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Male reproductive system inflammation after healing from coronavirus disease 2019

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Abstract

Background: There is evidence that, after severe acute respiratory syndrome coronavirus 2 infection, male reproductive function and semen quality may be damaged

Objectives: To evaluate a panel of inflammatory mediators in semen in patients recovered from coronavirus disease 2019.

Material and methods: Sexually active men with previous severe acute respiratory syndrome coronavirus 2 infection and proven recovery from coronavirus disease 2019 were enrolled in a prospective cohort study. Clinical, uro-andrological data and semen specimens were prospectively collected. For previously hospitalized coronavirus disease 2019 patients, data on serum inflammatory markers were retrospectively collected.

Results: A total of 43 men were enrolled in the study. Of these, 32 men were normozoospermic, three were oligozoospermic, and eight were crypto-azoospermic. Serum inflammatory markers (procalcitonin and C-reactive protein) were analyzed in previously hospitalized patients both at admission and at peak of infection. Levels at admission were statistically significantly higher in patients resulting in crypto-azoospermic with respect to those resulting in normozoospermic ($p = 0.05$; $p = 0.03$ and $p = 0.02$,

respectively) after healing. Seminal cytokine levels were similar among all groups. Interleukin-1β and tumor necrosis factor-α levels were significantly negatively related to sperm total number and concentration, whereas interleukin-4 was correlated with sperm motility.

Discussion and conclusion: Negative correlations between interleukin-1β and tumor necrosis factor-α and sperm number and the overall high levels of semen cytokines indicate a potential detrimental role of severe acute respiratory syndrome coronavirus 2 driven inflammation on spermatogenesis. Overall, our results indicate that male patients recovering from coronavirus disease 2019 deserve accurate follow-up for their fertility status.

KEYWORDS
COVID-19, cytokines, inflammation, men, SARS-CoV-2

1 | INTRODUCTION

The pandemic coronavirus disease 2019 (COVID-19), due to a novel virus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), caused one-hundred-millions of infections all over the word in the very first year of the outbreak.[1] The SARS-CoV-2 virus resulted more contagious and infected a higher percentage of young men during the second and third pandemic wave, as compared with the first one.[2] Angiotensin-converting enzyme 2 (ACE2) receptor is the primary receptor mediating the entry of SARS-CoV-2 into human cells. ACE2 receptor is present in spermatogonia, seminiferous tubules, Sertoli, and Leydig cells and is highly expressed in cell lines derived from male genitourinary epithelium of fertile young men.[3–5] After SARS-CoV-2 infection, changes in ACE2 signaling pathways can induce severe oxidative stress and inflammation that may cause significant damage to the male reproductive tract.[6] Therefore, clinical evidence of real COVID-19 impact on male reproductive function, even in the long term, is greatly advocated.[7]

In the last decade, new biomarkers of male genital tract inflammation have been reported as the most promising putative markers of infection.[8] We previously reported that pathological levels of interleukin (IL)-8 were present in semen from 33/43 of men after recovery from COVID-19.[9] Moreover, semen levels of IL-8 were related to the severity of illness (hospitalization and need for oxygen therapy or invasive ventilation)[9] suggesting an impairment of genital apparatus during the viraemic spread.

The high levels of semen IL-8 in the seminal fluid of COVID-19 patients suggest a direct involvement of genito-urinary epithelium in the inflammatory process. In fact, IL-8 production precedes the infiltration of inflammatory cells[9,10] and promotes natural killer (NK) cell recruitment and function.[11,13]

Increasing evidence on the impact of SARS-CoV-2 infection on semen parameters is reported.[9] However, a longitudinal analysis of semen collected by patients after COVID-19 recovery is still lacking.

To improve the knowledge of COVID-19 impact on male reproduction, we analyzed a panel of semen cytokines in COVID-19 patients after an average of 35 days of recovery from the infection.

2 | METHODS

2.1 | Study design and population

A prospective cohort study was designed following the Strengthening the Reporting of Observational Studies in Epidemiology guidelines. Due to the sudden and unexpected spread of the COVID-19 pandemic, no previous knowledge was present on the topic at the initial study design. Therefore, no a priori power analysis and sample dimension calculation were possible.

