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Focus on sex steroids in gender medicine: testosterone not only a male hormone

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ABSTRACT

Gender medicine is the study of the influence of biological and socio-economic and cultural differences on the state of health and disease of each person. Sex hormones play a predominant role in gender differences and, during my first year of PhD, my main interest was in studying the sexually dimorphic role of androgens in human metabolic disease. Specifically, female androgen excess and male androgen deficiency manifest with an overlapping adverse metabolic phenotype and my initial PhD project was to work on these patients to try to make my contribution in this "gender gap". Non-alcoholic fatty liver disease (NAFLD) is one of the conditions of this metabolic phenotype and, in the introduction of this thesis, I report an initial study in which a SHBG cut-off was identified to predict the risk of NAFLD in women with hirsutism and oligomenorrhea. With the onset of the COVID-19 pandemic, one of the earliest evidence was the greater involvement of male patients (in terms of infection, hospitalization and mortality). For this reason, in a context of gender medicine, many authors have investigated the possible determinants of these gender differences, proposing, among which, different lifestyles, different comorbidities and, last but not least, different levels of sex hormones. In this thesis I report the results of two studies on two different cohorts of patients (males and females, respectively) admitted in the Respiratory Intensive Care Unit (RICU) of Mantua, one of the epicenters of the first phase of the global SARS-CoV-2 pandemic in Italy. Specifically, I try to estimate the association between testosterone levels and the SARS-CoV-2 infection clinical and biochemical outcomes in these patients.

In the first study, a consecutive series of 31 male patients affected by SARS CoV-2 pneumonia and recovered in RICU were analyzed. In this cohort, lower total

testosterone (TT) and calculated Free Testosterone (cFT) were found in the transferred to Intensive Care Unit (ICU)/deceased in RICU group vs groups of patients transferred to Internal Medicine (IM) or maintained in the RICU in stable condition. Both TT and cFT showed a negative significant correlation with biochemical risk factors (ie, the neutrophil count, LDH, and PCT) but a positive association with the lymphocyte count. Likewise, TT was also negatively associated with CRP and ferritin levels. A steep increase in both ICU transfer and mortality risk was observed in men with TT < 5 nmol/L or cFT < 100 pmol/L. This study demonstrates that lower baseline levels of TT and cFT levels predict poor prognosis and mortality in SARSCoV-2-infected men admitted to RICU.

In the second study, a consecutive series of 17 women affected by SARSCoV-2 pneumonia and recovered in the same clinic were analyzed. TT and cFT were significantly and positively associated with PCT, CRP, and fibrinogen as well as with a worse hospital course. We did not observe any significant association between TT and cFT with LH; conversely, both TT and cFT showed a positive correlation with cortisol. By LOWESS analysis, a linear relationship could be assumed for CRP and fibrinogen, while a threshold effect was apparent in the relationship between TT and procalcitonin, LDH and ferritin. When the TT threshold value of 1 nmol/L was used, significant associations between TT and PCT, LDH or ferritin were observed for values above this value. For LDH and ferritin, this was confirmed also in an age-adjusted model. Similar results were found for the association of cFT with the inflammatory markers with a threshold effect towards LDH and ferritin with increased LDH and ferritin levels for values above cFT 5 pmol/L. Cortisol is associated with serum inflammatory markers with similar trends observed for TT;

conversely, the relationship between LH and inflammatory markers had different trends. Therefore, opposite to men, in women with SARS-CoV-2 pneumonia, higher TT and cFT are associated with a stronger inflammatory status, probably related to adrenal cortex hyperactivity.

INTRODUCTION

Gender medicine: an interesting emerging field

When we speak today of "precision medicine", of a medicine that treats the patient with a view to individual circumstances, gender also always plays a role. Gender medicine therefore always deals with both – with men and women.

By addressing the interrelation and integration of biological markers (i.e., sex) with indicators of psychological/cultural behavior (i.e., gender), gender medicine represents the crucial assumption for achieving the personalized health-care currently required in our clinics [*Gemmati et al., 2019*]

Therefore, the role of sex/gender in physiological and pathological processes is crucial in terms of efficient prevention, clinical signs' identification, prognosis definition, and therapy optimization.

In the third millennium it is necessary for the researchers (and also for the doctors, in my case) to be aware of how gender can impact different clinical conditions.

Gender and sex differences in several frequent diseases are more widespread than one may assume. In addition, they have significant yet frequently underestimated consequences on the daily practice of medicine, on outcomes and effects of specific therapies [*Regitz-Zagrosek et al., 2012*].

Gender differences are present in a number of clinical areas, such as in cardiovascular diseases, pulmonary diseases, gastroenterology and hepatology, in nephrology, autoimmune diseases, hematology, neurology and endocrinology.

Given the target of this thesis, in the next chapter I will focus on the impact of sex hormones in gender medicine.

Sex hormones in gender medicine

Sex hormones play a predominant role in gender differences. Historically, as a "gender vice", testosterone is considered the main regulator of male sexual and reproductive health and, on the contrary, estrogen is considered the fundamental regulator of female health.

During my first year of PhD, my main interest was in studying the sexually dimorphic role of androgens in human metabolic disease.

Specifically, female androgen excess and male androgen deficiency manifest with an overlapping adverse metabolic phenotype (including abdominal obesity, insulin resistance, type 2 diabetes mellitus, non-alcoholic fatty liver disease (NAFLD) and increased risk of cardiovascular disease) and my initial PhD project was to work on these patients to try to make my contribution in this "gender gap".

In the andrology unit of our hospital (Andrology, Women's Endocrinology and Gender Incongruence Unit, Careggi University Hospital – Director Prof. Linda Vignozzi) I already had a large available cohort of male patients with hypogonadotropic hypogonadism and metabolic syndrome so I focused my clinical and research activities more on the female population.

Figure 1 shows the objectives of my initial PhD project [**Figure 1**].

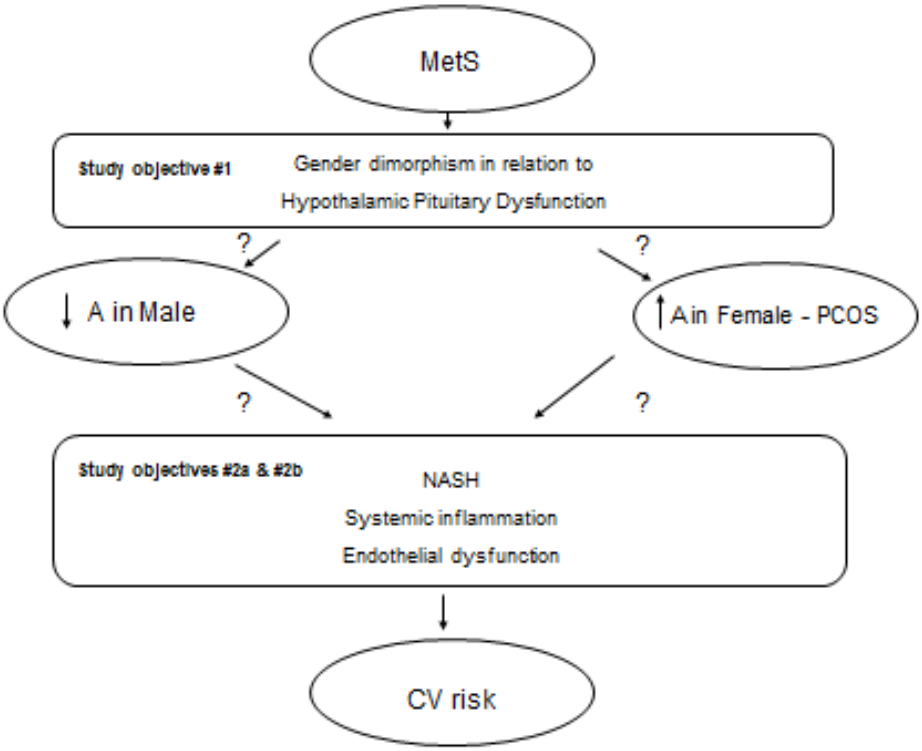

Figure 1 Objectives of my initial PhD project. MetS= Metabolic Syndrome; A= Androgens; PCOS= Polycystic Ovary Syndrome; NASH= Non Alcoholic SteatoHepatitis; CV= Cardio-Vascular

Androgen excess is one of the crucial finding of the Polycystic Ovary Syndrome (PCOS), one of the most frequent endocrine disease, affecting 5-10% of women in reproductive age [*Legro et al., 2013*]. In these patients, hyperandrogenemia presents clinically with hirsutism, acne and androgenetic alopecia, the former being the most frequent [*Bozdag et al., 2016*].

The role of androgens in the development of Metabolic Syndrome (MetS) -related cardiovascular risk factors such as NAFLD, systemic inflammation and endothelial dysfunction has not been investigated from a gender perspective.

NAFLD, and in particular Non-Alcoholic Steatohepatitis (NASH), is considered the hepatic hallmark of MetS. Androgens have a direct role in liver metabolism both in

hypogonadal men and in PCOS women [*Kim et al., 2012; Wu et al., 2018*]. NAFLD is a key factor in the development of CV diseases due to a pro-inflammatory, pro-coagulant and pro-fibrinogenic *milieu* [*Ballestri et al., 2014*].

For this reason, I investigated the presence of NAFLD in a group of female patients with oligomenorrhea and hirsutism referred by our outpatient clinic, with the ultimate aim of predicting individual cardio-metabolic risk through one of the most important risk factors.

However, it should be emphasized that the diagnosis of NAFLD is not a practical task and the condition is at risk of being overlooked. The use of simpler but still reliable surrogate markers is necessary to identify women with a high likelihood of NAFLD. The aim of the first study of my cohort of patients was to evaluate the clinical correlates of NAFLD Liver Fat Score (NAFLD-LFS) in women with oligomenorrhea and/or hirsutism. Furthermore, the study aimed to evaluate whether, among the hormonal parameters evaluated in such women, possible hallmarks of NAFLD may be identified. To this purpose, 66 women who attended our Outpatient Clinic for oligomenorrhea and/or hyperandrogenism were included in the study. In order to validate the results obtained in the first cohort, a second independent sample of 233 women evaluated for female sexual dysfunction (FSD) was analyzed. In cohort 1, NAFLD-LFS positively correlated with metabolic and inflammatory parameters. Among the hormone parameters, NAFLD-LFS showed no significant relationships with androgens but a significant negative correlation with sex hormone binding globulin (SHBG) ($p<0.0001$) that therefore appeared as a candidate hallmark for pathologic NAFLD-LFS. The ROC analysis showed a significant accuracy (81.1%, C.I.69.1-93.0, $p <0.0001$) for SHBG in identifying women with a

pathological NAFLD-LFS. In particular, a SHBG of 33.4 nmol/l was recognized as the best threshold, with a sensitivity of 73.3% and a specificity of 70.7% [**Figure 2**].

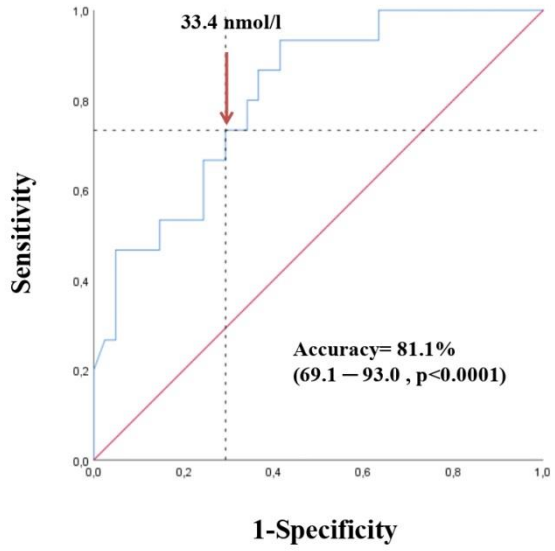

Figure 2. Receiver operating characteristic (ROC) curve for SHBG in detecting NAFLD risk according to NAFLD-LFS

In order to validate this value of SHBG as a marker of metabolic impairment possible related with the presence of NAFLD, we tested this threshold in cohort 2. FSD women with SHBG <33.4 nmol/l had worse metabolic parameters than women with SHBG ≥33.4 nmol/l and a significantly higher NAFLD-LFS even after adjusting for confounders (B=4.18 [2.05; 6.31], p=0.001). In conclusion, this study provides a new evidence in the diagnostic process of NAFLD, showing that the measurement of SHBG, which is routinely assessed in the workup of women referred for possible PCOS, could identify women at higher metabolic risk, thus detecting

those who may deserve further targeted diagnostic assessment [*Di Stasi et al., 2021 (a)*].

