

Low testosterone levels and high estradiol to testosterone ratio are associated with hyperinflammatory state and mortality in hospitalized men with COVID-19

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Abstract. – OBJECTIVE: Evidence supports a sex disparity in clinical outcomes of COVID-19 patients, with men exhibiting higher mortality rates compared to women. We aimed to test the correlation between serum levels of sex hormones [total testosterone, estradiol (E2), estradiol to testosterone (E2/T) ratio, progesterone], prolactin and 25-hydroxyvitamin D [25(OH)D] and markers of inflammation, coagulation and sepsis at admission in hospitalized men with COVID-19.

PATIENTS AND METHODS: We conducted an exploratory retrospective study including symptomatic men with confirmed SARS-CoV-2 infection who were consecutively admitted to our Institution between April 1 and May 31, 2020.

RESULTS: Patients were divided into survivors (n=20) and non-survivors (n=39). As compared to survivors, non-survivors showed significantly higher median neutrophil-to-lymphocyte ratio (NLR) values, D-dimer and procalcitonin (PCT) levels, along with significantly lower median 25(OH)D levels and total testosterone levels. Non-survivors exhibited significantly higher median values of E2/T ratio (a marker of aromatase activity). Spearman's correlation analysis revealed that total testosterone levels were significantly and inversely correlated with NLR, high-sensitivity C-reactive protein (hsCRP), interleukin-6, D-dimer and PCT. Conversely, E2/T ratio values were significantly and

positively correlated with the aforementioned markers and with white blood cell (WBC) count. In a multivariate analysis performed by a logistic regression model after adjusting for major confounders (age, body mass index, hypertension and cardiovascular disease, diabetes mellitus and malignancy), total testosterone levels were significantly and inversely associated with risk of COVID-19-related in-hospital mortality.

CONCLUSIONS: Low total testosterone levels and elevated E2/T ratio values at admission are associated with hyperinflammatory state in hospitalized men with COVID-19. Low total testosterone levels at admission represent an independent risk factor for in-hospital mortality in such patients. Therefore, total testosterone and E2/T ratio may serve as prognostic markers of disease severity in this population.

Key Words:

Testosterone, Estradiol, Progesterone, Estradiol to testosterone ratio, E2/T ratio, Aromatase activity, Vitamin D, Men, SARS-CoV-2, COVID-19 mortality.

Introduction

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome corona-

virus 2 (SARS-CoV-2) has been posing serious threats to global health since it was declared a pandemic on March 11, 2020. A dysregulated immune response called “cytokine storm” (a.k.a. “cytokine release syndrome”) and characterized by an exuberant increase in circulating levels of several pro-inflammatory cytokines [such as interferon- γ , tumor necrosis factor (TNF), interleukin (IL)-1, IL-2, IL-6] plays a pivotal role in the pathophysiology of the most severe cases of COVID-19, resulting in acute respiratory distress syndrome, disseminated intravascular coagulation, multiorgan failure and ultimately death^{1,2}.

Several studies on a global scale have suggested the existence of a sex disparity in clinical outcomes of COVID-19 patients³. Of note, hospitalized patients with severe COVID-19 exhibiting higher odds of death are more likely to be men than women⁴. Various comorbidities such as obesity, metabolic syndrome, type 2 diabetes, cardiovascular disease (CVD), hypertension, chronic kidney disease, chronic lung disease, hypovitaminosis D, as well as older age have emerged as independent risk factors for worse outcomes of COVID-19⁵⁻⁸.

The sexual dimorphism of COVID-19 morbidity and mortality has prompted researchers to hypothesize that the primary male sex hormone testosterone may represent an independent risk factor for COVID-19 severity, while estrogens (particularly the primary female sex hormone estradiol) may be protective^{9,10}. Sex-related hormonal milieu might influence immune response to the virus, virus-induced multiorgan dysfunction, development of the hyperinflammatory and hypercoagulable state, and COVID-19 morbidity and mortality after SARS-CoV-2 infection^{3,11}. Sex-associated differences in the expression and activity of the SARS-CoV-2 entry receptor angiotensin-converting enzyme 2 (ACE2) and its co-receptor transmembrane protease serine subtype 2 (TMPRSS2) have been suggested to contribute to the higher COVID-19 mortality rates observed in men^{12,13}. After ACE2 receptor binding and SARS-CoV-2 entry into host cells, there is a downregulation of ACE2 leading to an excessive accumulation and pro-inflammatory activity of angiotensin II (Ang II) that potentially results in the development of lung injury, pneumonia, acute respiratory distress syndrome, cardiac injury and/or myocarditis^{1,14}. A study conducted on isolated human airway smooth muscle cells demonstrated that testosterone upregulates ACE2 expression in both males

and females¹⁵, which may result in decreased accumulation of the pro-inflammatory molecule Ang II in the lungs. In addition, an expression patterns analysis of ACE2 in adult human testes indicated that ACE2 is predominantly enriched in spermatogonia and Sertoli and Leydig cells, providing evidence that the human testis is a potential target of SARS-CoV-2 infection¹⁶. Variability in COVID-19 severity may also be explained by differences in the host genome. For example, long polyQ alleles in the androgen receptor have been associated with severe clinical outcomes in men with COVID-19^{17,18}. Low testosterone levels have also been suggested to predispose to endothelial dysfunction, thrombosis and altered immune response, leading to impaired viral clearance and systemic inflammation¹⁹. However, whether the aforementioned mechanisms explain the sex disparity in disease severity and clinical outcomes of patients with COVID-19 still remains uncertain. It is also worth reminding that the abovementioned comorbidities (e.g. CVD, obesity, diabetes, chronic kidney disease, chronic lung disease etc.) associated with worse COVID-19 outcomes are often accompanied by lower serum testosterone concentrations in men^{20,21}. With regard to older age, testosterone concentrations in men decline continuously by 1% to 2% per year starting after 30 years of age^{22,23}. Nevertheless, retrospective and prospective studies have shown that low serum total testosterone levels are associated with exuberant systemic inflammation, disease severity and mortality in hospitalized men with COVID-19²⁴⁻²⁷.