Male patients with previous SARS-CoV-2 infection and subsequent proven recovery from COVID-19 (two consecutive negative NP swabs for SARS-CoV-2 ribonucleic acid) were screened for the present study. Besides male gender, inclusion criteria were: age between 18 and 65 years, active sexual life, and capability to express informed consent to participate in the current study. Exclusion criteria were ejaculatory disorders or refusal to participate.

The current study was approved by the Institutional Review Board of AOUC – Careggi Hospital, Florence Italy in June 2020, under code 17104. Subsequently, it was registered in clinicaltrials.gov with the identifier NCT04446169. It was designed and conducted according to the Declaration of Helsinki. All enrolled participants signed informed consent.

2.2 | Data recording and specimen collection

Clinical data and semen specimen collection were recorded according to the previously reported scheme.[9] Values of serum inflammatory markers (white blood cell count, procalcitonin, and C-reactive protein)

were retrospectively recorded for hospitalized patients at the time of admission, peak, and discharge.

Semen obtained with masturbation was collected in sterile jars. After liquefaction of semen and assessment of volume, semen samples were analyzed.

2.3 | Immunoplex assay and semen analysis

Detection and quantification of cytokines in semen were performed using the Milliplex Map kit Human Cytokine/Chemokine/Growth Factor Panel A Magnetic Bead Panel (Merck Kgaa, Darmstadt, Germany) following the manufacturer's protocols. The plate was analyzed with the Luminex 200 MAGPIX. The limit of sensitivity (MinDc = minimum detectable concentration) and the linear range of detection for the analyzed cytokines are reported in Table S1. Data were analyzed using a 5-parameter logistic curve-fitting method for calculating analyte concentrations in samples.

Semen collection was done after a period of abstinence 2–7 days prior to the collection was requested to all the enrolled patients, according to World Health Organization recommendations. Semen analysis was performed as reported.[9] Quantification of semen leukocytes was done by counting the number of round cells/ml with an improved Neubauer hemocytometer and evaluating the percentage of leukocytes and immature germ cells after May-Grunwald staining of the examined specimen. All analyses were performed by the same team of our academic center.

2.4 | Statistics

Patients' features were reported using medians and interquartile ranges (IQRs) for continuous variables and numbers and percentages for categorical variables. Patients were subsequently divided into different groups for comparisons according to their semen parameters or hospitalization. All analyses were age-adjusted. Differences were tested with the Kruskal-Wallis H test and Wilcoxon test for continuous variables whenever appropriate and with chi-square and Fisher's Exact tests for categorical variables according to sample dimension. Spearman test was applied to explore correlations between investigated variables. All statistical analyses were performed using IBM SPSS version 20.0 (SPSS Inc, Chicago, IL, USA). Statistical significance was set with a p-value < 0.05 and all statistical tests were two-tailed.

3 | RESULTS

3.1 | Patients' characteristics and clinical features

Of 160 male patients with a double-negative nasopharyngeal swab after SARS-CoV-2 infection invited to participate in the study, 43 men accepted and were enrolled (Figure S1).

Patients' clinical characteristics, including COVID-19 clinically relevant data, uro-andrological questionnaires, and seminal cytokines levels are summarized in Table 1. As expected, age was the main determinant of clinical management of COVID-19, with the youngest men (mean age: 44 years) not hospitalized as compared to the middle-aged men (mean age: 52 years) hospitalized, and elderly ones (mean age: 59 years) needing intensive care.

In our previous study, we found that, at semen analysis carried out on average 35 (IQR: 24–43) days after healing, 32 men were normozoospermic, three were oligozoospermic, and eight were crypto-azoospermic.[9] In addition, semen impairment was directly related to hospitalization requirement and severity of disease ($p < 0.01$).[9] Fifty percent of the recruited patients, admitted to the intensive care unit (ICU) due to COVID-19 complications, were crypto-azoospermic.

3.2 | Serum inflammatory markers at hospitalization

Data on serum inflammatory markers (white blood cells count, procalcitonin, and C-reactive protein), were retrospectively collected from 31 (72.1%) hospitalized patients, but data on procalcitonin were available only in 11 of them (25.6%). The values at admission, peak, and discharge, plus their decreasing time are shown in Table 2. Procalcitonin at admission and C-reactive protein at admission and at the peak were statistically significantly higher in men resulting in crypto-azoospermic after healing compared to normozoospermic ($p = 0.05$; $p = 0.03$ and $p = 0.02$, respectively).