Therefore in this study I was able to make a first point of my initial project of revealing the cardio-metabolic risk in female patients with androgen excess, estimating the presence of NAFLD by measuring a simple biochemical marker such as SHBG.

How COVID-19 changed my PhD program

Since March 2020, the COVID-19 pandemic has appeared in Italy. This pandemic has had a strong socio-health impact on our personal lives and also on our work as outpatient specialists and basic researchers.

In a gender medicine perspective, the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection has been shown to have a differential impact on women and men. Sex disaggregated data indeed showed greater mortality rates in men as compared with women. In particular, men are up at threefold higher odds of lethality than women [*Porcheddu et al., 2020*] thus indicating a firm gender inequality in SARS-CoV-2-associated sequelae.

During the first phase of the COVID-19 pandemic, I had the opportunity to collaborate with the colleagues of the Pneumology and Respiratory Intensive Care Unit (RICU) of the "Carlo Poma" Hospital in Mantua and, consequently, to contribute and deepen the knowledge on gender differences in SARS-Cov-2 infection.

The Global Health 5050 website provides updated sex-disaggregated data for COVID-19 indicators (http://globalhealth5050.org/covid19), which include at

August, 24th 2021, 134,343,423 cases, 5,332,290 hospitalizations, 242,107 Intensive Care Unit admissions, and 3,217,722 deaths. As reported by this website, men are more represented among COVID-19 patients requiring hospitalization (56.6% vs. 43.4%; http://globalhealth5050.org/covid19). The gap is even more evident if patients admitted to ICU are considered, of whom 67.3% are men (http://globalhealth5050.org/covid19). With the exception of a very limited number of countries, also mortality for COVID-19 is unbalanced towards men who represent 58.8% of deaths attributed to SARS-CoV-2 infection, irrespective of age (http://globalhealth5050.org/covid19).

The underlying reasons for these differences are not completely understood.

I and my colleagues studied different factors that may contribute to gender gap in COVID-19 [*Di Stasi & Rastrelli, 2021*]:

- *Lifestyle as a possible source of gender disparity*

The different exposition to SARS-CoV-2 infection due to lifestyle, including job position, may be a possible factor, which explains the gender disparity. Globally, men lead a life characterized by more intensive and various interpersonal exchanges, both for recreational activities and professional ones. Moreover, a study evaluating the attitudes and behaviors towards the pandemic in more than 21,000 subjects showed that women have a greater perception regarding the seriousness of COVID-19 [*Galasso et al., 2020*]. Accordingly, they showed a closer agreement with the strategies and policies for activity restraining and reported a higher compliance with public health and distancing rules [*Galasso et al., 2020*]. Based on this, one could expect that worse outcomes in men are secondary to a greater infection rate. This may be apparently the case for several African, Western, South and Southeast Asian

countries where, however, this excess is likely due to different opportunity of access to healthcare of men and women. For most countries, where studies on gender discrepancy in COVID-19 were mainly conducted, there is substantial similarity in the male to female ratio among the infected subjects (https://globalhealth5050.org/the-sex-gender-and-covid-19-project/). Moreover, when infections among healthcare workers are considered, two out of three are females (https://globalhealth5050.org/the-sex-gender-and-covid-19-project/). Overall, these proportions may suggest that women are even more prone to be infected by SARS-CoV-2 if exposed to risky environments. In light of this, the greater severity of the disease in men appears even more dramatic.

- *Comorbidities as possible source of gender disparity*

Among the putative conditions affecting the increased severity in men, different prevalence in risk factors and morbidities known to worsen the COVID-19 course may be hypothesized. A recent meta-analysis provided a comprehensive summary of the clinical characteristics of COVID-19 hospitalized patients and assessed the risk factors for mortality [*Corona et al., 2021*]. Male gender was confirmed as a risk factor for death. Hypertension, diabetes mellitus (DM), and cardiovascular diseases (CVD), were the most prevalent morbidities [*Corona et al., 2021*] with frequencies among hospitalized COVID-19 patients (40.8%, 22.3% and 18.5%, respectively) that exceeded two-to-three fold that observed in general population. Studies with higher representation of subjects with hypertension, DM, CVD, chronic obstructive pulmonary disease, chronic kidney disease, and active cancer reported higher mortality rates [*Corona et al., 2021*]. Since all the aforementioned morbidities

recognize male gender as a non-modifiable risk factor, this may partly explain the excess in male hospitalization and mortality for COVID-19. However, the comparison of COVID-19 patients in the different waves of outbreaks showed a change in their characteristics with a progressive decrease in age and prevalence of some comorbidities [*Roth et al., 2021*] but a roughly similar male to female ratio among subjects admitted to hospital [*Roth et al., 2021; Iftimie et al., 2021; Bongiovanni et al., 2021*]. This suggests that there are further factors, besides comorbidities, which may explain the adverse outcomes in men.

- *Sex hormones as possible source of gender disparities*

In each field of medicine and biology, when gender disparities are recognized, sex hormones are the major candidates for being the responsible. In fact, sex hormones circulate in men and women at different concentrations.

Focus on estrogens

Present literature has greatly underlined the data, which suggest a beneficial effect of estrogens and a detrimental one for androgens in COVID-19 patients. This is based on the assumption that estrogens are “female” and androgens are “male” hormones. Indeed, preclinical data on animal models of SARS and Middle East Respiratory Syndrome (MERS) caused by coronaviruses similar to SARS-CoV-2, in line with present epidemiological data on COVID-19, showed that female mice have better disease course and lower mortality than males [*Channappanavar et al., 2017*] and that ovariectomy or tamoxifen were associated with increased mortality [*Channappanavar et al., 2017*]. This may suggest that estrogens are protective towards coronavirus infection but it should not be forgotten that ovariectomy causes

not only estrogen but also androgen deprivation. Moreover, selective estrogen receptor inhibitors (SERMs), such as tamoxifen, have also tissue-specific pro-estrogenic effects and one of the most notorious adverse event with SERMs is venous thromboembolism, a life-threatening event in COVID-19. Hence, the role of sex hormones in COVID-19 is likely to be far more complicate than "estrogens are the good and androgens the bad". As an effect of estrogen levels, females mount a stronger immune response with higher levels of cytokines and antibodies that provides better protection from pathogens [*Mauvais-Jarvis et al., 2020*]. However, in COVID-19, the clinical worsening and life-threatening phase rely right in an exaggerate inflammatory response with the so called "cytokine storm" for which a hyperactive immune system could be even detrimental. In addition, some [*Zhang et al., 2021*], although not all [*Kalidhindi et al., 2020*], studies show that estrogens upregulate angiotensin-converting enzyme 2 (ACE2) that, on one hand, promotes the most favorable effects of renin-angiotensin system but, on the other hand, is the key enzyme that allow SARS-CoV-2 entering the pneumocytes and starting lung tissue damage and subsequent inflammatory events. The putative beneficial effects of estrogens on COVID-19 are also disproven by the observation that, in post-menopausal women, the lower risk of worse COVID-19 outcomes is still maintained on age-matched men (https://globalhealth5050.org/the-sex-gender-and-covid-19-project/). In addition, a study on 286 hospitalized COVID-19 male patients and 281 healthy controls have shown that patients have slightly but significantly higher estradiol levels and that higher estradiol is associated with worse, rather than better, COVID-19 clinical outcomes [*Salonia et al., 2021*]. Estradiol *per se* has not been confirmed a risk factor in another recent study (which used mass-spectrometry rather

than immunoassays for sex hormone measurement) [*Dhindsa et al., 2021*], although estradiol to T ratio was found higher among men with more severe COVID-19. These data do not allow attributing definitely a detrimental effect of estradiol in men with COVID-19 because they could be the reflection of severely decreased T levels; nonetheless, they allow excluding that estradiol may be beneficial for men with COVID-19.

Focus on androgens

In the dichotomy of estrogens=female and androgens=male, it is important to consider that, in premenopausal women, serum estradiol fluctuates considerably peaking at the mid-cycle up to 1000 pmol/L and declining progressively through the luteal phase by the lowest values around 50 pmol/L that are achieved in early follicular phase. In menopausal women, estradiol is commonly below 30 pmol/L. Adult men have stable estradiol levels ranging 30 to 150 pmol/L and, with ageing, only a slight decline is detected. Although there is, of course, substantial difference in estradiol levels between men and women, the abovementioned values show that its magnitude is smaller than it could be thought, at least for a considerable part of life. Conversely, the differences in testosterone (T) levels are more pronounced. Healthy premenopausal women have serum T levels around 0.5-1.0 nmol/L, which gradually decline with ageing. On the other hand, in healthy young men, total T has concentrations in the order of 15-25 nmol/L and, despite T declines with ageing, only in surgically or drug-induced castration, levels as low as in women are achieved. In this view, it is rationale to consider the role of T as a modulating factor of COVID-19 severity, responsible for the gender discrepancy. The transmembrane protease/serine subfamily member 2 (TMPRSS2) is, besides ACE2, another key step of the SARS-

CoV-2 infection. After the spike protein binds ACE2, it allows the fusion of viral and cell membranes thus promoting the internalization of SARS-CoV-2. TMPRSS2 is expressed not only in the airways but also in several other tissues, including the endothelium of micro- and macro-vessels where it favors clotting and, therefore, predisposes to the most frightening complications of COVID-19, namely the disseminated intravascular coagulation and pulmonary embolism. Before being considered in the pathogenesis of COVID-19 and its complications, TMPRSS2 has been extensively studied in the context of prostate cancer, where the androgen-dependence of its expression has been demonstrated. Therefore, it could be hypothesized that lower T levels could protect from SARS-CoV-2 infection and be the responsible for better outcomes in women. In an earlier phase of the pandemic, a retrospective study performed in Veneto, one of the most severely affected region in Italy and in the world at that time, provided data supporting the protective role of low T. By matching the data of regional registries of cancer patients with those on COVID-19 infected subjects, men with prostate cancer undergoing androgen deprivation therapy (ADT) were shown to have three to four fold higher risk of SARS-CoV-2 infection and of worse outcomes from COVID-19, as compared with men with cancer not upon ADT [*Montopoli et al. 2020*]. It should be however recognized that, due to the limited number of SARS-CoV-2 infections in the study population, the confidence intervals for the odds ratio were quite wide with borderline statistical significance in some cases. Later on, two Brazilian studies investigated the effect of antiandrogens in patients with mild to moderate COVID-19, showing a beneficial effect in terms of viral clearance, reduction of inflammatory markers and clinical remission, as compared with placebo [*Cadegiani et al., 2021*

(a); Cadegiani et al., 2021 (b)]. The putative detrimental role of T is in contrast with the physiologic decline of its levels in ageing men. In fact, the gap in adverse outcomes between men and women is particularly evident after the age of 60 years (http://globalhealth5050.org/covid19 last updated on August, 24th 2021), when men commonly have T levels significantly lower than healthy younger subjects and in 15-20% of cases achieve values consistent with hypogonadism. In ageing men, the drop of T in the hypogonadal range is favored by the presence of comorbidities, including obesity, CVD, DM and other chronic conditions that are highly prevalent among COVID-19 patients with worse outcomes. In this view, the role of low T levels, rather than high, should be considered as a risk factor in COVID-19.

As mentioned above, at the beginning of the first wave of SARS-CoV-2 infection in Italy I and my study group collected data on hospitalized men with COVID-19 with the aim of studying their gonadal function and the possible relationship with their clinical outcomes [*Rastrelli & Di Stasi et al., 2020*]. Subsequently, we investigated the role of testosterone also in female patients of the same clinic (RICU of Mantua) [*Di Stasi & Rastrelli et al., 2021*].

In this thesis I will illustrate and comment the results of these works on the role of testosterone in two cohorts of patients with SARS-Cov-2 infection.

PART 1: LOW TESTOSTERONE LEVELS PREDICT CLINICAL ADVERSE OUTCOMES IN MALE SARS-COV-2 PNEUMONIA PATIENTS

Objective of the study

To estimate the association between the T levels and the SARS-CoV-2 infection clinical outcomes (defined as conditions requiring to transfer to a higher or lower intensity of care units or even death) as well as the biochemical prognostic predictors of severe and fatal SARS-CoV-2 infection in a cohort of male patients admitted in the RICU of a single Hospital in Mantua, one of the epicenter of the first phase of global SARS-CoV-2 pandemic in Italy.

