); categorical variables are presented as frequency (%). For each variable, one-way ANOVA on ranks (Kruskal–Wallis test associated with Dunn's multiple comparison test) and Chi Square test were used for testing the difference among groups. Statistically significant p-values are indicated in bold.

Abbreviations: Asy, asymptomatic, BMI, Body Mass Index; CCI, Charlson Comorbidity Index; CRP, C-reactive protein; E_2, 17β-estradiol; ICU, intensive care unit; Sym, symptomatic; tT, total testosterone; WBC, white blood cells.

Critical-Illness (Ill) COVID-19 score was calculated as described in the methods.

TABLE 3 Multivariable logistic regression predicting death (with IL-10)

	OR	95%CI	P-value
IL-10	1.05	1.01, 1.09	**0.01**
tT	0.70	0.38, 1.27	0.2
E_2	1.01	0.97, 1.05	0.6
Age	1.13	1.00, 1.28	**0.04**

Statistically significant values are indicated in bold. tT, total testosterone; E_2, 17β-estradiol.

independent biomarkers of disease severity at early time points of SARS-CoV-2 infection.

3.5 | HLA-G5 and IL-10 inversely correlated with SARS-CoV-2S1/S2 IgG at hospital admission

At hospital admission, both HLA-G5 and IL-10 plasma levels correlated with tT, but HLA-G5 levels were not a predictive factor of death for men with COVID-19. In the hypothesis that IL-10 and HLA-G5 are induced to promote immune escape and viral spreading at an early stage of SARS-Cov-2 infection, we performed a linear regression analysis between SARS-CoV-2 IgG titer and HLA-G5 or IL-10 levels at hospital admission. Results revealed a weak but statistically significant negative correlation between SARS-CoV-2 IgG titer and HLA-G5 or IL-10 plasma levels ($p = 0.0205$, and $p = 0.0120$, respectively) (Figure 4A,B), suggesting that higher HLA-G5 and IL-10 plasma levels negatively impact on the immune responses against SARS-CoV-2. In 39 patients at follow-up, we also evaluated HLA-G5 plasma levels.