Other values, including white blood cell count at peak, white blood cell count decreasing time, and C-reactive protein decreasing time were slightly above statistical significance and showed higher values in crypto-azoospermic men compared to normozoospermic. A statistically significant negative correlation was found between procalcitonin values at admission and sperm concentration (Rho −0.738, $p < 0.01$) and between sperm total number and procalcitonin at admission (Rho −0.672, $p < 0.01$), and C-reactive protein at admission (Rho −0.360, $p = 0.04$) and at peak (Rho −0.411, $p = 0.03$) (Figure 1).

3.3 | Semen cytokines

The levels of IL-8, IL-4, IL-6, IL-17, tumor necrosis factor α (TNF-α), and IL-1β were measured in the seminal fluid of all patients. All cytokines were remarkably above the cut-off levels[13] in all semen samples (Figure 2). Significant differences among the groups were revealed only for IL-1β which was significantly higher in the group of crypto-azoospermic patients ($p = 0.01$) (Table 3). IL-8 values were statistically significantly higher in patients with severe COVID-19 infections requiring ICU recovery ($p = 0.01$). Both IL-17 and TNF-α showed a similar trend, without reaching statistical significance (Figure 3).

IL-1β and TNF-α values were negatively related to sperm total number (Rho −0.443, $p < 0.01$ and Rho −0.362, $p = 0.03$), respectively)

TABLE 1 Patients' clinical characteristics, globally and comparison according to semen analysis at enrollment

		Total ($n = 43$, 100%)	Normozoospermic ($n = 32$, 74.4%)	Oligozoospermic ($n = 3$, 7%)	Crypto-azoospermic ($n = 8$, 18.6%)	p
Age (years)	51 (45–58)	50 (44–57)	58 (43–60)	59 (53–63)	0.10	
BMI (kg/m^2)	26.3 (23.6–29.2)	26.2 (23.8–30.0)	23 (21.9–23.3)	27.5 (27.0–29.4)	0.06	
Smoking	Never	35 (81.4%)	27 (84.4%)	3 (100%)	5 (62.5%)	0.59
	Current	3 (7.0%)	2 (6.3%)	0	1 (12.5%)	
	Former	5 (11.6%)	3 (9.4%)	0	2 (25.0%)	
Period between first positive-oroparhyngeal swab and healing from COVID-19 (days)		31 (22–40)	32 (22–40)	31 (25–45)	33 (21–43)	0.99
Hospitalization for COVID-19	No	12 (27.9%)	11 (34.4%)	0	1 (12.5%)	**<0.01**
	Clinical ward	26 (60.5%)	20 (62.5%)	3 (100%)	3 (37.5%)	
	ICU	5 (11.6%)	1 (3.1%)	0	4 (50.0%)	
Uro-andrologic questionnaires	IPSS	4 (2–8)	3 (1–7)	5 (2–9)	7 (5–9)	0.20
	NIH-CPSI	0 (0–2)	0 (0–2)	0 (0–2)	1 (0–4)	0.78
	MSHQ-EJD	24 (22–25)	25 (22-25)	23 (15–24)	22 (22–24)	0.70

Abbreviations: BMI, body mass index; ICU, intensive care unit; IPSS, international prostate symptom score; NIH-CPSI, National Institute of Health-chronic prostatitis symptom index; MSHQ:EjD, male sexual health questionnaire - ejaculatory dysfunction.
All values are expressed as median (interquartile range) or *n* (%). Statistical analyses are Kruskal-Wallis H test and Fisher's Exact test accordingly.