In view of the above, we conducted an exploratory retrospective study among male patients with COVID-19 admitted to our Institution during the first Italian COVID-19 outbreak, comparing the levels of circulating sex hormones [total testosterone, 17 β -estradiol (E2), progesterone, estradiol/testosterone (E2/T) ratio] and prolactin (PRL) at admission between survivors and non-survivors. Our study primarily aimed to evaluate the association between circulating hormones (sex hormones and PRL) and: i) different markers of inflammation, coagulation and sepsis (including vitamin D), and ii) clinical outcomes of survival and death in this population. The choice to study sex hormones, PRL and vitamin D was based on the potential immunomodulatory role of these hormones and on their subsequent influence on COVID-19 pathophysiology and clinical course²⁸⁻³³.

Patients and Methods

Study Design and Participants

We conducted an exploratory retrospective study including symptomatic male patients with confirmed SARS-CoV-2 infection who were consecutively admitted to our Institution (Tor Vergata University Hospital-PTV, Rome, Italy) during the first Italian wave of COVID-19 (between April 1 and May 31, 2020). Participants were divided into two groups based on the outcomes of survival and death after hospital admission, namely: survivors and non-survivors.

Informed Consent and Data Collection

The study was reviewed and approved by the Ethics Committee of University of Rome Tor Vergata (Registration Number: 141/20, July 23, 2020). At admission, all participants and/or their legal guardians provided written informed consent to anonymous data collection and analysis for research purposes. Patients' medical records were independently reviewed by two members of our research team. Demographic, clinical and laboratory data were collected via electronic medical records (Modulab®) and recorded in an anonymous database containing unambiguous and alphanumeric codes that were progressively assigned to all hospitalized patients with COVID-19. This study and data collection were performed in accordance with the principles of the Declaration of Helsinki.

Clinical, Biochemical and Hormonal Assessment

Initial diagnosis of COVID-19 was made by an infectious disease specialist based on the presence of clinical symptoms (fever, cough, dyspnea and/or anosmia) and imaging tests (chest X-ray and/or computed tomography) suggestive of acute respiratory tract infection and COVID-19-related pneumonia. Laboratory confirmation of SARS-CoV-2 infection was made through nasopharyngeal swab samples obtained at hospital admission and analyzed via real-time reverse transcription polymerase chain reaction (RT-PCR) for 2019-nCoV RNA extraction according to the manufacturer's instructions (RT-PCR kit Seegene Allplex™ 2019-nCoV Assay, Seegene Inc, Seoul, South Korea). Hematological, biochemical and hormonal parameters were measured on venous blood, serum and plasma samples collected at

admission to the emergency department or infectious disease unit, when patients had not yet taken any steroids or antivirals.

White blood cell (WBC) count was measured by using an automated hematological analyzer (Dasit-Sysmex, Milan, Italy). Neutrophil-to-lymphocyte ratio (NLR) was also measured as a marker of systemic inflammation (reference range: 0.78-3.53)³⁴. Serum levels of high-sensitivity C-reactive protein (hsCRP; reference range: 0-5 mg/L) were determined by using an immunoturbidimetric method (Abbott Diagnostics, Milan, Italy). Serum levels of IL-6 (reference range: 0-50 pg/mL) were measured by using a chemiluminescence method (IMMULITE 2000 instrument, Siemens, Milan, Italy), whereas serum levels of TNF- α (reference range: 0-12.4 pg/mL) were measured by using the enzyme-linked immunosorbent assay (ELISA) technique (DRG, International Instruments GmbH, Marburg, Germany). Serum levels of procalcitonin (PCT; reference range: 0.01-0.50 ng/mL) were measured by using a chemiluminescence method (Architect Instrument, Abbott, Milan, Italy). Plasma fibrinogen (reference range: 200-400 mg/dL) and D-dimer (reference range: 0-500 ng/mL) levels were measured through the Clauss method and the immunometric method, respectively, by using the same automated analyzer (ACL-TOP 750, Instrumentation Laboratory, Werfen, Milan, Italy). Total serum 25-hydroxyvitamin D [25(OH)D] levels were measured by electrochemiluminescence (Abbott Architect Instrument, Milan, Italy), with the limit of quantitative value at 2.2 ng/mL at 20% coefficient variation. We measured total serum 25(OH)D levels, as they represent the most reliable biomarker of vitamin D status³⁵. Vitamin D status was defined according to the Endocrine Society guidelines³⁵.

For the specific purposes of this study, on each sample for every patient at admission we measured circulating hormones by using commercially available analytical methods. In all participants, total testosterone, E2, progesterone and PRL were measured by a direct chemiluminescent immunoassay (CLIA; Abbott Architect Instrument, Milan, Italy). Reference ranges for circulating hormones in men were the following: total testosterone, 240.24-870.68 ng/dL; E2, 11-44 pg/mL; progesterone, <0.2 ng/mL; PRL, 3.46-19.4 ng/mL. In order to evaluate the balance between E2 and testosterone, we calculated the E2/T as a marker of aromatase activity, as it has previously been described even in the context of

COVID-19^{26,36}. The formula $10 \times E2/T$ was used to calculate the E2/T ratio and to make the resulting ratio unit-free. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (expressed in meters squared, m^2).

Statistical Analysis

With regard to statistical analysis, continuous variables with normal distribution were presented as mean and standard deviation (SD), whereas non-normal variables were reported as median and interquartile range (IQR). Comparisons of continuous variables between two groups were performed through *t*-test or Wilcoxon test in case of non-normal data. Chi-squared test and Fisher's exact test were used to compare categorical variables between groups. Comparisons between percentages were performed using the chi-squared test. Correlation between two continuous variables was tested by Spearman's rank correlation coefficient. Multivariate analysis performed by a logistic regression model was used to determine the independent association of sex hormone, PRL and 25(OH)D levels and risk of COVID-19-related in-hospital mortality. The following covariates were initially included in the logistic regression model: (1) continuous variables: age, BMI, E2, total testosterone, E2/T ratio, progesterone, PRL and 25(OH)D levels, and (2) categorical variables: hypertension and CVD, diabetes mellitus, and malignancy (active malignancy or history of previous malignancy). After backward elimination process, variables associated with a *p*-value of >0.1 were excluded from the model. For all the statistical analyses performed, a *p*-value of <0.05 was considered statistically significant. All statistical analyses were performed using MedCalc Version 18.2.18 (MedCalc Software Ltd, Ostend, Belgium).