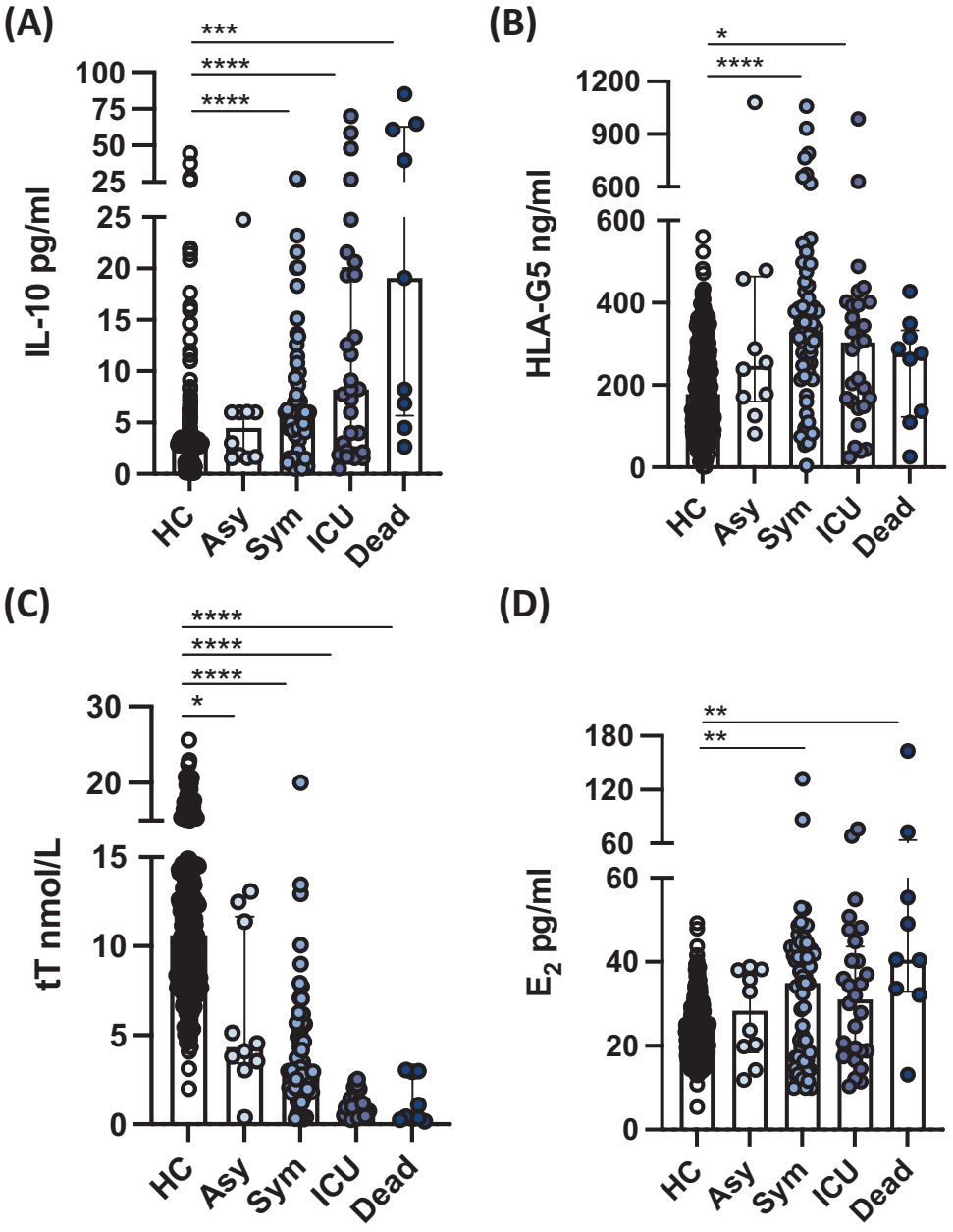

FIGURE 1 Interleukin (IL)-10 and human leukocyte antigen G5 (HLA-G5), testosterone and 17β-estradiol plasma levels in coronavirus disease 2019 (COVID-19) patients with different disease severity at hospitalization. IL-10 and HLA-G5 were evaluated by enzyme-linked immunosorbent assay (ELISA) and total testosterone (tT) and 17β-estradiol (E_2) were evaluated by chemiluminescence immunoassay (CLIA) in plasma of healthy donors (HC, $n = 292$) and of COVID-19 patients asymptomatic (Asy, $n = 10$), symptomatic (Sym, $n = 63$), admitted to the intensive care unit (ICU, $n = 29$), and patients subsequently deceased (Dead, $n = 9$). The levels of IL-10 (A), of soluble HLA-G5 (B), tT (C), and E_2 (D) in the indicated cohorts are shown. Each dot represents a single donor; bars indicate medians with an interquartile range. *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001. The *p*-values of all comparisons are reported in Table S2

Notably, HLA-G5 was significantly reduced at follow-up ($p < 0.0001$) compared to the admission time point, being in all the samples tested below the detection limit of the assay (Table 4 and Figure S2), thus supporting the hypothesis that elevated HLA-G5 levels at hospital admission are associated with viral active infections.

4 | DISCUSSION

Although in a limited number of patients, by investigating the combined impact of sex hormones, IL-10, and HLA-G5 on COVID-19 outcomes, we showed, for the first time, an inverse correlation between

TABLE 4 Demographic, clinical, and laboratory parameters of subjects included in the study at 7-month follow-up

Variables	Admission ($N = 53$)	Follow up ($N = 53$)	*p*-Value
Age	60.0 (48.0, 66.5)	60.0 (48.0, 66.5)	1.000
Ethnicity			
White-European	47 (88.6)		N/A
Latin-American	3 (5.7)		N/A
African	2 (3.8)		N/A
Asian-Far East Asian	1 (1.9)		N/A
BMI	28.7 (25.9, 31.4)	29.8 (26.1, 31.8)	1.0000
Comorbidities			
CCI	0.0 (0.0, 1.0)	0.0 (0.0, 0.0)	**0.0071**
CCI-age	2.0 (0.0, 3.5)	2.0 (0.0, 4.0)	**0.0001**
CCI (score)			
0	33 (62.3)	42 (79.3)	0.0867
1	9 (17.0)	5 (9.4)	0.3903
≥ 2	11 (20.7)	6 (11.3)	0.2897
Arterial hypertension	20 (37.7)	Not tested	N/A
CRP (mg/L)	115.8 (20.15, 3.5)	Not tested	N/A
Laboratory parameters			
WBC (count/ul)	7.0 (5.6, 8.6)	6.2 (5.5, 8.4)	0.2702
Neutrophils (count/ul)	5.6 (4.2, 6.5)	3.6 (2.9, 4.7)	<**0.0001**
Lymphocytes (count/ul)	1.0 (0.7, 1.5)	2.3 (1.9, 2.8)	<**0.0001**
IL-6 (pg/ml)	30.4 (7.72, 83.05)	27.7 (4.38, 86.5)	0.5561
IL-10 (pg/ml)	4.9 (2.0, 7.9)	5.6 (3.4, 7.8)	0.3887
HLA-G5 (ng/ml)	324.7 (215.3, 394.5)	<15.0 (< 15.0, < 15.0)	<**0.0001**
tT (nmol/L)	2.5 (1.3, 4.4)	9.8 (3.7, 10.3)	<**0.0001**
E_2 (pg/ml)	32.48 (17.4, 41.3)	30.4 (25.4, 35.3)	0.9807
SARS-CoV-2 IgG (AU/ml)	35.6 (5.5, 105)	134.0 (87.6, 224.0)	<**0.0001**