TABLE 2 Patients' inflammatory markers during admission for coronavirus disease 2019 (COVID-19) infection, globally and according to semen analysis

	Total ($n = 31$)	Normozoospermic ($n = 21$, 67.7%)	Oligozoospermic ($n = 3$, 9.7%)	Crypto-azoospermic ($n = 7$, 22.6%)	p
WBC at admission (1000/ml)	5.5 (5.1–7.6)	5.5 (5.1–7.4)	5.4 (5.4–10.1)	7.3 (3.5–8.4)	0.84
WBC at peak (1000/ml)	7.4 (6.0–12.6)	6.7 (6.0–11.2)	5.4 (5.1–10.1)	12.8 (7.7–18.3)	0.08
WBC at discharge (1000/ml)	5.7 (4.4–6.5)	6.0 (4.9–6.6)	4.6 (4.1–5.6)	4.7 (3.0–6.7)	0.12
WBC decreasing time (days)	16 (8–21)	17 (11–21)	4 (2–4)	16 (8–22)	0.07
Procalcitonin at admission (mg/ml)	0.16 (0.10–0.35)	0.12 (0.07–0.18)	NA	0.33 (0.26–0.36)	**0.05**
Procalcitonin at peak (mg/ml)	0.15 (0.10–0.42)	0.14 (0.10–0.17)	0.10 (0.08–0.12)	0.42 (0.36–0.56)	0.16
Procalcitonin at discharge (mg/ml)	0.08 (0.04–0.12)	0.08 (0.04–0.12)	0.18 (0.18–0.18)	0.07 (0.03–0.10)	0.47
Procalcitonin decreasing time (days)	19 (11–24)	19 (11–23)	NA	22 (14–31)	0.48
C-reactive protein at admission (mg/ml)	79 (28–120)	56 (13–93)	58 (31–120)	142 (86–158)	**0.03**
C-reactive protein at peak (mg/ml)	79 (44–142)	56 (18–106)	58 (38–120)	206 (134–291)	**0.02**
C-reactive protein at discharge (mg/ml)	12 (4–39)	9 (4–39)	17 (12–38)	0 (0–43)	0.46
C-reactive protein decreasing time (days)	17 (8–24)	17 (12–24)	4 (4–4)	22 (8–27)	0.07

WBC = white blood cells.
All values are expressed as median (interquartile range) or *n* (%). Statistical analyses are Kruskal-Wallis H test and Fisher's Exact test accordingly.

and with sperm concentration (Rho −0.414, $p = 0.01$ and Rho −0.372, $p = 0.02$, respectively) (Figure S2). In contrast, IL-4 levels were significantly positively correlated with sperm motility (Rho +0.346, $p = 0.05$).

A trend of positive correlation which however did not reach the statistical significance was also present between IL-4 and sperm total number.

4 | DISCUSSION

Infection of male genital reproductive apparatus has been reported in the course of numerous bacterial and viral infections including COVID-19.[14] We report here that high levels of inflammatory cytokines are present in the semen of men recently (median time, 35 days) recovered