Results

Participant Demographics and Length of Hospital Stay

A total of 59 consecutive Caucasian men admitted to our Institution (between April 1 and May 31, 2020) were enrolled into this retrospective single-center study. All patients were laboratory-confirmed cases of COVID-19. Table I shows participant demographics and length of hospital stay, whereas Table II lists the prevalence of major comorbidities in the study population.

Patients were divided into two groups based

on the outcomes of survival and death after hospital admission, namely: survivors ($n=20$; 34%) and non-survivors ($n=39$; 66%). The mean age of study participants was 66.83 ± 12.87 years. Mean age was lower in survivors compared to non-survivors (64.1 ± 13 vs. 68.2 ± 12.7 years, respectively), although this difference was not statistically significant ($p=0.24$). Median BMI values were comparable between survivors and non-survivors (27.7 vs. 27.9 Kg/m^2 , respectively; $p=0.63$) (Table I). Among survivors, there were 2 patients (10%) with obesity, 6 patients (30%) with hypertension and CVD, 1 patient (5%) with diabetes mellitus, and 2 patients (10%) with active or previous malignancy. Among non-survivors, there were 10 patients (25.6%) with obesity, 18 patients (46.1%) with hypertension and CVD, 5 patients (12.8%) with diabetes mellitus, and 7 patients (17.9%) with active or previous malignancy. All diabetic patients had type 2 diabetes mellitus. There was no statistically significant difference in the prevalence of obesity (expressed as a BMI value of ≥ 30 kg/m^2), hypertension and CVD, diabetes mellitus, and active or previous malignancy between survivors and non-survivors (Table II).

As per our institutional protocol, all patients received the same standard care for the treatment of COVID-19, which consisted of dexamethasone plus hydroxychloroquine and/or lopinavir/ritonavir administered shortly after the admission and the initial blood collection. The mean length of hospital stay was significantly longer in survivors than in non-survivors (34 ± 19 days vs. 14 ± 7 days, respectively; $p<0.0001$) (Table I). As compared to survivors, a higher proportion of non-survivors required intensive care unit (ICU) admission, which is in line with the greater disease severity in the non-survivor group. Of note, a significantly higher proportion of non-survivors required ICU admission compared to survivors: 31 out of 39 non-survivors (79.4%) vs. 7 out of 20 survivors (35%) ($p<0.0001$).

Markers of Inflammation, Coagulation and Sepsis in Survivors and Non-Survivors

Different markers of inflammation, coagulation, and sepsis were measured at admission in survivors and non-survivors. Values of hematological and biochemical parameters in survivors and non-survivors at the time of hospital admission are shown in Table III. Non-survivors exhibited significantly higher median NLR values

Table I. Participant demographics and length of hospital stay. *p*-values refer to the comparison between survivor and non-survivor groups.

| Total number of patients: 59 | | | | | | | |
|-------------------------------------|---------|---------|-------|--------|-------|--|---|
| Mean age: 66.83 ± 12.87 years | | | | | | | |
| Survivors, N (%) 20 (34%) | Minimum | Maximum | Mean | Median | SD | 25 th -75 th percentile | <i>p</i> -value compared to non-survivors |
| Age (years) | 38 | 89 | 64.10 | 61 | 13.02 | 55-76 | <i>p</i> = 0.24 |
| BMI (Kg/m ²) | 25.4 | 33 | 28.03 | 27.7 | 1.95 | 26.6-28.7 | <i>p</i> = 0.63 |
| Length of hospital stay (days) | 7 | 75 | 34.1 | 28.5 | 19.6 | 20-47 | <i>p</i> < 0.0001 |
| Non-survivors, N (%) 39 (66%) | Minimum | Maximum | Mean | Median | SD | 25 th -75 th percentile | <i>p</i> -value compared to survivors |
| Age (years) | 39 | 88 | 68.23 | 72 | 12.74 | 58.5-79 | <i>p</i> = 0.24 |
| BMI (Kg/m ²) | 23.5 | 36 | 28.5 | 27.9 | 3.0 | 26.9-30.3 | <i>p</i> = 0.63 |
| Length of hospital stay (days) | 4 | 32 | 14.0 | 13 | 7.0 | 8.25-17.75 | <i>p</i> < 0.0001 |

BMI, body mass index; SD, standard deviation.

compared to survivors (14.1 vs. 5.6, respectively; *p*=0.008). There was no significant difference in median WBC count values between survivors and non-survivors (Table III).

With regard to markers of coagulation, non-survivors showed significantly higher median levels of D-dimer as compared to survivors: 1348 ng/mL vs. 949.5 ng/mL, respectively (*p*=0.03). Median levels of fibrinogen were also higher in non-survivors compared to survivors (701 mg/dL vs. 692 mg/dL, respectively), although there was no statistically significant difference in median fibrinogen levels between the two groups (Table III). With regard to markers of inflammation and sepsis, non-survivors showed a trend toward higher median hsCRP levels compared to survivors (141.2 mg/L vs. 96.9 mg/L, respectively; *p*=0.07). Median values of PCT were significantly higher in non-survivors compared to survivors (0.47 ng/mL vs. 0.10 ng/mL; *p*=0.002). Median IL-6 and TNF- α values were comparable between survivors and non-survivors (Table III).

Hormonal Assessment in Survivors and Non-Survivors

Values of sex hormones and PRL in survivors and non-survivors at the time of hospital admission are shown in Table IV. Non-survivors showed significantly lower median total testosterone levels com-

pared to survivors (52.12 ng/dL vs. 140.31 ng/dL, respectively; *p*=0.005). The percentage of patients with severely low total testosterone levels (<100 ng/dL) was significantly higher in the non-survivor group compared to the survivor group: 71.8% of non-survivors (28 out of 39 non-survivors) vs. 35% of survivors (7 out of 20 survivors) (*p*<0.0001).