Materials and methods

Study population

Data from a consecutive series of 31 male patients with SARS-CoV-2 pneumonia and recovered in the RICU of the "Carlo Poma" Hospital in Mantua, Italy, were analyzed.

Diagnosis of SARS-Cov-2 infection and other performed exams

A laboratory pharyngeal-nose swab positivity of SARS-CoV-2 infection was confirmed by chest X-ray. Acute respiratory distress syndrome (ARDS) was

defined by the "Berlin definition," and patients were segregated into mild ARDS (PaO2/FiO2 ≤ 300 mmHg and > 200 mmHg), moderate ARDS (PaO2/FIO2 ≤ 200 mmHg and > 100 mmHg), and severe ARDS (PaO2/FiO2 ≤ 100 mmHg) (*Definition Task Force ARDS, 2012*).

According to the protocol (approved by the Local Ethics Committee Val Padana, Mantua, Italy) and as for clinical practice, each patient underwent a standardized diagnostic workup. Specifically, blood samples were drawn on the first morning after the admission to the RICU within 8.00 AM, in fasting condition, for the determination of blood count and leukocyte formula, creatinine, uric acid, electrolytes, transaminases, albumin, creatine phosphokinase (CPK), C-reactive protein (CRP), procalcitonin (PCT), lactate dehydrogenase (LDH), ferritin, D-dimer, fibrinogen, interleukin 6 (IL-6), TT, sex hormone–binding globulin (SHBG), cortisol and luteinizing hormone (LH). Hematological and biochemical analyses were performed in the central laboratory of the "Carlo Poma" Hospital (Mantua, Italy) with commercially available kits routinely used for hospital clinical practice. TT was measured once by an immunoassay (Electrochemiluminescence Immunoassay, ECLIA), and free T was calculated by the Vermeulen formula [*Vermeleun et al., 1999*]. Patients were divided according to the outcome throughout the hospitalization in RICU. For the first analysis, patients have been segregated in four groups: (a) The first group composed by patients with improved clinical conditions overtime who have been transferred to the internal medicine (IM) units; clinically, they no longer needed non-invasive ventilation (NIV) but only low-flow oxygen therapy; (b) the

second group composed by patients still in RICU and under NIV at the time of the current analysis (data on the definitive outcomes are not available at the time of the study drafting); (c) the third composed by patients transferred to intensive care unit (ICU) for intubation; and (d) the fourth group composed by patients who eventually died.

Statistical analysis

Differences among groups with different prognoses were evaluated eventually merging the two groups with the worst outcomes (the aforementioned third and fourth groups in male cohort). Thereafter, a sensitivity analysis was performed to compare the groups of which post-RICU outcome was known at the time of the analysis (thus excluding the aforementioned second group). Either Mann-Whitney U test or one-way ANOVA on ranks (Kruskal-Wallis test) was used to test the differences concerning continuous variables between two or more than two groups, respectively. Differences between continuous categorical variables were assessed by the likelihood-ratio test. Data were expressed as medians [interquartile ranges] or percentage for continuous and categorical variables, respectively. Univariate relationships were firstly assessed by Spearman's rank correlation and afterward checked for non-linearity by the locally weighted scatterplot smoothing (LOWESS) analysis. When linearity could not be assumed, threshold levels for TT or calculated free T (cFT) were identified by the LOWESS analysis and further confirmed by linear regression models with linear spline functions for TT and cFT levels. This analysis allowed identifying threshold levels at which a significant change in the slope of the association between T and other blood markers occurred. In male cohort,

the relationship between the clinical outcomes (transferred to IM, in charge to RICU, transferred to ICU or deceased) and TT or cFT was assessed by ordinal logistic regressions. The probability of each outcome based on T according to this regression model was calculated and fitted in a LOWESS curve as a function of TT or cFT.

All the analyses were performed by Statistical Package for the Social Sciences Statistics 26 (IBM Corporation). Spline linear functions were carried out by Stata MP 13 (StataCorp).

Results

Of 31 patients, 21 (67.7%) were transferred from RICU to IM after overall improvement of the respiratory conditions, six (19.4%) were stable at time of the present analysis and maintained in RICU, and four (12.9%) worsened their conditions and were either transferred to ICU (n = 2) or eventually died (n = 2).

Table 1 details descriptive statistics of the whole cohort of patients as segregated according to the different outcomes [**Table 1**].

	Reference range	Transferred to IM (n=21)	In charge in RICU (n=6)	Transferred to ICU/deceased (n=4)	p for trend	p[1]
Demographics and previous medical history						
Age (years)	---	63.0 [55.0-66.5]	72.0 [33.0-83.5]	74.5 [59.5-85.0]	0.162	*0.068*
Smoking habits (%)						
Former smoker	---	42.9	0.0	50.0	0.128	0.823
Current smoker	---	4.8	0.0	0.0		
Obesity (%)	---	42.9	16.7	0.0	0.860	**0.046**
Hypertension (%)	---	57.1	33.3	50.0	0.584	0.793
Dyslipidemia (%)	---	23.8	33.3	25.0	0.899	0.959
Diabetes (%)	---	28.6	33.3	0.0	0.267	0.119
Hypothyroidism (%)	---	14.3	0.0	25.0	0.347	0.610
Chronic Renal Failure (%)	---	4.8	0.0	0.0	0.672	0.550
Arrhythmia (%)	---	0.0	0.0	25.0	0.114	**0.048**
Psychiatric Diseases (%)	---	0.0	33.3	25.0	**0.023**	**0.048**
Hematologic Diseases (%)	---	4.8	16.7	0.0	0.501	0.550
CVD (%)	---	4.8	0.0	0.0	0.672	0.550
Liver Diseases (%)	---	4.8	0.0	0.0	0.672	0.550
Parameters during Hospitalization in RICU						
Time in RICU (days)	---	7.0 [4.0-9.0]	10.0 [7.0-14.3]	5.0 [4.0-12.0]	0.338	0.456
PaO2/FiO2 (mm Hg)	<300 for ARDS	130.4 [104.8-165.4]	119.5 [99.5-155.0]	87.3 [80.8-157.9]	0.226	0.132
Severe ARDS (%)	PaO2/FiO2 ≤100 mm Hg	14.3	16.7	75.0	**0.050**	**0.016**
WBC (10^3/µL)	4.4-11	7.3 [4.3-9.0]	7.2 [5.3-8.6]	11.6 [7.7-17.3]	0.115	0.056
Neutrophils (10^3/µL)	2.0-7.5	4.0 [2.6-6.7]	6.3 [4.2-7.2]	10.6 [6.4-16.4]	*0.085*	**0.027**
Lymphocytes (10^3/µL)	1.3-4.8	1.2 [0.9-1.6]	0.7 [0.5-0.9]	0.7 [0.5-0.9]	**0.017**	**0.035**
Hemoglobin (g/dl)	13.5-17-5	11.8 [9.4-14.0]	12.4 [10.3-14.0]	11.6 [9.4-13.0]	0.849	0.794
Hematocrit (%)	40-50	34.8 [28.4-42.1]	37.4 [33.1-41.8]	36.2 [30.2-42.1]	0.771	0.911
Platelets (10^3/µL)	150-400	278.5 [173.5-387.3]	299.5 [229.0-346.8]	284.0 [197.3-391.0]	0.993	0.911
Creatinine (mg/dl)	0.6-1.3	0.9 [0.8-1.1]	1.1 [0.8-1.2]	0.8 [0.7-2.3]	0.592	0.592
Uric acid (mg/dl)	2.5-7.2	4.0 [2.9-5.1]	3.4 [2.7-5.9]	4.2 [2.7-8.4]	0.973	0.803
Sodium (mEq/L)	135-145	137.0 [132.0-138.5]	137.5 [131.5-139.5]	137.5 [133.0-154.8]	0.726	0.452

Potassium (mEq/L)	3.4-4.7	3.8 [3.6-3.9]	3.7 [3.4-4.0]	4.4 [3.8-4.5]	*0.088*	**0.047**
AST (U/L)	10-33	35.0 [30.5-63.0]	31.0 [27.5-45.0]	46.5 [21.8-90.8]	0.530	0.748
ALT (U/L)	5-37	47.0 [32.5-65]	28.5 [21.5-51.8]	79.0 [28.0-114.3]	0.108	0.231
CPK (U/L)	25-200	56.0 [28.0-95.3]	39.5 [22.3-143.8]	94 [53.5-582.3]	0.284	0.183
CRP (mg/L)	0-5	15.7 [3.4-64.2]	24.9 [10.5-72.5]	143.1 [46.1-257.2]	*0.060*	**0.023**
Procalcitonin (ng/ml)	0-0.09	0.08 [0.05-0.15]	0.10 [0.05-0.33]	1.33 [0.46-2.88]	**0.006**	**<0.001**
LDH (U/L)	150-450	414.5 [347.0-515.0]	621.0 [563.3-954.8]	935.5 [623.8-1070.3]	**0.002**	**0.003**
Fibrinogen (mg/dl)	150-450	426.0 [303.0-535.0]	455.5 [386.0-617.5]	471.0 [152.3-756.0]	0.811	0.969
D-Dimer (ng/ml)	<500	1836.0 [515.0-3697.0]	1343.5 [631.0-3197.3]	2108.5 [858.0-8171.0]	0.867	0.667
Ferritin (ng/ml)	30-400	993.0 [656.0-1365.0]	1679.5 [511.0-3791.0]	1809.0 [876.0-2199.8]	0.167	0.081
IL-6 (pg/ml)	<7	50.3 [13.1-86.0]	56.1 [30.1-77.6]	137.2 [50.3-205.8]	0.291	0.148
Total T (nmol/L)	8.6-29	8.8 [4.1-16.2]	5.0 [1.8-7.6]	1.0 [0.2-1.9]	**0.005**	**0.001**
Calculated free T (pmol/L)	<225	146.5 [93.8-287.0]	118.0 [40.8-133.5]	17.5 [5.8-37.0]	**0.006**	**0.001**
SHBG (nmol/L)	18.3-54.1	35.6 [22.0-59.0]	24.0 [19.6-37.4]	21.3 [12.2-39.6]	0.159	0.157
LH (U/L)	1.7-8.6	6.6 [4.6-9.6]	16.3 [7.9-20.3]	11.2 [9.0-19.3]	**0.043**	**0.037**

Table 1. Characteristics of the study population according to clinical outcome

Data are reported as median and interquartile range for continuous variables and as percentage for categorical variables. Differences in continuous variables were assessed by one-way ANOVA on ranks (Kruskal–Wallis test) for comparison between the three groups or by Mann–Whitney U test for comparison between the groups with better (transferred to IM) or adverse (transferred to ICU/deceased) outcomes. Differences in categorical variables were evaluated by the likelihood-ratio test.

p for trend refers to the comparisons between all the groups

p^1 refers to comparison between men transferred to IM Inpatients Clinics and men transferred to ICU/deceased

Abbreviations: IM= Internal Medicine; RICU= Respiratory Intensive Care Unit; ICU= Intensive Care Unit; CVD= cardiovascular disease; ARDS= Acute Respiratory Distress Syndrome; WBC= White Blood Cell; AST= Alanine transaminase; AST= aspartate transaminase; CPK= creatine phosphokinase; CRP= C-Reactive Protein; LDH= Lactate Dehydrogenase; IL-6= Interleukin-6; T= testosterone; SHBG= Sex hormone Binding Globulin; LH= luteinizing hormone

Overall, significant difference among groups was found in terms of previous history of psychiatric diseases and of prevalence of severe ARDS at RICU admission. Moreover, lymphocyte count was the lowest in both RICU and ICU/deceased patients, whereas PCT and LDH had the highest values in the ICU/deceased group. Of note, no differences in D-dimer serum levels were observed. LH, TT, and cFT were significantly different among groups, with higher LH and lower TT and cFT in the ICU/deceased group. These differences were confirmed when comparing the groups transferred from RICU because of either improved respiratory conditions or adverse outcomes, respectively [**Table 1**]. In addition, the comparison of these two groups revealed that men in the adverse outcome group (ie, transferred to ICU or deceased) more frequently had a history of arrhythmia and were less frequently obese. Besides the aforementioned biochemical and hormonal parameters, men in the ICU/deceased group had significantly higher neutrophil count, potassium, and CRP levels, as compared with men transferred to IM.