Continuous variables are presented as medians (interquartile range); categorical variables are presented as frequency (%). For each variable, the two-tailed Mann-Whitney test and Chi Square test were used for testing the difference among groups. Statistically significant p-values are indicated in bold.
Abbreviations: BMI, Body Mass Index; CCI, Charlson Comorbidity Index; CRP, C-reactive protein; E_2, 17β-estradiol; tT, total testosterone; WBC, white blood cells.

circulating IL-10 or HLA-G5 and tT in men with COVID-19 at the time of hospital admission. High IL-10 combined with low tT emerged as predictors of death in COVID-19 patients, whilst soluble HLA-G5 was not. We proposed that these parameters represent important and innovative biomarkers of disease severity. Interestingly, the correlation between IL-10 and tT was lost at the 7-month follow-up. Notably, soluble HLA-G5 and IL-10 positively correlated with SAR-CoV-2 infection, supporting their role in promoting immune escape and viral spreading.

The critical increase of IL-10 in patients with COVID-19 is currently indicated as a feature of hyperinflammation during infection,[1,10] and a number of studies reported that IL-10 levels, as well as the presence of high frequency of IL-10-producing regulatory T cells, represent a predictor marker for disease severity.[7–9,46] We confirmed the finding of significantly elevated levels of IL-10, and IL-6, in male patients with COVID-19 at hospital admission compared to HC, which progressively increased according to disease severity, with ICU admission or death outcomes showing the highest levels. Age-related differences in plasma cytokine levels demonstrated that IL-10 is stable throughout life, while IL-6 is increased in elderly people.[47–50] The elevated levels of IL-6 in COVID-19 patients compared to HC reflect the age difference; however, the detected IL-6 levels in patients were 10-fold higher compared to those reported in age-matched healthy volunteers,[47] indicating that the high levels of IL-6 detected in patients are related not only to the age but also to the disease. Conversely, the observed increased levels of IL-10 are disease-related.

We reported an inverse correlation between IL-10 and tT levels in men with COVID-19. Moreover, we observed that the risk of death in those men with the lowest tT levels increased in the presence of high IL-10 levels. These results are in line with previous data where low tT levels were indeed associated with a greater risk of ICU admission and death outcomes.[38,40] Pre-clinical and clinical studies showed that low testosterone is associated with an increase in pro-inflammatory

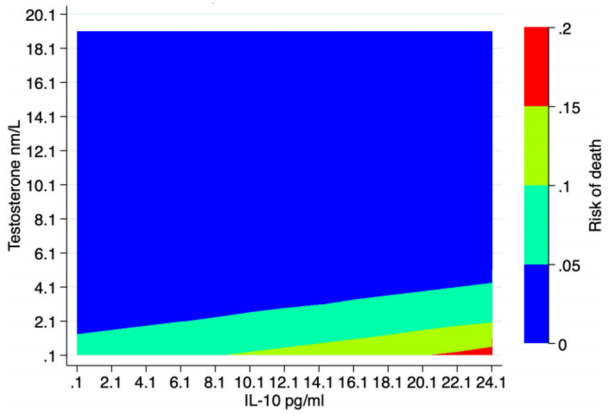

FIGURE 2 Risk of death by testosterone and interleukin (IL)-10 levels. Interaction test assessing the hypothesis that circulating IL-10 levels could differently impact the relationship between the total testosterone levels and the risk of death