Non-survivors also exhibited higher median E2 levels compared to survivors (28 pg/mL vs. 26 pg/mL, respectively), although this difference was not statistically significant (Table IV). However, non-survivors showed significantly higher median E2/T ratio values compared to survivors (6.77 vs. 2.08, respectively; *p*=0.006). Moreover, non-survivors showed a trend toward higher median progesterone levels compared to survivors (0.20 ng/mL vs. 0.10 ng/mL, respectively; *p*=0.07). Median PRL values were within the reference range in both survivors and non-survivors (12.15 ng/mL vs. 17.33 ng/mL, respectively), and there was no statistically significant difference in median PRL values between the two study groups (Table IV).

Correlation Between Sex Hormones, PRL and Markers of Inflammation, Coagulation and Sepsis

Spearman's correlation analysis did not reveal any significant correlation between age, BMI,

Table II. Prevalence of major comorbidities in the study population. Percentages refer to the total number of patients within each group (survivor group and non-survivor group).

| Comorbidity | Survivors (N = 20) | Non-survivors (N = 39) | p-value |
|---|--------------------|------------------------|-----------------|
| Obesity (BMI \geq 30 Kg/m ²), n (%) | 2 (10%) | 10 (25.6%) | <i>p</i> = 0.15 |
| Hypertension and CVD, n (%) | 6 (30%) | 18 (46.1%) | <i>p</i> = 0.23 |
| Diabetes mellitus*, n (%) | 1 (5%) | 5 (12.8%) | <i>p</i> = 0.34 |
| Active or previous malignancy, n (%) | 2 (10%) | 7 (17.9%) | <i>p</i> = 0.42 |

BMI, body mass index; CVD, cardiovascular disease. *All diabetic patients had type 2 diabetes mellitus.

sex hormones and PRL (**Supplementary Table I**). However, we found a trend toward a significant positive association between BMI and E2/T ratio values (ρ : 0.240; p =0.06) (**Supplementary Table I**).

Spearman correlations between sex hormones, PRL and markers of inflammation, coagulation and sepsis at the time of hospital admission are shown in Table V. Total testosterone levels were significantly and inversely correlated with

NLR (ρ : -0.451; p =0.0003), hsCRP (ρ : -0.350; p =0.006), IL-6 (ρ : -0.266; p =0.04), D-dimer (ρ : -0.327; p =0.01) and PCT (ρ : -0.551; p <0.0001). We also observed an inverse correlation between total testosterone levels and WBC count that approached statistical significance (ρ : -0.255; p =0.05) (Table V).

E2 levels were significantly and positively correlated with WBC count (ρ : 0.449; p =0.0004) and NLR values (ρ : 0.306; p =0.01). Also, there

Table III. Values of hematological and biochemical parameters in survivors and non-survivors at the time of hospital admission.

| Hematological and biochemical parameters | Survivors (N = 20) | Non-survivors (N = 39) | p-value |
|--|---|--|--------------|
| WBC count ($\times 10^3/\mu\text{L}$) | Median: 7.04 95% CI: 6.20 to 7.79 IQR: 6.11 to 8.19 | Median: 8.29 95% CI: 7.05 to 9.82 IQR: 5.73 to 10.80 | 0.18 |
| NLR | Median: 5.58 95% CI: 3.48 to 9.40 IQR: 3.22 to 11.73 | Median: 14.10 95% CI: 8.40 to 17.41 IQR: 5.82 to 21.09 | 0.008 |
| D-dimer (ng/mL) | Median: 949.50 95% CI: 614.09 to 1487.09 IQR: 598.50 to 1558.00 | Median: 1348.00 95% CI: 1059.09 to 3539.87 IQR: 830.25 to 16892.75 | 0.03 |
| Fibrinogen (mg/dL) | Median: 692.00 95% CI: 531.90 to 806.51 IQR: 510.00 to 829.50 | Median: 701.00 95% CI: 582.42 to 817.31 IQR: 529.75 to 896.00 | 0.68 |
| hsCRP (mg/L) | Median: 96.95 95% CI: 39.41 to 145.56 IQR: 31.70 to 163.25 | Median: 141.20 95% CI: 100.14 to 192.71 IQR: 79.72 to 227.50 | 0.07 |
| IL-6 (pg/mL) | Median: 53.50 95% CI: 37.74 to 164.27 IQR: 31.90 to 194.00 | Median: 50.80 95% CI: 29.59 to 85.10 IQR: 23.20 to 149.25 | 0.79 |
| TNF- α (pg/mL) | Median: 26.31 95% CI: 11.67 to 48.07 IQR: 10.62 to 65.29 | Median: 21.82 95% CI: 14.97 to 35.70 IQR: 9.24 to 39.95 | 0.68 |
| PCT (ng/mL) | Median: 0.10 95% CI: 0.04 to 0.18 IQR: 0.04 to 0.19 | Median: 0.47 95% CI: 0.17 to 1.46 IQR: 0.10 to 2.19 | 0.002 |
| 25(OH)D (ng/mL) | Median: 10.95 95% CI: 9.05 to 14.72 IQR: 8.10 to 15.25 | Median: 8.10 95% CI: 6.05 to 11.52 IQR: 5.22 to 14.22 | 0.04 |

25(OH)D, 25-hydroxyvitamin D; 95% CI, 95% confidence interval for the median; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; IQR, interquartile range; NLR, neutrophil-to-lymphocyte ratio; PCT, procalcitonin; TNF- α , tumor necrosis factor-alpha; WBC, white blood cell.

was a trend toward a significant positive correlation between E2 and D-dimer levels (ρ : 0.246; $p=0.06$).

E2/T ratio values were significantly and positively correlated with WBC count (ρ : 0.416; $p=0.001$), NLR values (ρ : 0.501; $p=0.0001$), and hsCRP (ρ : 0.385; $p=0.002$), IL-6 (ρ : 0.273; $p=0.03$), D-dimer (ρ : 0.402; $p=0.001$) and PCT levels (ρ : 0.611; $p<0.0001$) (Table V).

Progesterone levels were significantly and positively correlated with WBC count (ρ : 0.310; $p=0.01$). We also observed a positive correlation between progesterone levels and NLR values that approached statistical significance (ρ : 0.249; $p=0.05$) (Table V).

PRL levels were significantly and positively associated with PCT levels (ρ : 0.266; $p=0.04$).