In order to further explore the trends of hormonal levels according to the severity of the outcome, hormones were analyzed in each category after splitting the adverse outcome group into men transferred to ICU and men eventually deceased. Figure 3 confirms the aforementioned differences and shows a stepwise decrease in both TT and cFT according to the severity of the outcome at the time of the final assessment [**Figure 3**]. Despite not achieving the statistical significant, LH had higher values in men with stable or adverse outcome categories. SHBG had comparable values among the four groups [**Figure 3**]. Besides the outcome classes, LH, TT, and cFT were significantly different in patients with or without severe ARDS (LH = 12.0 U/L [8.5-21.7] vs 6.9 U/L [5.0-10.4]; TT = 2.19 nmol/L [1.25-4.58] vs 7.0 nmol/L [4.07-

13.8] and cFT = 50.5 pmol/L [18.6-84.0] vs 138.0 pmol/L [102.0-221.0], respectively; all P < .05). No differences in SHBG were found (data not shown). In this cohort, TT and cFT were not significantly associated with age (B= −0.93 [−0.287; 0.101], P = .337 and B= −2.84 [−5.96; 0.274], P = .072, respectively); similarly, TT and cFT did not differ in obese and non-obese men (TT = 6.75 nmol/L [3.19-14.86] vs 4.61 nmol/L [2.12-11.65], P = .633 and cFT = 127.0 pmol/L [58.1-167.0] vs 120.0 pmol/L [46.8-204.5], respectively; P = .929).

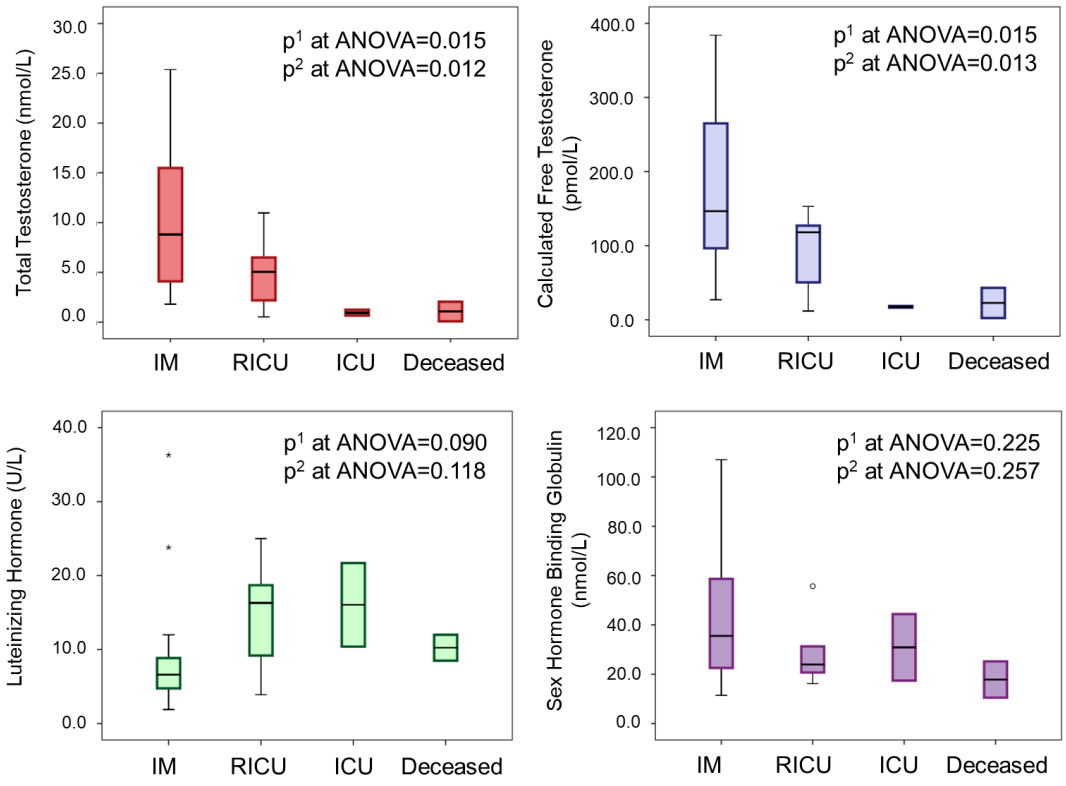

Figure 3. Hormone and sex hormone–binding globulin levels according to different clinical outcomes.
Data are expressed as box plot, with median and interquartile range; whiskers represent minimum and maximum values; circles and asterisks represent the outliers and extreme outliers. p1 is the significance level for one-way ANOVA on ranks (Kruskal-Wallis test) for testing the differences among the four groups; p2 is the significance level for one-way ANOVA on ranks (Kruskal-Wallis test) after excluding the RICU group. Abbreviations: IM = internal medicine inpatient clinics; RICU = respiratory intensive care unit; ICU = intensive care unit

As both TT and cFT showed a significant and progressive decline according to worsening outcomes, we evaluated the relationship between T and markers of disease severity, as emerged by table 2 [**Table 2**].

Table 2 shows the correlation for TT and cFT with markers of disease severity. Both TT and cFT showed a negative significant correlation with the neutrophil count, LDH, and PCT levels and a positive one with the lymphocyte count. TT was also negatively correlated with CRP and ferritin; cFT showed a similar but not statistically significant trend. Neither TT nor cFT was correlated with potassium.

	Total testosterone (nmol/L)		**Calculated Free Testosterone (pmol/L)**	
	Spearman's rho	**p**	**Spearman's rho**	**p**
Neutrophils (10^3/µL)	-0.462	**0.012**	-0.450	**0.014**
Lymphocytes (10^3/µL)	0.493	**0.007**	0.461	**0.012**
CRP (mg/L)	-0.385	**0.035**	-0.357	0.053
Procalcitonin (ng/ml)	-0.448	**0.015**	-0.454	**0.013**
LDH (U/L)	-0.490	**0.006**	-0.465	**0.010**
Ferritin (ng/ml)	-0.401	**0.031**	-0.320	0.091
Potassium (mEq/L)	-0.134	0.471	-0.202	0.285

Table 2. Correlation between total and free testosterone with parameters associated with different outcomes in SARS-CoV-2 pneumonia patients
Data derive from Spearman's rank correlation test.Abbreviations: CRP= C-Reactive Protein; LDH= Lactate Dehydrogenase; SARS-CoV-2= Severe Acute Respiratory Syndrome Coronavirus 2.

In order to further explore these relationships, we conducted LOWESS analyses to check for their non-linearity. Figures 4 and 5 show the results for TT and cFT, respectively [**Figures 4-5**]

Several non-linear relationships were observed with TT. The visual inspection of LOWESS curves allowed hypothesizing a possible threshold for TT levels around 5 nmol/L [**Figure 4**].

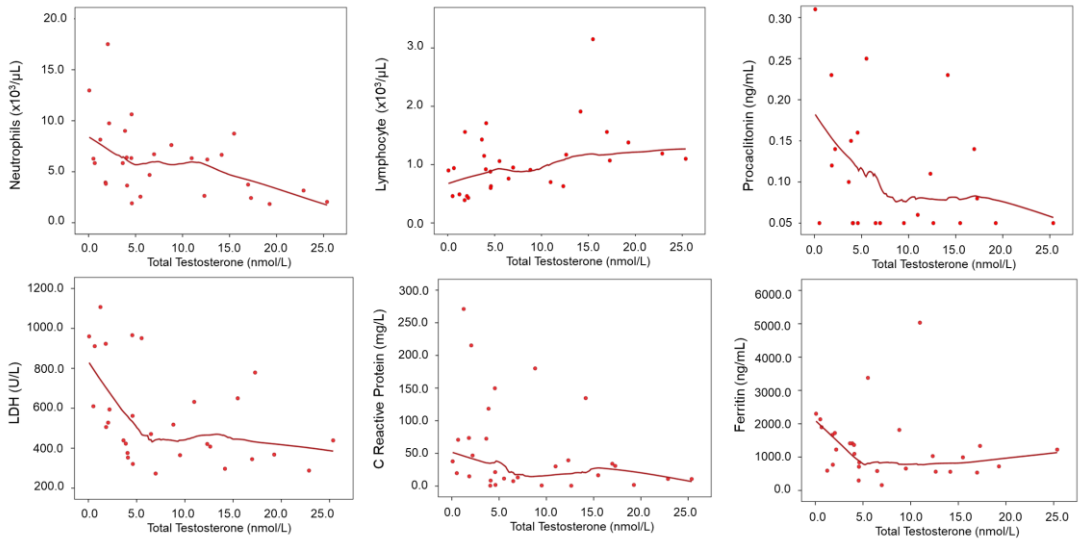

Figure 4. Relationship between total testosterone and blood inflammatory markers of severity of SARS-CoV-2 pneumonia.
The smooth curves were carried out by locally weighted scatterplot smoothing (LOWESS) analysis. Abbreviations: LDH = lactate dehydrogenase

For cFT, the linearity could be assumed for all of the variables but LDH, which showed a steeper increase for cFT levels below 100 pmol/L [**Figure 5**].

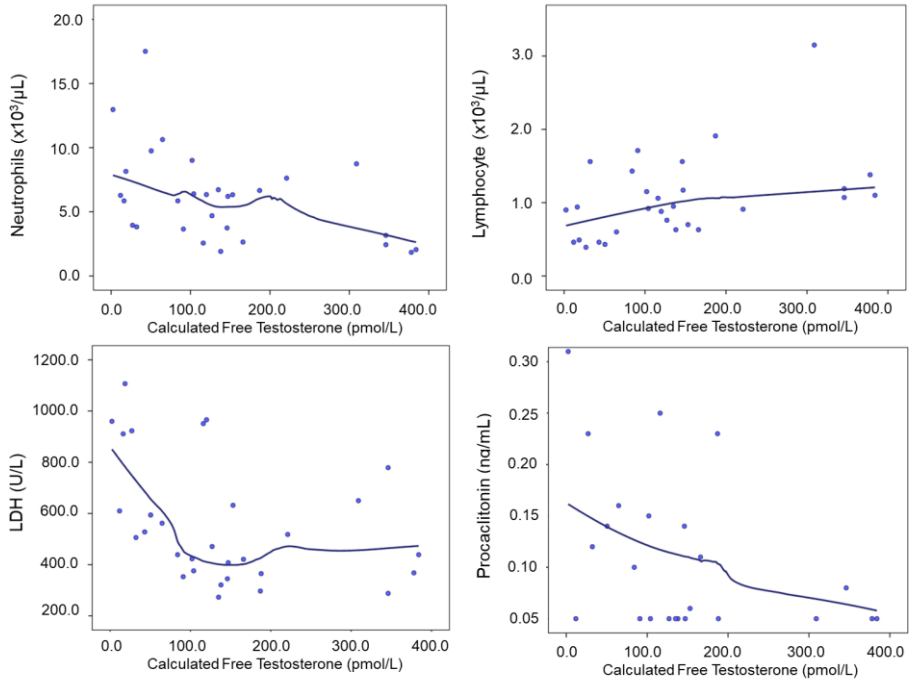

Figure 5 Relationship between calculated free testosterone and blood inflammatory markers of severity of SARS-CoV-2 pneumonia. The smooth curves were carried out by locally weighted scatterplot smoothing (LOWESS) analysis. Abbreviations: LDH = lactate dehydrogenase

In order to check formally these putative thresholds, linear regressions with linear spline functions were performed.

Table 3 reports the results of these analyses: PCT, LDH, and ferritin levels depicted different trends above and below TT 5 nmol/L [**Table 3**]. In particular, for TT < 5 nmol/L, PCT, LDH, and ferritin increased on average of 0.18 ng/mL, 72.72 U/L, and 232.17 ng/mL, for each nmol/L decrease in TT, respectively; these values were higher than those predicted assuming linearity. This was not the case for the neutrophil and lymphocyte counts for which a linear association with TT may be assumed. The association between TT and CRP was not confirmed in this analysis. After adjustment for age and comorbidities, all the above-mentioned linear relationships were confirmed (neutrophils: B= −0.19 [−0.36;-0.03], P = .026; lymphocyte: B = 0.03 [0.00;0.06]; P = .043; PCT: B= −0.03 [−0.07; 0.01]; P = .137; LDH: B= −16.45 [−28.22;-4.69], P = .008; CRP: B= −2.81 [−6.57;0.94], P = .135; ferritin: B= −21.76 [−80.36;36.83]; P = .451). A threshold effect for cFT of 100 pmol/L was confirmed for PCT and LDH that decreased on average of 0.09 ng/mL and 41.46 U/L, for each 10 pmol/L increase in cFT, respectively; again, these values were higher than those predicted by assuming linearity. Conversely, linearity could be assumed for the relationship between cFT and neutrophil and lymphocyte counts. After adjustment for age and comorbidities, all the above-mentioned linear relationships were confirmed (neutrophils: B= −0.10 [−0.21;0.00], P = .064; lymphocyte: B = 0.02 [0.00;0.03]; P = .045; PCT: B= −0.02 [−0.04; 0.00]; P = .135; LDH: B= −10.93 [−18.58;-3.29], P = .007).