cytokines (e.g., IL-6 and TNF),[51] and testosterone supplementation in hypogonadal men suppressed IL-1β and TNF and potentiated the expression of IL-10.[52] In contrast, in men with COVID-19 we observed that lower tT levels were associated with higher IL-10. Thus, we can postulate that the SARS-CoV-2 infection induces the release of IL-10, which, in the presence of severe inflammation, promotes an acute serum testosterone drop, compatible with a hypogonadal status,[53] along with an increase in E_2 levels. Further supporting our hypothesis are results from a limited number of patients, for which viral load levels were available, indicating that in the presence of high SARS-CoV-2 viral load the levels of IL-10 increased. A direct correlation between viral load and tT was not evidenced (data not shown). Overall, our data support the hypothesis that the hormonal milieu observed in men with severe COVID-19 is compatible with a condition of secondary hypogonadism,[38] where we may speculate that low tT levels are a consequence of SARS-CoV-2 infection[54] rather than a predisposing factor for SARS-CoV-2 infection[55] and the consequent COVID-19 clinical severity. In this context, findings at the 7-month follow-up support this conclusion. Notwithstanding not in all patients,[54] at the 7-month assessment, the trend toward normalization of circulating tT was observed, along with a reduction of IL-10 plasma levels, and the inverse correlation between IL-10 e tT was eventually lost.

We reported that dead patients were older than the asymptomatic ones, but their age distribution was comparable to that of symptomatic and ICU patients. It is known that testosterone peaks at age 19 years and fall by age 40 years, but no evidence for a further fall with increasing age through to old age was reported.[45] The 53 COVID-19 patients analyzed at hospital admission and 7-month follow-up are comprised of between 36 and 75 years, an age for which on average 13 nmol/L of serum testosterone has been reported in age-matched healthy subjects.[45] The overall levels of tT in COVID-19 patients were below 5 nmol/L, and at 7-months follow-up in most of the patients increased and, in a large fraction of patients, reached the normal levels. Thus, this evidence supports the conclusion that tT variations in our patients were not associated with aging, but with the disease. Age-related immune changes with the functional decline of both innate and adaptive immunity with an overall increase in pro-inflammatory cytokines, IL-6 and TNF, also called "inflammaging",[56] have been associated with increased infections, including SARS-CoV-2,[57] and other diseases.[58] Comorbidities, evaluated as CCI, were comparable among the COVID-19 patients analyzed excluding a link between the reported results with inflammation and disease associated with aging. Overall, we can conclude that the observed associations between IL-10, tT, and risk of death are not linked to age nor associated with comorbidities.

It is still debated whether IL-10 elevation at an early stage of the infection, induced to counteract the increase of IL-6 and the inflammatory responses, fails to control the disease because it acts as a pro-inflammatory molecule, thus promoting the further production of other cytokines.[11] It is well known that viral infections promote IL-10 release as a mechanism to favor immune escape and viral spreading.[5,59] In this context, IL-10 has been shown to be indeed critically involved in inhibiting immune responses by promoting the expression of HLA-G and its receptors, and regulatory cell expansion.[23,26,60,61] At hospital admission, in male patients with COVID-19 we observed a significant increase in soluble HLA-G5 levels, with the highest HLA-G5 levels detected in symptomatic and ICU patients. The involvement of HLA-G has been previously reported in COVID-19 patients showing downregulation of membrane-bound HLA-G in T and B cells during SARS-CoV-2 infections, which returned to basal levels when viral RNA returned negative,[20] and significantly increased levels of soluble HLA-G at the time of patients hospitalization.[21,62] Here, we depicted a weak but significant negative correlation between SARS-CoV-2 IgG titer and HLA-G5 (or IL-10) plasma levels in men at hospital admission, suggesting that high HLA-G5 and IL-10 levels negatively impact the immune responses against SARS-CoV-2 infection. These data are in contrast with previous findings showing that membrane-bound HLA-G levels decreased during the replication phase of SARS-CoV-2 and increased after clearance[20] and soluble HLA-G levels increased when clinical outcomes improve in COVID-19.[62] These discrepancies might be ascribed to the different cohorts of patients analyzed: COVID-19 female ICU admitted patients[20] and COVID-19 female and male patients[62] as compared with a cohort of COVID-19 male patients in our study. Moreover, conclusions are based on results obtained by analyzing HLA-G levels at early time points after COVID-19 diagnosis: 23 days[20] and 19 days,[62] respectively. In the present study we showed novel evidence that HLA-G5 plasma levels were significantly reduced at 7-month follow-up, thus supporting the hypothesis that the up-regulation of HLA-G at the early stage after SARS-CoV-2 infection represents a mechanism to prevent immune responses against the virus, eventually promoting viral spreading.[63] Although we cannot demonstrate a direct effect of IL-10 in promoting the induction of soluble HLA-G5, we may propose that the dramatic elevation of IL-10 during COVID-19 is indeed a negative feedback mechanism induced to suppress excessive inflammation that acts also as positive feedback for viral spreading and survival. It has been proposed that IL-10 acts as a pro-inflammatory cytokine promoting $CD8^+$ T cell activation, which on the one hand leads to the increase of IFN-γ levels, which in turn activate tissue macrophages, and on the other promotes $CD8^+$ T cell