Vitamin D Status in Survivors and Non-Survivors

Vitamin D status was defined according to the Endocrine Society guidelines as follows: i) vitamin D deficiency, defined as serum 25(OH)D levels <20 ng/mL; ii) vitamin D insufficiency, defined as serum 25(OH)D levels between 20 and <30 ng/mL; iii) vitamin D sufficiency, defined as serum 25(OH)D levels ≥ 30 ng/mL. Vitamin D deficiency was further divided into two different categories: i) severe vitamin D deficiency, defined as serum 25(OH)D levels <10 ng/mL; and ii) mild-to-moderate vitamin D deficiency, defined

as serum 25(OH)D levels between 10 and <20 ng/mL.

Median total serum 25(OH)D levels were significantly lower in non-survivors compared to survivors (8.1 ng/mL vs. 10.9 ng/mL, respectively; $p=0.04$) (Table III). In the entire study cohort, 56% of patients (33/59) had severe vitamin D deficiency, 34% (20/59) had mild-to-moderate vitamin D deficiency, 7% (4/59) had vitamin D insufficiency, and 3% (2/59) had vitamin D sufficiency. Among survivors ($n=20$), 45% of patients (9/20) had severe vitamin D deficiency, 40% (8/20) had mild-to-moderate vitamin D deficiency, 10% (2/20) had vitamin D insufficiency, and 5% (1/20) had vitamin D sufficiency. Among non-survivors ($n=39$), 61.5% of patients (24/39) had severe vitamin D deficiency, 31% (12/39) had mild-to-moderate vitamin D deficiency, 5% (2/39) had vitamin D insufficiency, and 2.5% (1/39) had vitamin D sufficiency.

Correlation Between 25(OH)D Levels and Markers of Inflammation, Coagulation and Sepsis

Spearman correlations between 25(OH)D levels and markers of inflammation, coagulation and sepsis at the time of hospital admission are shown in Table VI. Total serum 25(OH)D levels were significantly and inversely correlated with PCT (ρ : -0.397; $p=0.001$) and PRL levels (ρ : -0.292; $p=0.02$), while significantly and positively

Table IV. Values of sex hormones and PRL in survivors and non-survivors at the time of hospital admission.

| Hormonal parameter | Survivors (N = 20) | Non-survivors (N = 39) | p-value |
|----------------------------|---|---|--------------|
| Total testosterone (ng/dL) | Median: 140.31 95% CI: 59.94 to 217.02 IQR: 50.70 to 228.56 | Median: 52.12 95% CI: 35.25 to 89.55 IQR: 25.61 to 113.79 | 0.005 |
| E2 (pg/mL) | Median: 26.00 95% CI: 21.33 to 38.98 IQR: 21.00 to 42.00 | Median: 28.00 95% CI: 24.00 to 36.08 IQR: 23.00 to 43.75 | 0.60 |
| E2/T ratio | Median: 2.08 95% CI: 1.13 to 4.26 IQR: 1.10 to 4.36 | Median: 6.77 95% CI: 3.75 to 9.50 IQR: 2.57 to 13.40 | 0.006 |
| Progesterone (ng/mL) | Median: 0.10 95% CI: 0.10 to 0.18 IQR: 0.10 to 0.20 | Median: 0.20 95% CI: 0.10 to 0.20 IQR: 0.10 to 0.37 | 0.07 |
| PRL (ng/mL) | Median: 12.15 95% CI: 7.84 to 14.95 IQR: 7.16 to 16.5 | Median: 17.33 95% CI: 12.97 to 21.79 IQR: 11.16 to 23.63 | 0.08 |

95% CI, 95% confidence interval for the median; E2, 17 β -estradiol; E2/T ratio, estradiol to testosterone ratio; IQR, interquartile range; PRL, prolactin.

Table V. Spearman correlations between sex hormones, PRL and markers of inflammation, coagulation and sepsis at the time of hospital admission.

| Continuous variable | Total testosterone | E2 | E2/T ratio | Progesterone | PRL |
|---------------------|--|--|---|--|--|
| WBC | ρ : -0.255 <i>p</i> -value: 0.05 | ρ : 0.449 <i>p</i> -value: 0.0004 | ρ : 0.416 <i>p</i> -value: 0.001 | ρ : 0.310 <i>p</i> -value: 0.01 | ρ : 0.0179 <i>p</i> -value: 0.8 |
| NLR | ρ : -0.451 <i>p</i> -value: 0.0003 | ρ : 0.306 <i>p</i> -value: 0.01 | ρ : 0.501 <i>p</i> -value: 0.0001 | ρ : 0.249 <i>p</i> -value: 0.05 | ρ : 0.0748 <i>p</i> -value: 0.5 |
| hsCRP | ρ : -0.350 <i>p</i> -value: 0.006 | ρ : 0.158 <i>p</i> -value: 0.2 | ρ : 0.385 <i>p</i> -value: 0.002 | ρ : -0.219 <i>p</i> -value: 0.09 | ρ : 0.116 <i>p</i> -value: 0.3 |
| IL-6 | ρ : -0.266 <i>p</i> -value: 0.04 | ρ : 0.0802 <i>p</i> -value: 0.5 | ρ : 0.273 <i>p</i> -value: 0.03 | ρ : 0.0220 <i>p</i> -value: 0.8 | ρ : 0.0215 <i>p</i> -value: 0.8 |
| TNF- α | ρ : -0.194 <i>p</i> -value: 0.1 | ρ : 0.166 <i>p</i> -value: 0.2 | ρ : 0.220 <i>p</i> -value: 0.09 | ρ : 0.129 <i>p</i> -value: 0.3 | ρ : 0.145 <i>p</i> -value: 0.2 |
| D-dimer | ρ : -0.327 <i>p</i> -value: 0.01 | ρ : 0.246 <i>p</i> -value: 0.06 | ρ : 0.402 <i>p</i> -value: 0.001 | ρ : -0.0707 <i>p</i> -value: 0.5 | ρ : 0.144 <i>p</i> -value: 0.2 |
| Fibrinogen | ρ : -0.0561 <i>p</i> -value: 0.6 | ρ : -0.0475 <i>p</i> -value: 0.7 | ρ : 0.076 <i>p</i> -value: 0.5 | ρ : -0.153 <i>p</i> -value: 0.2 | ρ : 0.0160 <i>p</i> -value: 0.9 |
| PCT | ρ : -0.551 <i>p</i> -value: < 0.0001 | ρ : 0.211 <i>p</i> -value: 0.1 | ρ : 0.611 <i>p</i> -value: < 0.0001 | ρ : 0.0696 <i>p</i> -value: 0.6 | ρ : 0.266 <i>p</i> -value: 0.04 |

ρ , Spearman's rank correlation coefficient; E2, 17 β -estradiol; E2/T ratio, estradiol to testosterone ratio; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; NLR, neutrophil-to-lymphocyte ratio; PCT, procalcitonin; PRL, prolactin; TNF- α , tumor necrosis factor-alpha; WBC, white blood cell.

associated with total testosterone levels (ρ : 0.264; $p=0.04$).