	Total Testosterone			Calculated Free Testosterone		
	Linear	**<5 nmol/L**	**≥5 nmol/L**	**Linear**	**<100 pmol/L**	**≥100 pmol/L**
Neutrophils (10^3/µL)	**B=-0.23 [-0.40;-0.06] p=0.012**	B=-0.63 [-1.53;0.27] p=0.161	B=-0.15 [-0.40;0.09] p=0.200	**B=-0.14 [-0.25;-0.03] p=0.012**	B=-0.32 [-0.73;0.09] p=0.121	B=-0.10 [-0.25;0.05] p=0.191
Lymphocytes (10^3/µL)	**B=0.03 [0.01;0.06] p=0.023**	B=0.07 [-0.08;0.22] p=0.355	B=0.03 [-0.01;0.07] p=0.175	**B=0.02 [0.00;0.04] p=0.017**	B=0.04 [-0.03;0.11] p=0.234	B=0.02 [-0.01;0.042] p=0.160
Procalcitonin (ng/ml)	B=-0.03 [-0.07;0.00] 0.084	**B=-0.18 [-0.35;-0.01] p=0.042**	B=-0.00 [-0.05;0.05] 0.920	B=-0.02 [-0.04;0.00] p=0.069	**B=-0.09 [-0.16;-0.01] p=0.026**	B=-0.00 [-0.03;0.03] p=0.884
LDH (U/L)	**B=-14.49 [-26.49;-2.49] p=0.020**	**B=-72.72 [-130.02;-15.43] p=0.015**	B=-3.39 [-18.98;12.20] 0.659	**B=-8.06 [-15.78;-0.35] p=0.041**	**B=-41.46 [-67.18;-15.73] p=0.003**	B=0.71 [-8.81;10.22] p=0.880
CRP (mg/L)	B=-3.00 [-6.67;0.68] p=0.106	B=-10.02 [-28.78;8.75] p=0.283	B=-1.66 [-6.76;3.45] 0.511	---	---	---
Ferritin (ng/mL)	B=-21.68 [-80.77;37.40] p=0.458	**B=-232.17 [-397.37;-66.97] p=0.010**	B=-49.76 [-196,47;96,94] p=0.474	---	---	---

Table 3 Confirmation of thresholds for total and free testosterone towards blood inflammatory markers using linear regressions with linear spline functions

Data are reported as B coefficients and 95% confidence interval. Data derive from linear regressions ("Linear" columns) with total and free testosterone used as continuous variables using the whole range of values. Columns reporting thresholds (< and ≥ 5 nmol/L; < and ≥ 100 pmol/L) report data derived from linear regressions linear spline functions.
Significant associations from linear regressions with the whole range of testosterone values without significant associations with spline functions denote that linearity could be assumed. Significant and non-significant associations from linear regressions with the whole range of testosterone values with significant associations with spline functions denote that linearity could not be assumed and confirm the indicated threshold value. Non-significant associations from linear regressions with the whole range of testosterone values with non-significant associations with spline functions denote a lack of assoctiation.
Abbreviations: LDH= Lactate Dehydrogenase; CRP= C-Reactive Protein.

After confirming the association between TT and cFT with several markers of disease severity, the relationship of these parameters with the clinical outcomes was evaluated. Ordinal linear regressions showed that for each nmol/L decrease in TT and for each 10 pmol/L decrease in cFT, the probability of having worse outcomes significantly increased (OR = 1.42 [1.06;1.89]; P = .017 and OR = 1.25 [1.06;1.48];

P = .007 for TT and cFT, respectively) being unaffected by the adjustment for age and CCI (OR = 1.35 [1.03;1.76]; P = .029 and OR = 1.23 [1.04;1.46]; P = .015 for TT and cFT, respectively).

The estimated probability of being transferred to IM and ICU, or to die based on T levels is reported in figure 6 [**Figure 6**].

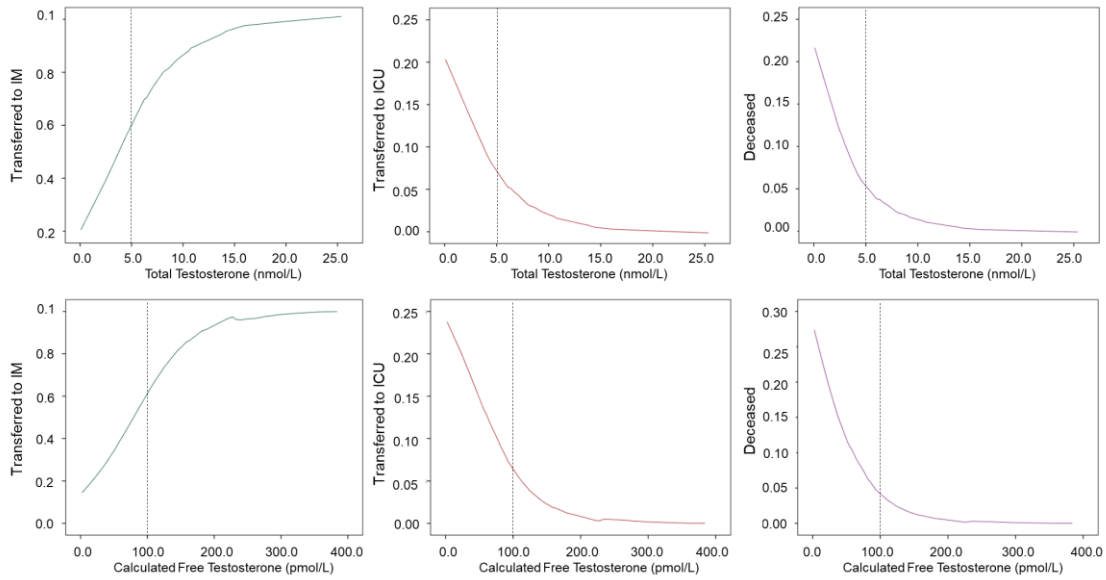

Figure 6. Relationship between total or calculated free testosterone and estimated probability of different clinical outcomes in SARSCoV-2 pneumonia patients based on T levels.
The smooth curves were carried out by locally weighted scatterplot smoothing (LOWESS) analysis. Abbreviations: IM = internal medicine inpatient clinics; ICU = intensive care unit

A non-linear change in the probability of different outcomes is well recognizable. When applying the aforementioned thresholds, the probability of being transferred to IM was 36.51% [29.28-52.86] and 95.44% [78.05- 99.09] below and above TT 5 nmol/L, respectively (P < .0001). The probability of being transferred to the ICU or dying below and above TT 5 nmol/L was 14.18% [8.89-17.03] or 0.60% [0.12-3.32], P < .0001 or 12.40% [6.77-16.43] and 0.39% [0.07-2.26], P < .0001, respectively. As

for actual cases, eight out of 21 (38.1%) transferred to IM, two out of two (100%) transferred to ICU, and two out of two (100%) deceased had TT < 5 nmol/L (P = .035). As for cFT, the probability of being transferred to IM, ICU, or dying in men with cFT below and above 100 pmol/L was 25.58% [19.35-41.76] vs 84.08% [74.58-99.45], 18.43% [11.80-21.43] vs 2.10% [0.06-3.65], and 16.39% [8.59-21.93] vs 1.26% [0.04-2.25], respectively; all P < .0001. As for actual cases, five out of 21 (23.8%) transferred to IM inpatient clinics, two out of two (100%) transferred to ICU, and two out of two (100%) deceased had cFT < 100 pmol/L (P = .010).

Discussion

This study demonstrates for the first time that lower levels of TT and cFT (assessed the first day after the admission in RICU) are novel predictors of poor prognosis in SARS-CoV-2 men admitted in RICU for pneumonia. Noteworthy, we found a longitudinal relationship between lower TT and cFT levels and a higher risk of clinical deterioration, thus leading to ICU transfer or to death.

Accordingly, we found that lower TT and cFT are significantly associated with higher serum LDH, ferritin, PCT, as well as to an increased level of neutrophils and decrease in lymphocyte count.

This latter finding confirms the relevant clinical importance of our observations as all the above-mentioned inflammatory biomarkers had emerged as poor prognostic factors for SARS-CoV-2 infection [*Ruan et al., 2020*]. Of note, no differences in D-dimer, an important parameter for the diagnosis of disseminated intravascular coagulation, were observed. Interestingly, for several of these markers (i.e., PCT,

LDH, and ferritin) a non-linear association with T was found with an apparent threshold effect (namely, TT at 5 nmol/L or cFT at 100 pmol/L). For TT < 5 nmol/L, the increase in PCT, LDH, or ferritin associated with one nmol/L decline in TT emerged to be sixfold, fivefold, and 10-fold higher than the linear prediction. Similar results were obtained for cFT below 100 pmol/L for PCT and LDH. Overall, this means that below these threshold values, a further decrease in TT or cFT of 0.5-1.3 nmol/L or 10-23 pmol/L, respectively, could be sufficient to cause an increase in these prognostic markers from the lower limit of normal range above the levels that were found associated with SARS-CoV-2-associated in-hospital death [*Zhou et al., 2020*].

Also, in this case, statistical modeling for estimating probability demonstrated a non-linear relationship with mortality or ICU transfer risk. Below TT 5 nmol/L or cFT 100 pmol/L, a steep increase in mortality or ICU transfer risk was observed, with 20- to 30-fold or 10- to 15-fold higher estimated average risk of adverse outcomes as compared to TT or cFT above thresholds, respectively. In contrast, a gradual improvement of clinical outcomes (transfer to non-intensive care IM) with increasing TT or cFT levels was observed. We also showed that low baseline TT and cFT levels are related to a more severe ARDS at RICU admission. Noteworthy, these data highlight a novel potential mechanism of frailty and mortality by identifying low T as a risk factor for the severe respiratory failure and inflammatory storm in SARS-CoV-2 infections. Our observations are further substantiated by several epidemiological evidence demonstrating that male hypogonadism represents a risk factor for a higher morbidity and mortality [*Corona et al., 2018; Araujo et al., 2011*]. In particular, it has been well demonstrated that the so-called functional

hypogonadism is associated with conditions like obesity and inflammation in males [*Corona et al., 2020*].