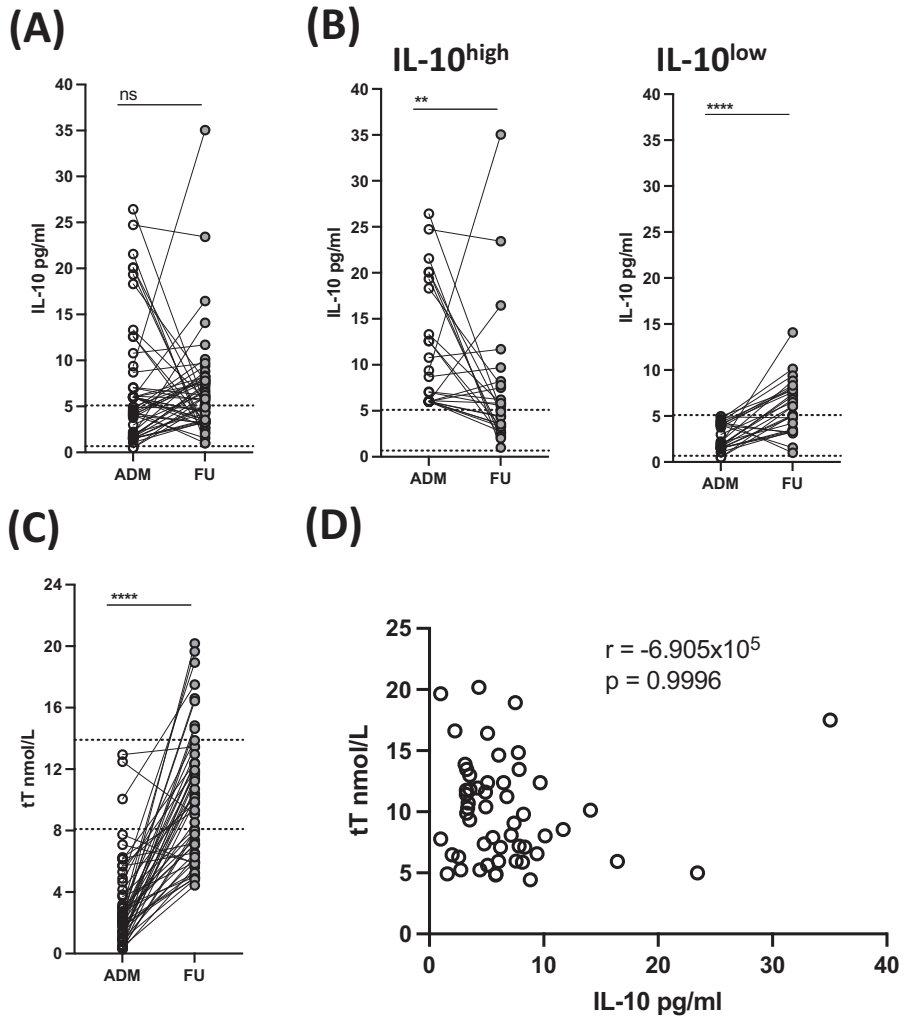

FIGURE 3 Interleukin (IL)-10 and testosterone levels in coronavirus disease 2019 (COVID-19) patients at follow-up. IL-10 (A,B) and total testosterone (tT) (C) were evaluated in the plasma of 53 COVID-19 patients at first hospital admission (ADM) and 7-month follow-up (FU). Each dot represents a single donor; lines connect the data of the same donor. Dot lines indicate the 95% CI of the indicated analyte in HC ($n = 292$). Ns, not significant, **≤0.01, and ****≤0.0001 are *p*-values of paired Wilcoxon's test. (D) Scatterplot and linear regression analyses between the levels of IL-10 and tT of patients at 7-month follow-up in the entire cohort. Each data point represents a donor, data distribution, Pearson's rank correlation coefficient (r), and p-value are shown