Relationship Between Sex Hormone, PRL and 25(OH)D Levels and COVID-19-Related in-Hospital Mortality

We also evaluated sex hormone, PRL and 25(OH)D levels through a logistic regression analysis to determine the independent association between these continuous variables and COVID-19-related in-hospital mortality. In our final logistic regression model after adjusting for major confounders (age, BMI, hypertension and CVD, diabetes mellitus and malignancy), we found a significant inverse association between serum total testosterone levels and risk of in-hospital mortality (odds ratio [OR], 0.99; 95% confidence interval [CI] 0.98-0.99; $p=0.008$; [Supplementary Table II](#)).

Discussion

In our study non-survivors exhibited significantly higher levels of various markers of inflammation, coagulation and sepsis (NLR, D-dimer and PCT) compared to survivors. This finding is in line with the development of the typical hyper-inflammatory and hypercoagulable state associated with the most severe cases of COVID-19^{1,2}. In both survivors and non-survivors, median total

testosterone levels were indicative of hypogonadism, the latter being defined as total testosterone levels of ≤ 265 ng/dL (≤ 9.2 nmol/L)³⁷. However, non-survivors exhibited significantly lower median total testosterone levels compared to survivors (52.12 ng/dL vs. 140.31 ng/dL). Although the mean age was higher in non-survivors than in survivors (68 vs. 64 years), this difference was not statistically significant. In addition, total testosterone levels did not correlate with age. Thus, a contribution of age-related testosterone decline to the significant difference observed in total testosterone levels between survivors and non-survivors can be excluded.

In our cohort, a significantly higher proportion of non-survivors showed severely low total testosterone levels (< 100 ng/dL) compared to survivors (71.8% vs. 35%, respectively). Accordingly, Lanser et al²⁵ have recently found that hospitalized COVID-19 men with total testosterone levels < 100 ng/dL exhibited a more than 18-fold higher in-hospital mortality risk compared to men with total testosterone levels > 230 ng/dL, as well as a 12-fold higher risk for ICU admission compared to men with total testosterone levels ≥ 100 ng/dL. Importantly, we found that total testosterone levels were significantly associated with the risk of COVID-19-related in-hospital mortality, independent of age, BMI and major comorbidities such as hypertension and CVD, diabetes mellitus and malignancy (which are often associated with

Table VI. Spearman correlations between 25(OH)D levels and markers of inflammation, coagulation and sepsis at the time of hospital admission.

| Continuous variable | 25(OH)D | |
|---------------------|---------|-----------------|
| | ρ | <i>p</i> -value |
| WBC | 0.0443 | 0.7 |
| NLR | -0.0887 | 0.5 |
| hsCRP | -0.0548 | 0.6 |
| IL-6 | -0.0902 | 0.5 |
| TNF- α | -0.0877 | 0.5 |
| D-dimer | -0.0668 | 0.6 |
| Fibrinogen | 0.139 | 0.3 |
| PCT | -0.397 | 0.001 |
| Total testosterone | 0.264 | 0.04 |
| E2 | 0.0297 | 0.8 |
| E2/T ratio | -0.228 | 0.08 |
| Progesterone | -0.209 | 0.1 |
| PRL | -0.292 | 0.02 |

ρ , Spearman's rank correlation coefficient; 25(OH)D, 25-hydroxyvitamin D; E2, 17 β -estradiol; E2/T ratio, estradiol to testosterone ratio; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; NLR, neutrophil-to-lymphocyte ratio; PCT, procalcitonin; PRL, prolactin; TNF- α , tumor necrosis factor-alpha; WBC, white blood cell.

low testosterone levels)^{20,21,38}. These findings suggest a low total testosterone level at admission as an independent risk factor for COVID-19-related mortality in hospitalized men.

We also found a significant inverse correlation between total testosterone levels and values of different markers of inflammation, coagulation and sepsis, namely NLR, hsCRP, IL-6, D-dimer and PCT. These findings indicate that low serum testosterone levels may predispose to an impaired immune response resulting in the development of the hyperinflammatory and hypercoagulable state that negatively affects the prognosis of men with COVID-19 (as it has previously been suggested)¹⁹. Even though the pathophysiological mechanisms by which low testosterone levels may trigger and/or exacerbate COVID-19-related systemic inflammation are unknown, these might involve the immunomodulatory properties exerted by testosterone²⁸. Yet, the relationship between hypogonadism, critical illness and systemic diseases are complex since these clinical conditions may interact with each other in a bidirectional interplay, thus making challenging to interpret low testosterone levels under such circumstances. In this regard, it is worth mentioning that low testosterone levels are frequently found in male patients with critical illness complicated by

hyperinflammatory state and sepsis, as a likely consequence of decreased androgen production (resulting from primary hypogonadism, secondary hypogonadism, or both) and shunting of androgen to estrogen synthesis due to increased aromatase activity^{39,40}. Aromatase is the enzyme responsible for the conversion of testosterone and androstenedione into E2 and estrone, respectively⁴¹. In men, aromatase is expressed in testes (mainly in Leydig cells)⁴² as well as in a number of extragonadal sites, including adipose tissue, bone, breast and brain⁴¹. Upregulation of aromatase enzyme in adipose tissue during critical illness (as a possible consequence of the excessive production of pro-inflammatory cytokines) may promote a substantial increase in the conversion of testosterone to estradiol^{43,44}. Local production of estrogens mediated by aromatase peripherally leads to the suppression of the hypothalamic-pituitary-gonadal (HPG) axis. Therefore, combined hypothalamic-pituitary-gonadal origins of hypogonadism and hypoandrogenism have been suggested in critically ill men⁴⁴. This process may involve different mechanisms other than the hyperinflammatory state, including the use of certain medications (e.g. corticosteroids) able to affect the function of the HPG axis.