Therefore, it could be also speculated that obesity and low T could even foster the cytokine storm aggravating further the clinical condition. Consistently, over the last decades it has become clear that T is involved in a multitude of biological processes in males, other than reproduction and sexuality. In particular, a novel aspect of the physiology of T is its anti-inflammatory role [*Vignozzi et al., 2012 (a); Vignozzi et al., 2012 (b)*]. Several preclinical and clinical evidence showed that low T boosted pro-inflammatory cytokines and that T treatment blunted their levels [*Vignozzi et al., 2012 (a); Kelly et al., 2013; Mohamad et al., 2019*]. There is evidence for an immunomodulatory and protective effect of T by regulating differentiation of T lymphocytes [*Vignozzi et al., 2012 (a); Fijak et al., 2011; Vignozzi et al., 2013*]. Interestingly, we observed that lymphocyte count increased, while neutrophil levels decreased, as a function of increasing TT and cFT. Accumulating evidence suggests that SARS-CoV-2 infection severity is influenced by a dysregulation of the immune response. A drastic lymphopenia with reduction in numbers of CD4 + T cells, CD8 + T cells, B cells, and natural killer (NK) cells is a common feature in patients with severe SARS-CoV-2 infection, but not in patients with mild disease [*Cao, 2020*], with neutrophil-to-lymphocyte ratio reflecting an enhanced inflammatory process and a poor prognosis [*Lagunas-Rangel, 2019*]. AR is expressed on CD4 + T lymphocytes, CD8 + T lymphocytes, and macrophages supporting the possibility of direct action of T on these cells [*Liva et al., 2001*]. A worsening of clinical status was coupled not only with reduced T level, but also with increased LH levels, even though the latter association did not maintain significance when the four groups were

compared, thus supporting the presence of a primary hypogonadism. Accordingly, an orchitis-like syndrome has been hypothesized in SARS-CoV-2 men [*Isidori et al., 2014*]. Several pathogenic mechanisms occurring during the SARS-CoV-2 infection might be responsible for the impairment of testicular function. First, ACE2 is highly expressed within the human testis, being a constitutive product of adult-type Leydig cells [*Douglas et al., 2004; Wang et al., 2020*]. Because angiotensin II reduced both basal and LH stimulated testosterone synthesis by Leydig cells, ACE2 has been hypothesized to modulate the steroidogenic activity of these cells and to shield testis by limiting angiotensin II detrimental effects [*Dufau et al., 1987; Khanum et al., 1988*]. ACE2 on Leydig cells could also alter local microvascular flow and permeability [*Douglas et al., 2004*] and favoring inflammation [*Xu et al., 2006*]. Moreover, increased LH levels could contribute further to testicular alterations by modulating testicular blood flow, endothelial cell permeability, and fluid accumulation within the testis [*Widmark et al., 1986*]. Finally, in our study, SARS-CoV-2 men are affected by a severe form of hypogonadism associated with a primary testicular impairment. A number of well-designed longitudinal studies have shown that late-onset hypogonadism (LOH) represents a common clinical entity among aging males. In most men, there is a slow decline in serum TT levels with aging, even in the absence of disease [*Wu et al., 2008*]. However, in our cohort of men T level was sharply reduced without showing any significant age-dependent modulation, further supporting the view of a potential direct effect of SARS-CoV-2 on testicular function. A study on 6 men (mean age 39 years; ranged from 20 to 58 years old) has previously reported LH and T levels in SARS men [*Xu et al., 2006*]. The authors compared SARS men with age-matched healthy men, showing a rise of

LH similar to our study, but associated with normal T level [Xu et al., 2006]. Our study is the first to report novel evidence of a clear-cut reduction in T level along with an LH increase in SARS-CoV-2 men; however, while TT and cFT were associated with a higher risk of clinical deterioration, increased LH levels tended to have a similar relationship, without reaching a statistical significance. We would like to recognize that low serum T levels might also be a consequence and not a reason for the patients' condition. However, the causal effect relationship could only be tested by randomized trials with testosterone treatment vs placebo in hypogonadal men upon submission to the ICU. Also, the presence of either a psychiatric disorder or cardiac arrhythmias was associated with a higher risk of a poor prognosis, whereas obese patients were paradoxically less prone to worse outcomes. Some limitations should be recognized. Firstly, the sample size is relatively small with a limited number of adverse outcomes. However, a small sample size represents a concern for lack of significant associations, more than for significant ones. Secondly, six patients were still in charge of the RICU at the time of the present analysis; hence, we do not know their definitive outcome. However, the days passed in RICU are comparable among the groups. Unfortunately, information on the onset of infection before RICU was not collected. Moreover, this study lacks of a control group of patients not affected by SARS-CoV-2 but hospitalized and evaluated in the same way. This comparison could have strengthen our study as the prevalence of hypogonadism in elderly male patients admitted to the hospital for acute illness is reported to be close to 50% [*Iglesias et al., 2014; Nakashima et al., 2017*]. Finally, T was assessed only one time and not by the gold standard method (such as mass spectrometry, which was not available in this clinical setting) but it was measured by a commercially

available immunoassay used in a high-volume hospital and undergoing quality control programs. Free T was calculated rather than measured.

PART 2: HIGHER TESTOSTERONE IS ASSOCIATED WITH INCREASED INFLAMMATORY MARKERS IN WOMEN WITH SARS-COV-2 PNEUMONIA: PRELIMINARY RESULTS FROM AN OBSERVATIONAL STUDY

Objective of the study

To perform a preliminary study to assess the association between T levels and biochemical markers in a cohort of female patients admitted for SARS-CoV-2 infection in the RICU of a single Hospital in Mantua, one of the epicenters of first phase of the SARS-CoV-2 epidemic in Italy. This study reports the findings obtained on a small sample of 17 patients with SARS-CoV-2 infection followed to the discharge to lower intensity care units or death.

Materials and methods

Study population

Data from a consecutive series of 17 female patients with SARS-CoV-2 pneumonia and recovered in the RICU of the "Carlo Poma" Hospital in Mantua, Italy, were analyzed.

Diagnosis of SARS-Cov-2 infection and other performed exams

A laboratory pharyngeal-nose swab positivity of SARS-CoV-2 infection was confirmed by chest X-ray. ARDS was defined by the "Berlin definition," and patients were segregated into mild ARDS (PaO2/FiO2 ≤ 300 mmHg and > 200 mmHg), moderate ARDS (PaO2/FIO2 ≤ 200 mmHg and > 100 mmHg), and severe ARDS (PaO2/FiO2 ≤ 100 mmHg) (Definition Task Force ARDS, 2012).

According to the protocol (approved by the Local Ethics Committee Val Padana, Mantua, Italy) and as for clinical practice, each patient underwent a standardized diagnostic workup. Specifically, blood samples were drawn on the first morning after the admission to the RICU within 8.00 AM, in fasting condition, for the determination of blood count and leukocyte formula, creatinine, uric acid, electrolytes, transaminases, albumin, CPK, CRP, PCT, LDH, ferritin, D-dimer, fibrinogen, IL-6, TT, SHBG, cortisol and LH. Hematological and biochemical analyses were performed in the central laboratory of the "Carlo Poma" Hospital (Mantua, Italy) with commercially available kits routinely used for hospital clinical practice. TT was measured once by an immunoassay (Electrochemiluminescence Immunoassay, ECLIA), and free T was calculated by the Vermeulen formula [14]. Cortisol was measured only in patients (n=12) not receiving steroid therapy.

Statistical analysis

Data were expressed as medians [interquartile ranges] or percentage for continuous and categorical variables, respectively. Univariate relationships among T and biochemical markers were firstly assessed by Spearman's rank correlation. Afterwards, unadjusted and age-adjusted linear regression models were carried out. Non-linearity of the relationships were explored by the locally weighted scatterplot smoothing (LOWESS) analysis and formally checked by linear regression models with linear spline functions for T. This analysis allowed identifying threshold levels at which a significant change in the slope of the association between T and other blood markers occurred.

All the analyses were performed by Statistical Package for the Social Sciences Statistics 26 (IBM Corporation). Spline linear functions were carried out by Stata MP 13 (StataCorp).

Results

Table 4 details descriptive statistics of the cohort including information collected at the admission to RICU [**Table 4**].

	Reference range	**Values in the cohort (n=17)**
Demographics and previous medical history		
Age (years)	---	69.0 [57.5-74.0]
Menopause (%)	---	94.1%
Smoking habits (%)		
Former smoker	---	11.8
Current smoker	---	11.8

Obesity (%)	---	52.9
Hypertension (%)	---	76.5
ACEi (%)		17.6
ARBs (%)		35.3
Dyslipidemia (%)	---	17.6
Diabetes (%)	---	29.4
Hypothyroidism (%)	---	5.9
Chronic Renal Failure (%)	---	0.0
Arrhythmia (%)	---	5.9
Psychiatric Diseases (%)	---	11.8
Hematologic Diseases (%)	---	11.8
CVD (%)	---	11.8
Liver Diseases (%)	---	0.0
Parameters during Hospitalization in RICU		
Time in RICU(days)	---	6.0 [3.5-12.5]
PaO2/FiO2 (mm Hg)	<300 for ARDS	152.0 [113.5-178.1]
Severe ARDS (%)	PaO2/FiO2 ≤100 mm Hg	17.6
Moderate ARDS (%)	PaO2/FiO2 100-200 mm Hg	82.4
WBC (10^3/µL)	4.4-11	8.4 [5.3-11.1]
Neutrophils (10^3/µL)	2.0-7.5	6.6 [3.6-9.0]
Lymphocytes (10^3/µL)	1.3-4.8	1.3 [0.8-1.7]
Hemoglobin (g/dl)	13.5-17-5	11.2 [10.0-11.9]
Hematocrit (%)	40-50	35.8 [33.1-37.4]
Platelets (10^3/µL)	150-400	356.0 [253.5-449.0]
Creatinine (mg/dl)	0.6-1.3	0.7 [0.6-1.0]
Uric acid (mg/dl)	2.5-7.2	4.3 [3.0-6.9]
Sodium (mEq/L)	135-145	139.0 [137.0-141.0]
Potassium (mEq/L)	3.4-4.7	3.6 [3.4-3.7]
AST (U/L)	10-33	28.0 [19.5-31.8]
ALT (U/L)	5-37	21.0 [11.5-33.5]
CPK (U/L)	25-200	50.0 [38.5-75.5]
CRP (mg/L)	0-5	25.6 [15.8-62.5]
Procalcitonin (ng/ml)	0-0.09	0.05 [0.05-0.22]
LDH (U/L)	150-450	471.0 [420.0-528.0]

Fibrinogen (mg/dl)	150-450	467.5 [331.5-643.5]
D-Dimer (ng/ml)	<500	1712.0 [987.5-3683.0]
Ferritin (ng/ml)	30-400	400.0 [233.0-967.5]
IL-6 (pg/ml)	<7	71.7 [55.0-109.5]
TT (nmol/L)	0-1.5	0.5 [0.2-1.2]
cFT(pmol/L)	---	5.2 [2.8-15.1]
SHBG (nmol/L)	27.1-128.0	32.7 [27.4-54.5]
LH (U/L)	7.7-58.5	16.7 [4.3-27.0]
Cortisol (µg/dl)	6.0-18.4	20.1 [14.3-24.8]

Table 4. Descriptive statistics of the cohort
Data are reported as median and interquartile range for continuous variables and as percentage for categorical variables. Abbreviations: ACEi, ACE inhibitors; ARBs, Angiotensin Receptors Blockers; ARDS, acute respiratory distress syndrome; AST, alanine transaminase; AST, aspartate transaminase; CPK, creatine phosphokinase; CRP, C-reactive protein; CVD, cardiovascular disease; IL-6, interleukin 6;LDH, lactate dehydrogenase; LH, luteinizing hormone; RICU, respiratory intensive care unit; SHBG, sex hormone–binding globulin; T, testosterone; FAI, Free Androgen Index; WBC, white blood cell.

Of the 17 patients only one was not in menopause. She was 50 years old and her hormone values on the first morning after RICU admission were as follow: TT = 0.52 nmol/l, albumin = 3.12 g/dL , SHBG = 97.8 nmol/l , cFT = 4.3 pmol/l , LH = 8.9 mU/l , cortisol = 18.5 mcg/dL. She was discharged from RICU after 26 days of hospitalization for better clinical conditions.

The relationship between T and biochemical inflammatory markers was firstly assessed by univariate correlation tests. Table 5 shows that TT and cFT measured on the first morning after RICU admission were significantly and positively associated with inflammatory markers assessed at the same time point, including procalcitonin, CRP, and fibrinogen [**Table 5**]. Accordingly, higher TT and cFT were related with a worse hospital course, being associated with longer hospitalization in RICU (where the present observations were collected) and ICU (transferred from RICU for deteriorated clinical conditions).

In order to further explore the relationship of T with adverse biochemical and clinical

parameters in women with SARS-CoV-2 pneumonia, we checked the relationship of TT or cFT with the releasing hormone (LH) and, surprisingly, we did not observe any significant association [**Table 5**]. On the contrary, both TT and cFT showed a strong positive correlation with cortisol level [**Table 5**], which, in turn, was negatively associated with LH (rho=-0.748, p=0.013). None of the aforementioned hormones correlated significantly with age (rho= -0.205, 0.132, -0.093, -0.024; p=0.429, 0.626, 0.732, 0.947, for TT, cFT, LH and cortisol, respectively).

	Total testosterone (nmol/l)		**Calculated free testosterone (pmol/l)**	
	Spearman's rho	**p**	**Spearman's rho**	**P**
Procalcitonin (ng/ml)	0.727	**0.001**	0.710	**0.003**
LDH (U/L)	0.396	0.115	0.333	0.208
Ferritin (ng/ml)	0.454	*0.067*	0.294	0.268
CRP (mg/l)	0.673	**0.003**	0.647	**0.007**
Fibrinogen (mg/dl)	0.669	**0.005**	0.513	**0.042**
Days in RICU	0.571	**0.017**	0.379	0.148
Days in ICU	0.593	**0.012**	0.493	*0.052*
LH (U/L)	-0.317	0.232	-0.280	0.294
Cortisol (μg/dl)	0.954	**<0.0001**	0.842	**0.002**

Table 5. Correlation between total and free testosterone with hormones, biochemical inflammatory markers and days in RICU.
Data derived from Spearman's rank correlation test. Abbreviations: LDH, lactate dehydrogenase; CRP. C-reactive protein; RICU, Respiratory Intensive Care Unit; ICU, Intensive Care Unit; LH, Luteinizing Hormone.