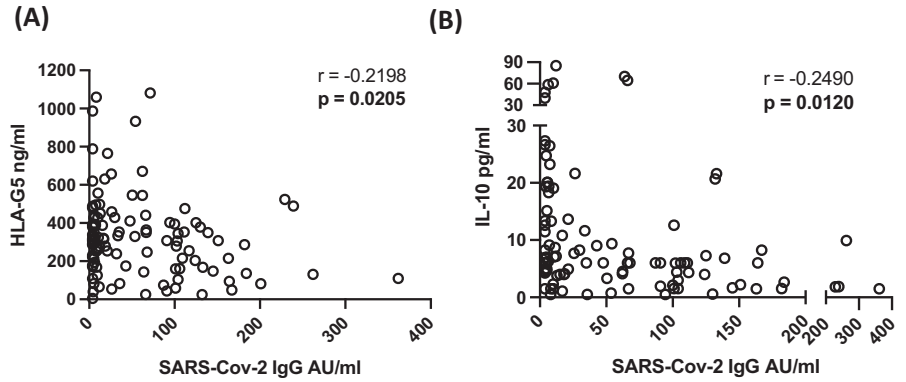

FIGURE 4 Human leukocyte antigen G5 (HLA-G5) and interleukin (IL)-10 inversely correlated with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) immunoglobulin G (IgG) in coronavirus disease 2019 (COVID-19) patients at hospitalization. SARS-CoV-2 IgG, HLA-G5, and IL-10 were evaluated in the plasma of COVID-19 patients at hospital admission. Scatterplot and linear regression analyses between the levels of (A) SARS-CoV-2 IgG and HLA-G5, and (B) SARS-CoV-2 IgG and IL-10. Each data point represents a donor, data distribution, Pearson's rank correlation coefficient (*r*), and *p*-value are shown

exhaustion.[10,64,65] However, one feature of SARS-CoV-2 infection is lymphopenia,[66,67] which in severe disease patients is associated with low clonal expansion of $CD8^+$ T cells with the exhausted phenotype and reduced ability to secrete cytokines.[67,68] This functional exhaustion of $CD8^+$ T cells has been correlated with viral persistence and disease progression in other settings.[69] Thus, our findings support the central role of IL-10 at the early stage of SARS-CoV-2 infection in promoting T-cell exhaustion and viral spreading.

In conclusion, we show that circulating IL-10, soluble HLA-G5, and testosterone are linked to SARS-CoV-2 infection, acting as biomarkers of disease severity. High levels of IL-10 and soluble HLA-G5 and low tT levels at the time of hospital admission are associated with more severe clinical outcomes in male patients with COVID-19, with both IL-10 and tT levels being relevant predictors of the risk of death. These results pave the way for additional longitudinal studies, based on longer follow-up and including larger cohorts of subjects, to validate the early concomitant evaluation of IL-10 and testosterone as a prognostic factor of disease outcome.

AUTHOR CONTRIBUTIONS

Giada Amodio, Paolo Capogrosso, and Francesco Montorsi analyzed data and drafted the report; Giuseppe A. Ramirez, Antonella Castagna, Alberto Zangrillo, Francesco De Cobelli, Michela Tassara, Giovanni Landoni, Moreno Tresoldi, Patrizia Rovere-Querini, and Fabio Ciceri took care of patients; Giada Amodio, Marina Pontillo, Cristina Tresoldi, Massimo Locatelli, Luca Santoleri, Michela Tassara, Luca Boeri, Cristina Carenzi, Anna Maria Ferrara, Daniele Cignoli acquired the data. Andrea Salonia designed and led the study, and Giada Amodio, Andrea Salonia, and Silvia Gregori analyzed the data and wrote the report.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

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

ORCID

Giada Amodio https://orcid.org/0000-0002-2457-387X
Paolo Capogrosso https://orcid.org/0000-0003-2347-9504
Luca Boeri https://orcid.org/0000-0003-0040-784X
Daniele Cignoli https://orcid.org/0000-0001-7873-7318
Antonella Castagna https://orcid.org/0000-0002-8338-9714
Alberto Zangrillo https://orcid.org/0000-0002-7687-7648
Moreno Tresoldi https://orcid.org/0000-0003-1541-6650
Giovanni Landoni https://orcid.org/0000-0002-8594-5980
Fabio Ciceri https://orcid.org/0000-0003-0873-0123
Andrea Salonia https://orcid.org/0000-0002-0595-7165
Silvia Gregori https://orcid.org/0000-0002-3517-9683

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SUPPORTING INFORMATION

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

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