With regard to COVID-19, it has also been hypothesized that SARS-CoV-2 may directly target ACE2-positive spermatogonia, Sertoli cells and Leydig cells, resulting in the disruption of spermatogenesis and male gonadal function^{16,45}. Notably, levels of testosterone and luteinizing hormone (LH) suggestive of secondary hypogonadism (central hypogonadism due to hypothalamic-pituitary axis failure), primary hypogonadism (primary testicular failure) and compensated hypogonadism (normal total testosterone and elevated LH levels) have recently been reported in approximately 85%, 9% and 1% of hospitalized men with COVID-19, respectively²⁴.

Although we found a significant positive association between E2 levels and WBC count and NLR values, median E2 levels were within the reference range in both survivors and non-survivors (26 and 28 pg/mL, respectively), and no statistically significant difference in median E2 levels was found between the two study groups. However, we found that median E2/T ratio, which serves as a marker of aromatase activity³⁶, was significantly higher in non-survivors compared to survivors (6.77 vs. 2.08, respectively). Furthermore, E2/T ratio was significantly and positively associated with WBC count and NLR values,

along with different inflammatory and coagulation markers such as hsCRP, IL-6, D-dimer and PCT. Notably, pro-inflammatory cytokines such as IL-6 and TNF- α have been found to stimulate aromatase activity^{46,47}. Thus, these findings suggest a potential role of inflammation-induced aromatase activity in the exacerbation of disease severity among hospitalized men with COVID-19.

The trend toward a significant positive association between BMI and E2/T ratio values (**Supplementary Table I**) is in line with the correlation observed in humans between obesity, increased BMI values and increased aromatase expression in subcutaneous fat⁴⁸. In critically ill men, an increased aromatase activity in white adipose tissue could represent a compensatory mechanism aimed to raise estrogen levels, which may result in reduced local inflammation⁴⁹ in light of the possible anti-inflammatory and immunomodulatory properties exerted by estrogens (even in the context of COVID-19)²⁹. However, the contribution of an enhanced adipose tissue aromatase activity to the increase in circulating estrogen levels remains questionable.

Other studies are in line with our findings. In a prospective cohort study involving 152 consecutive patients with COVID-19 (including 143 hospitalized patients), Dhindsa et al²⁶ showed that testosterone concentrations in men (but not in women) at the time of presentation and on day 3 were inversely associated with disease severity and circulating levels of various pro-inflammatory cytokines and chemokines such as IL-6, CRP and interferon- γ inducible protein 10. In keeping with our findings, this study also found that men with severe COVID-19 (among which 37.9% died), as compared to those with mild disease, exhibited significantly lower median testosterone concentrations at admission (by 64.9%; 53 vs. 151 ng/dL; $p=0.008$), at day 3 (by 82.9%; 19 vs. 111 ng/dL; $p=0.006$) and at day 7 (by 84.1%; 20 vs. 126 ng/dL; $p=0.02$). In addition, estradiol concentrations did not differ between men with severe and mild COVID-19, and they were not significantly associated with cytokine levels²⁶. Nonetheless, median E2/T ratio was significantly higher in men with severe COVID-19 at days 0, 3 and 7 compared to men with mild COVID-19. On multivariate linear regression analyses using age, BMI, and Charlson Comorbidity Index score, E2/T ratios were positively associated with IL-6 levels at days 0 and 3²⁶.

A retrospective explorative analysis of 377 hospitalized COVID-19 patients found that lower

total and free testosterone levels and lower total testosterone to E2 (tT/E2) ratio at hospital admission correlated with greater disease severity in men²⁵. Furthermore, total and free testosterone levels and tT/E2 ratio negatively correlated with CRP, IL-6, PCT, fibrinogen and ferritin levels. Multivariate logistic regression analysis showed that E2 and tT/E2 ratio significantly predicted in-hospital mortality in men²⁵.

In a case-control study conducted by Salonia et al²⁴, a cohort of 286 symptomatic men with laboratory-confirmed COVID-19 at hospital admission was compared with a cohort of 281 healthy men. Authors found significantly lower levels of LH and total testosterone in patients with COVID-19 compared to healthy controls, while healthy controls exhibited significantly lower circulating E2 levels. SARS-CoV-2 infection status was also independently associated with lower total testosterone levels and higher risk of hypogonadism (defined as total testosterone level ≤ 9.2 nmol/L), after accounting for age, BMI, Charlson Comorbidity Index and IL-6 values. After accounting for clinical and laboratory parameters, lower total testosterone levels were also associated with greater risk of ICU admission and death outcomes²⁴.

The trend toward higher median progesterone levels observed in non-survivors warrants further investigation in large prospective studies, as it has been suggested that progesterone may exert a protective role in men with COVID-19 in light of its anti-inflammatory and immunomodulatory properties^{30,31}.

With regard to vitamin D status, in our study non-survivors showed significantly lower median serum 25(OH)D levels as well as a higher frequency of severe vitamin D deficiency at hospital admission compared to survivors (8.1 vs. 10.9 ng/mL and 61.5% vs. 45%, respectively). In addition, Spearman's correlation analysis revealed a significant inverse correlation between 25(OH)D and PCT levels. Besides being a well-known biomarker of infection, systemic inflammation and sepsis, PCT has been suggested as a reliable indicator of disease severity in patients with COVID-19⁵⁰.