In order to assess the possible non-linearity of the relationship between androgens and the inflammatory markers, we conducted LOWESS analyses. Figure 7 suggests that, while a linear relationship could be assumed for CRP and fibrinogen, a threshold effect is apparent in the relationship between TT and procalcitonin, LDH and ferritin [**Figure 7**].

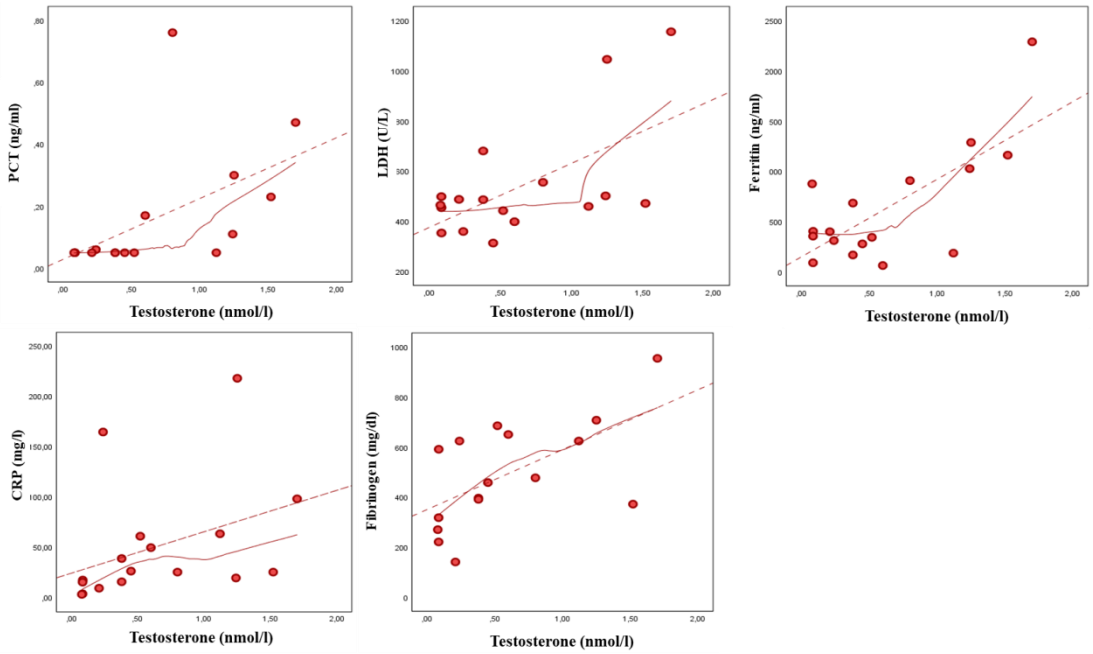

Figure 7. Relationship between total testosterone and biochemical inflammatory markers of severity of SARS-CoV-2 pneumonia.
The smooth curves were carried out by locally weighted scatterplot smoothing (LOWESS) analysis. Abbreviations: PCT= Procalcitonin, LDH = lactate dehydrogenase, CRP= C-reactive protein

Data from spline regression analyses are shown in table 6 [**Table 6**].

	Unadjusted model			Age- adjusted model		
	Total Testosterone					
	Linear	**Non linear**		**Linear**	**Non linear**	
		<1 nmol/L	**≥1 nmol/L**		**<1 nmol/L**	**≥1 nmol/L**
PCT (ng/mL)	**B= 0.2 [0.0;0.4] p=0.036** R^2=0.277	B=0.5 [-0.1;1.3] p=0.076	**B=0.6 [0.1;1.1] 0.016**	**B=0.2 [0.0;0.4] p=0.044** R^2=0.279	B=0.3 [-0.2;0.7] p=0.196	B=0.1 [-0.7;0.8] p=0.827
		R^2=0.287			R^2=0.287	
LDH (U/L)	**B=255.7 [69.7;441.7] p=0.010** R^2=0.364	B=42.0 [-325.2; 409.3] p=0.810	**B=675.9 [21.9;1329.8] p=0.044**	**B=264.3 [67.3;461.4] p=0.012** R^2=0.373	B=21.1 [-355.2;397.3] p=0.905	**B=768.2 [67.4;1468.9] p=0.034**
		R^2=0.445			R^2=0.477	
Ferritin (ng/mL)	**B=771.8 [368.8;1174.9] p=0.001** R^2=0.526	B=-62.6 [-713.9;588.6] p=0.840	**B=2412.6 [1253.0;3572.2] p=0.001**	**B=727.1 [314.2;1139.9] p=0.002** R^2=0.563	B=-41.4 [-722.0;639.2] p=0.898	**B=2318.9 [1051.2;3586.6] p=0.002**
		R^2=0.723			R^2=0.729	
CRP (mg/L)	B=41.2 [-14.8;97.3] p=0.138 R^2=0.141	B= 57.2 [-60.9;175.3] p=0.317	B=9.9 [-200.4;220.2] p=0.921	B=46.7 [-11.3;104.7] p=0.106 R^2=0.192	B=50.7 [-70.6;172.0] p=0.383	B=38.3 [-187.6;264.2] p=0.720
		R^2=0.147			R^2=0.192	
Fibrinogen (mg/dL)	**B=239.3 [56.3;422.3] p=0.014** R^2=0.360	B= 292.3 [-96.3;680.8] p=0.128	B=140.1 [-522.0;802.3] p=0.655	**B=218.5 [36.7;400.2] p=0.022** R^2=0.441	B=339.4 [-39.6;718.5] p=0.075	B=-19.2 [-694.2;655.8] p=0.952
		R^2=0.365			R^2=0.469	
	Calculated free Testosterone					
	Linear	**Non linear**		**Linear**	**Non linear**	
		<5 pmol/L	**≥5 pmol/L**		**<5 pmol/L**	**≥5 pmol/L**
PCT (ng/mL)	B= 0.0 [-0.0;0.0] p=*0.057* R^2=0.252	B= 0.0 [-0.1;0.1] p=0.568	B= 0.0 [-0.0;0.0] p=0.133	B= 0.0 [-0.0;0.0] p=0.062 R^2=0.263	B= 0.0 [-0.1;0.1] p=0.549	B=0.0 [-0.0;0.0] p=0.145
		R^2=0.262			R^2=0.276	

LDH (U/L)	**B= 13.9 [6.1;21.6] p=0.002** R^2=0.512	B= -6.2 [-90.0; 77.6] p=0.875	**B= 15.0 [5.7;24.2] p=0.004** R^2=0.522	**B= 13.9 [5.9;22.0] p=0.003** R^2=0.519	B= -6.2 [-93.5;81.2] p=0.881	**B= 15.0 [5.4;24.7] p=0.005** R^2=0.528
Ferritin (ng/mL)	**B= 34.2 [15.0;53.5] p=0.002** R^2=0.509	B= -44.4 [-24.91;160.3] p=0.647	**B= 38.5 [16.0;61.0] p=0.003** R^2=0.534	**B= 35.0 [17.7;52.3] p=0.001** R^2=0.638	B= -43.4 [-226.2;139.5] p=0.615	**B= 39.3 [19.1;59.4] p=0.001** R^2=0.663
CRP (mg/L)	**B= 3.0 [0.8;5.3] p=0.013** R^2=0.368	B= 11.1 [-13.2;35.4] p=0.343	*B= 2.6 [-0.1;5.3] p=0.057* R^2=0.392	**B= 3.0 [0.6;5.4] p=0.017** R^2=0.377	B= 11.0 [-14.3;36.4] p=0.361	B= 2.6 [-0.2;5.4] p=0.068 R^2=0.401
Fibrinogn (mg/dL)	**B= 9.1 [0.5;17.6] p=0.039** R^2=0.270	B= 40.1 [-51.4;131.6] p=0.361	B= 7.4 [-2.7;17.5] p=0.137 R^2=0.299	**B= 9.4 [1.6;17.3] p=0.022** R^2=0.441	B=40.5 [-42.9;124.0] p=0.311	B= 7.7 [-1.5;16.9] p=0.092 R^2=0.471

Table 6. Association between total and free testosterone with biochemical inflammatory markers
Data derived from spline regression analysis.
Abbreviations PCR: procalcitonin; LDH: lactate dehydrogenase; CRP: C-reactive protein.

When the TT threshold value of 1 nmol/L was used, significant associations between TT and PCT, LDH or ferritin were observed for values above but not below this value. This was confirmed for the relationship between TT and LDH or ferritin in an age-adjusted model [**Table 6**]. Similar results were found for the association of cFT with the inflammatory markers with a threshold effect towards LDH and ferritin with significantly increased LDH and ferritin levels for values above – but not below - cFT 5 pmol/L [**Table 6**]. The number of days spent in ICU but not in RICU showed a linear association with TT (B=7.99 [1.95; 14.02], p=0.013 and 4.66 [-3.10; 12.43], p=0.219, for ICU and RICU, respectively) and cFT (B=0.22 [0.02; 0.41], p=0.035

and 0.09 [-0.28; 0.48], p=0.584, for ICU and RICU, respectively), even after adjusting for age.

LOWESS curves were carried out for the association between cortisol or LH with the same inflammatory parameters [**Figures 8 and 9, respectively**]. Figure 8 shows that cortisol is associated with serum inflammatory markers with similar trends observed for TT although without reaching statistically significant relationships; conversely, as shown by figure 9, the relationship between LH and inflammatory markers had different trends than those observed for TT and cortisol.

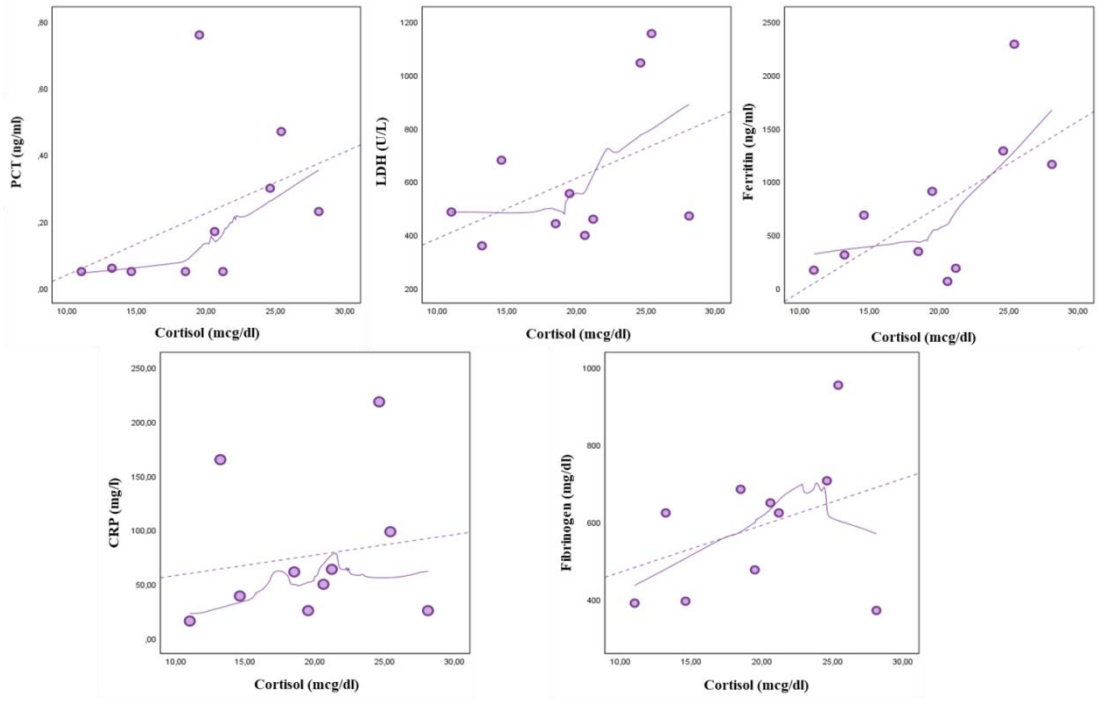

Figure 8. Relationship between cortisol and biochemical inflammatory markers of severity of SARS-CoV-2 pneumonia.
The smooth curves were carried out by locally weighted scatterplot smoothing (LOWESS) analysis. Abbreviations: PCT= Procalcitonin, LDH = lactate dehydrogenase, CRP= C-reactive protein