In a recent retrospective study conducted on 137 consecutive hospitalized patients with COVID-19 admitted to our Institution, we showed that serum 25(OH)D levels are significantly and inversely associated with the risk of COVID-19-related in-hospital mortality, independently of major comorbidities (CVD, hypertension, obesity and diabetes mellitus)⁶. Moreover, several studies conducted on COVID-19

patients showed that low vitamin D levels are significantly associated with an increase in various markers of inflammation and coagulation (e.g. IL-6, ferritin, TNF- α , D-dimer) and represent an independent predictor of disease severity and mortality^{6,8,51-54}. Interestingly, vitamin D supplementation has also been shown to significantly reduce PCT levels in hospitalized patients with ventilator-associated pneumonia⁵⁵. Hence, these findings suggest that severe hypovitaminosis D may contribute to elicit the development of the hyperinflammatory state and sepsis in COVID-19 patients on account of the anti-inflammatory and immunomodulatory properties exerted by vitamin D, which acts on immune cells expressing the vitamin D receptor (VDR)^{33,56}. Notably, we also found a significant positive association between 25(OH)D and total testosterone levels. Similarly, Peruzzu et al⁵⁷ found a significant positive correlation between 25(OH)D and testosterone levels in elderly hospitalized men with COVID-19, supporting a potential role of testosterone in maintaining 25(OH)D levels. Data from human primary testicular cell culture models suggest that calcitriol (the biologically active form of vitamin D) may play a role in testosterone biosynthesis *in vitro*⁵⁸. On the other hand, data from animal and human models showed that the microsomal vitamin D 25-hydroxylase (the major enzyme involved in vitamin D 25-hydroxylation) is highly expressed in the Leydig cells of the testis and its expression is under the control of LH⁵⁹. Accordingly, clinical conditions characterized by testicular damage are frequently associated with reduced serum 25(OH)D levels⁵⁹. Testis has been suggested as a potential target of SARS-CoV-2 infection^{16,45}, and primary and secondary hypogonadism have been reported in men with COVID-19²⁴. Therefore, both primary and secondary hypogonadism may partly contribute to the high prevalence of hypovitaminosis D observed in this population⁶. Altogether, these findings may suggest a synergistic contribution of vitamin D and testosterone deficiencies to the adverse outcomes of hospitalized men with COVID-19.

Interestingly, calcitriol has also been shown to downregulate the production of pro-inflammatory cytokines (IL1- β , IL-6 and TNF- α) in human activated macrophages by significantly decreasing the aromatase activity⁶⁰. In our cohort, 25(OH)D levels were inversely correlated with E2/T ratio, although this association was not statistically significant (ρ : -0.228; $p=0.08$).

The main strengths of our study include the assessment of the E2/T ratio as well as the assessment of sex hormones (including progesterone) and PRL levels upon admission and their correlation with different markers of inflammation, coagulation and sepsis. We also evaluated vitamin D status at admission and its correlation with sex hormones and markers of inflammation, coagulation and sepsis. In addition, all patients in our cohort had the blood immediately drawn at hospital admission before steroids and/or antivirals were started, therefore excluding the potential interference of these drugs with the HPG axis. Our results could also be analyzed in future meta-analyses of observational studies assessing serum sex hormone levels and vitamin D status in hospitalized men with COVID-19.

However, we acknowledge that our study has many limitations. First, this was a small single-center cohort study, thus limiting the generalizability of our findings. Second, the retrospective database design of the study and the lack of a control group consisting of hospitalized men with comorbidities other than COVID-19 do not allow us to prove a causative relationship between low serum total testosterone levels, elevated E2/T ratio values and adverse clinical outcomes of COVID-19. In this regard, low serum total testosterone levels may simply be a marker of critical illness (including acute viral infections) without playing a role in the pathophysiology of COVID-19. Large prospective case-control studies are required to assess whether the magnitude of HPG axis suppression in men correlates with COVID-19, in particular, or with the severity of acute illness (including other viral infections), in general. Also, we do not have information about the hormonal status of the study participants before SARS-CoV-2 infection. Thus, the existence of male hypogonadism prior to SARS-CoV-2 infection in the study participants cannot be excluded or clarified. Third, the lack of assessment of LH prevented us from distinguishing between different types of hypogonadism (primary hypogonadism, compensated hypogonadism and secondary hypogonadism). Another major limitation of our study is the lack of assessment of sex hormone binding globulin (SHBG) and free or bioavailable testosterone levels. Nevertheless, given the almost 3-fold difference in total testosterone levels between survivors and non-survivors, it is highly likely that free testosterone levels would also be lower in non-survivors compared to survivors.

Conclusions

In conclusion, both testosterone and E2/T ratio at admission may serve as prognostic markers of disease severity in hospitalized men with COVID-19 independently of the hypogonadism etiology. Specifically, low total testosterone levels at admission may represent a major risk factor for poor prognosis of COVID-19 in hospitalized men, independent of age, BMI and major comorbidities. In this context, the present study outlines the importance of assessing serum testosterone and E2 levels in men hospitalized due to COVID-19. Indeed, these markers might be particularly useful to identify men at higher risk for adverse outcomes of COVID-19 and to subsequently prioritize the initiation or the implementation of effective therapeutic interventions aimed to reduce disease morbidity and mortality. Yet, large prospective studies are warranted to establish whether low testosterone levels and high E2/T ratios in hospitalized men with COVID-19 are merely the consequence of inflammation-induced HPG axis suppression and aromatase activation or if they play an actual mechanistic role in COVID-19 pathophysiology and disease severity. In the latter case, the safety profile and the effects of testosterone replacement therapy and/or aromatase inhibitors on clinical outcomes in hypogonadal men with severe COVID-19 should be evaluated in future randomized controlled trials.

Conflict of Interest

The Authors declare that they have no conflict of interest to disclose.

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Authors' Contribution

Marco Infante and Maria Morello conceived the research project, wrote the paper, interpreted data, supervised the project and equally contributed to the manuscript. Loredana D'Amore and Santina Lupisella collected and retrieved clinical and biochemical data, analyzed results and contributed to the research project. Massimo Pieri performed statistical analysis. Sergio Bernardini, Andrea Fabbri, Marco Iannetta and Massimo Andreoni supervised the research project, revised and approved the final version of the manuscript. All authors read, edited and approved the final version of the manuscript.

Ethics Approval

This research study was conducted retrospectively from data obtained for clinical purposes. This study involving human participants was in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was reviewed and approved by the Ethics Committee of University of Rome Tor Vergata (Registration Number: 141/20, July 23, 2020). Informed consent was obtained from all participants and/or legal guardians involved in the study. All participants and/or legal guardians signed informed consent regarding the publication of their data.

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