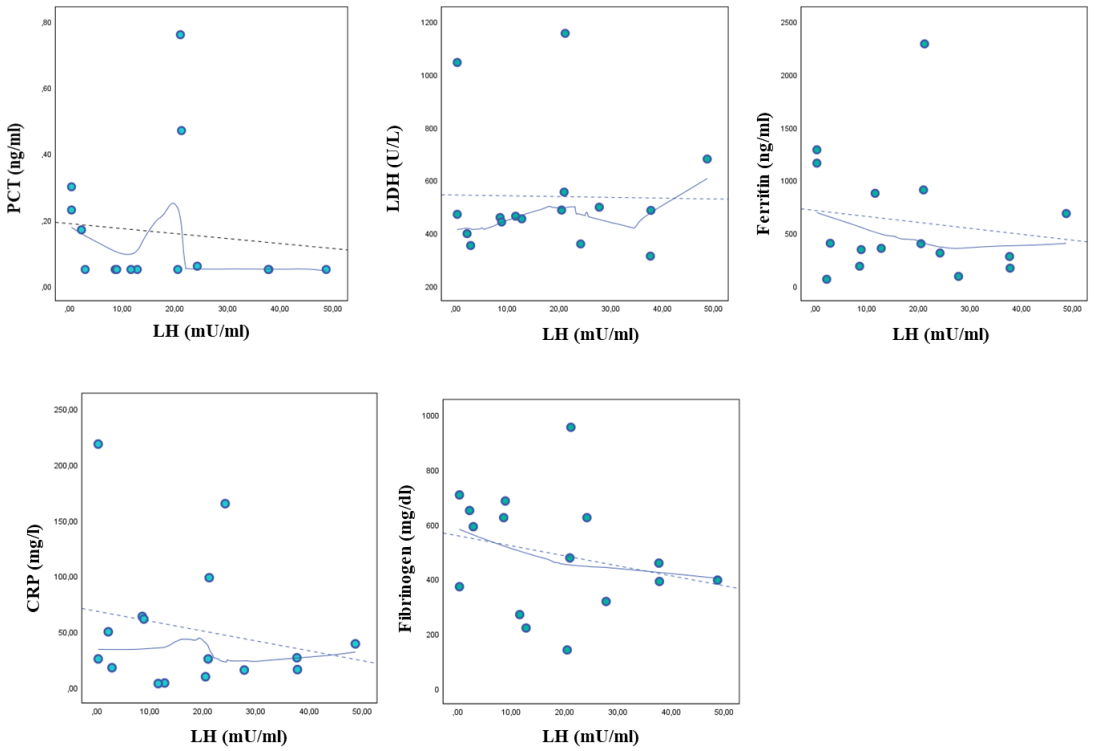

Figure 9. Relationship between LH and biochemical inflammatory markers of severity of SARS-CoV-2 pneumonia.
The smooth curves were carried out by locally weighted scatterplot smoothing (LOWESS) analysis. Abbreviations: PCT= Procalcitonin, LDH = lactate dehydrogenase, CRP= C-reactive protein

When comparing women with different ARDS severity, we confirmed that those with severe ARDS had significantly higher TT than women with moderate ARDS (1.5 [0.4-1.7] vs. 0.4 [0.1-1.3] nmol/L, p=0.05). No differences were found in LH and cortisol levels.

During the follow-up period, only one of the observed women with SARS-CoV-2 pneumonia deceased. She was 83 years old and her hormone values on the first morning after RICU admission were as follow: TT = 1.25 nmol/L, albumin = 3.0 g/dL, SHBG = 14.8 nmol/L, cFT = 40.4 pmol/L, LH = 0.3 U/L, cortisol = 24.6

mcg/dL. She deceased after 6 days in RICU. The remaining patients were transferred to internal medicine clinic for improved clinical conditions after 6.0 [3.3-12.8] days in RICU.

Discussion

Our study evaluates for the first time the role of testosterone in a cohort of women with SARS-CoV-2 pneumonia. Despite based on a small sample size, the present results show that higher TT and cFT are associated with a greater severity of the disease, as suggested by higher serum pro-inflammatory markers and longer stay in RICU before the transferral to lower intensity care units.

This is in complete opposition to recent findings in men from the same [*Rastrelli & Di Stasi et al., 2020*] and other, although similar, clinical settings [*Cayan et al, 2019; Ma et al., 2020; Salciccia et al., 2020*], which showed that low rather than high T level is associated with worse inflammatory marker profile and poorer clinical outcomes.

The role of T in inflammation in men and women is a challenging and still incompletely unravelled topic for Gender Medicine. In both animal models and men [*Fijak et al., 2011; Bobjer et al., 2013*], low T is associated with organ-specific as well as with systemic inflammation, as denoted by a rise in pro-inflammatory cytokines [*Vignozzi et al., 2014; Mohamad et al., 2019*]. Accordingly, T therapy is able to improve the inflammatory features and to decrease circulating pro-inflammatory markers [*Kalinchenko et al., 2010*]. The evidence in female is scanty and contradictory. In women with PCOS, the androgen excess is associated with a pro-inflammatory status [*González et al., 2012; Hatziagelaki et al., 2019*]. Furthermore, in the context of some autoimmune diseases such as rheumatoid

arthritis, women with lower number of CAG repeats in the androgen receptor gene, that confers higher androgen sensitivity, develop a more severe clinical course [*Dziedziejko et al., 2012*]. On the other hand, androgen treatment has been associated with clinical improvement in some autoimmune disorders. In post-menopausal women with active rheumatoid arthritis, one year of treatment with 50 mg of T propionate every two weeks resulted in improved general wellbeing [*Yu et al., 2007*]. Similarly, 12 weeks of treatment with DHEA 200 mg daily, as compared with placebo, improved the clinical manifestations of systemic lupus erythematosus in women receiving also standard therapy [*Petri et al., 2004*]. Recently, the anti-inflammatory mechanisms of T have been studied in vaginal smooth muscle cell from rats and humans. The activation of the androgen receptor by the androgen receptor super-agonist DHT inhibits the secretion of several pro-inflammatory factors while counteracting the chronic and self-perpetuating inflammatory features [*Maseroli et al., 2020*]. Overall, these data indicate how complex- and still not completely unravel – the role of T is in women.

While the results in SARS-CoV-2 infected men described in our [*Rastrelli & Di Stasi et al., 2020*] and other cohorts [*Cayan et al, 2019; Ma et al., 2020; Salciccia et al., 2020*] are in keeping with the expected anti-inflammatory role of T, the results hereby described on SARS-CoV-2 infected women are in apparent contrast. The increased levels of pro-inflammatory markers that are associated with higher T levels suggest a pro-inflammatory effect of T in this population.

In the attempt of hypothesizing possible mechanisms for explaining this observation, we analyzed the hormone levels in deeper detail. A noteworthy result, emerging from the observation of this cohort, is that T levels are surprisingly high for menopausal

women. In women, T is mainly secreted by the ovary, driven by LH stimulation of theca cells in the reproductive age. In the post-menopausal period, similar to estradiol or other ovarian sex hormones, T levels physiologically decline [*Davison et al., 2005*]. The post-menopausal decline in ovarian hormones is accompanied by an increased gonadotropins level. It is therefore surprising that, in this cohort of women, relatively low LH levels were observed. In fact, one out of four women admitted to RICU had LH levels below 4.3 U/L (lower limit of the interquartile range) largely below the values expected in menopausal women. Therefore, it could be hypothesized that the high level of T in women with COVID-19, being strongly associated with cortisol level, could be mostly produced by the adrenal gland. High testosterone from the adrenal source, in turn, will then exert a negative feedback on pituitary, resulting in an inhibitory effect on the post-menopausal-associated LH increase. However, low LH could reflect not only the T-related negative feedback but also the direct suppressive effect of systemic inflammatory status itself on gonadotropins. Noteworthy, the adrenal source of T in women is often overlooked and considered negligible. However, its role may get important in stressful conditions, such as those related to acute and critical illnesses, including i.e. SARS-CoV-2 pneumonia, which might induce an adrenal cortex hyperactivity and cortisol surge. The hyperactivation of the hypothalamic-pituitary-adrenal axis is not specific to the zona fasciculate because it activates also the zona reticularis with a potential overproduction of androgens. The tight positive association of circulating T with cortisol levels, whilst lacking an association with serum LH, further substantiate the hypothesis of an adrenal origin of the increased T level. Moreover, stress-related cortisol excess may also explain the inappropriately low LH levels; in fact, a

significant negative relationship links cortisol and LH in the present cohort. If confirmed, the observed association between T and cortisol level may be of paramount importance since cortisol has been recently claimed as a better independent predictor of an increased mortality in COVID-19 patients than other laboratory markers [*Tan et al., 2020*]. In this large cohort of COVID-19 patients admitted to three teaching hospitals in London UK (n=535), baseline cortisol concentration higher than 744 nmol/L was predictive of a reduced median survival [*Tan et al., 2020*]. In our study cohort, which is characterized by a greatly lower sample size, we were not able to select an optimal cut-off for cortisol, as opposed to TT and calculated free T.

The data hereby presented suggest that, in a severe acute and critical illness, such as SARS-CoV-2 pneumonia, the inflammatory response elicits the activation of stress hormone response, as suggested by increased cortisol levels. The relatively high T levels in menopausal women are, similar to cortisol, the expression of the adrenal cortex activation. This explains the relationship between higher T levels and increased inflammatory markers. Therefore, it might be conceivable that T is a mirror, more than a pathogenic factor, of the inflammatory burst in SARS-CoV-2 women. Nevertheless, as the cross-sectional design of the study does not allow defining the cause-effect relationship, a contribution of T in worsening COVID-19 related inflammatory status could not be ruled out. The transmembrane serine protease 2 (TMPRSS2) that is involved in the proteolytic processing of the SARS-CoV-2 spike protein thus facilitating the viral entry within the cells, has been previously studied in malignant prostatic tissue [*Mollica et al., 2020*]. In this context, TMPRSS2 has been proven as an androgen dependent protein [*Mollica et al., 2020*]

with androgen receptor as a promoter for TMPRSS2 gene transcription [*Mohamed et al., 2021*]. Accordingly, it may be speculated that higher testosterone levels in this study population may contribute to cause a more severe disease also through this pathogenic mechanism.

An understanding of the gender-related differences in adrenal hyperactivation during SARS-CoV-2 infection would be noteworthy. In men, increased T of adrenal source is not documentable because testis activity, although impaired in SARS-CoV-2 infection [*Rastrelli & Di Stasi et al., 2020*; *Cayan et al, 2019; Ma et al., 2020; Salciccia et al., 2020*], dilutes the effect and, eventually T appears as very low for adult males.

Some limitations should be recognized. Firstly, the sample size is very small and the results here reported need to be interpreted cautiously and confirmed in wider populations. For the moment, only one non-peer-reviewed report has been published on a cohort of SARS-CoV-2 women with a similar sample size and characteristics, which found results comparable with ours [*https://www.medrxiv.org/content/10.1101/2020.05.07.20073817v2*]. Further studies are advocated. Moreover, for simplicity's sake, only a single baseline testosterone and cortisol concentration measurement was analysed, thus without accounting for intra- and inter-individual variations of hormones response to stress. Thirdly, among our women, only a patient experienced an adverse outcome. This prevents from evaluating the predicting role of T on clinical outcomes; however, her hormone levels (high cortisol, inappropriately high T and low LH for an 83-years old woman) are in keeping with our hypothesis. Moreover, the study is a retrospective one and information on ACTH, or other adrenal glands hormones i.e. androstenedione,

DHEAS and 17OH-progesterone are not available. These would have been useful to support or disprove our hypothesis. However, we found a tight association between cortisol level and all the pro-inflammatory markers, but a threshold for predicting a worst clinical presentation or course was not found. In addition, T was not assessed by the gold standard method. However, the commercially available immunoassay used in a high-volume hospital, which undergoes quality control programs makes the measurement reliable. Finally, information on the onset of infection before RICU is not available.

CONCLUSIONS

In this thesis I try to summarize my PhD period and the works that I realized in this time. Sex hormones have a pivotal role in gender medicine and their levels correlate with different metabolic and inflammatory patterns in male and female populations. Specifically, the gender gap in COVID-19 pandemic is still far from being fully understood but I hope that these preliminary studies will be followed by further studies on this interesting topic.

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