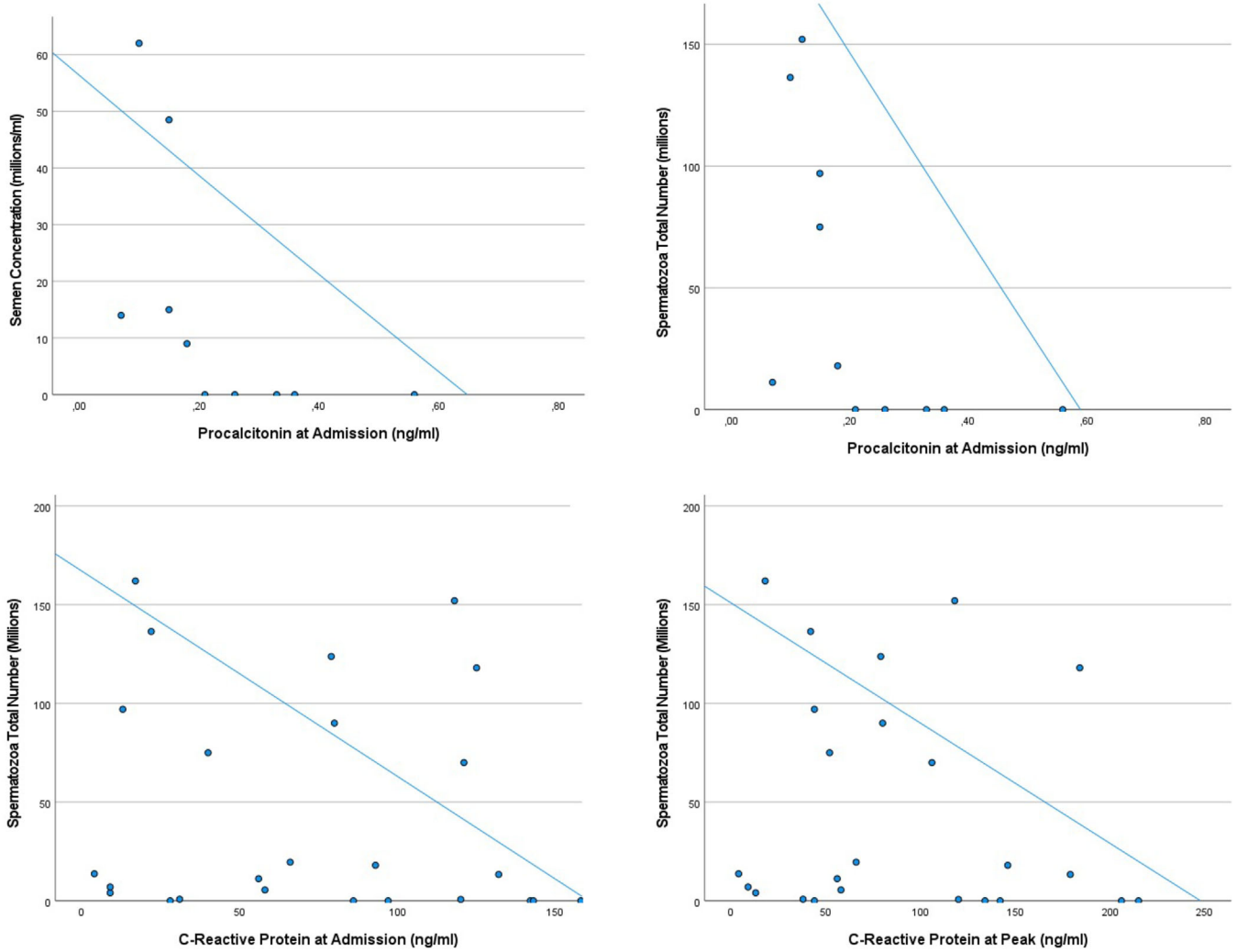

FIGURE 1 Correlation of procalcitonin and C-reactive protein values at admission with sperm concentration and sperm total number

from COVID-19, indicating the presence of an inflammatory condition in the male genital tract.

Viruses typically reach the male reproductive organs through hematogenous spread. The blood-testis barrier protects germ cells in most infections. However, some viruses acquire the property to cross the barrier and, in some cases, can infect testicular cells and/or induce an immune-inflammatory response within the testis.[7,15] Fifteen percent of mumps-infected males undergo bilateral orchitis with severe impairment of testicular function.[16] HIV and Zika viruses have been detected in the semen and can lead to orchitis.[17] Immune-inflammatory response against virally infected cells is largely mediated by macrophages, T cells, NK cells, and to a lesser extent, by B cells. These cells release pro-inflammatory cytokines (e.g., IL-17, IL-15, IL-8, and interferon-γ) which in turn increase the expression of several growth factors including fibroblast growth factor (FGF) and FGF-2, resulting in abnormal proliferation of either epithelial and stromal cells. The increased oxygen due to cell proliferation often leads to local hypoxia producing low levels of reactive oxygen species promoting angiogenesis and the production of additional growth factors (i.e., vascular endothelial growth factor, IL-8, FGF-2, FGF-7, and transforming growth factor ß).[14]

Spermatogenesis impairment was observed in different clinical conditions, including autoimmune orchitis, and it has been repeatedly associated with the increased concentration of inflammatory cytokines in seminal plasma.

IL-6 and TNF-α, in particular, were involved in the damage of the blood-testis barrier.[18–20] IL-8 was found significantly increased in patients with prostatitis.[13]

Patients with severe COVID-19 frequently undergo sepsis[21] and the presence of high viral load in the blood is supposed to facilitate dissemination in different organs and apparatuses including the male reproductive tract and, rarely, semen.[22,23] Several molecular mechanisms may be involved in testicular damage in severe COVID-19 patients. A high concentration of IL-6 is likely involved in the damage of the blood-testis barrier. The viral load may directly induce the testicular damage[24] or indirectly through the immune response, either innate

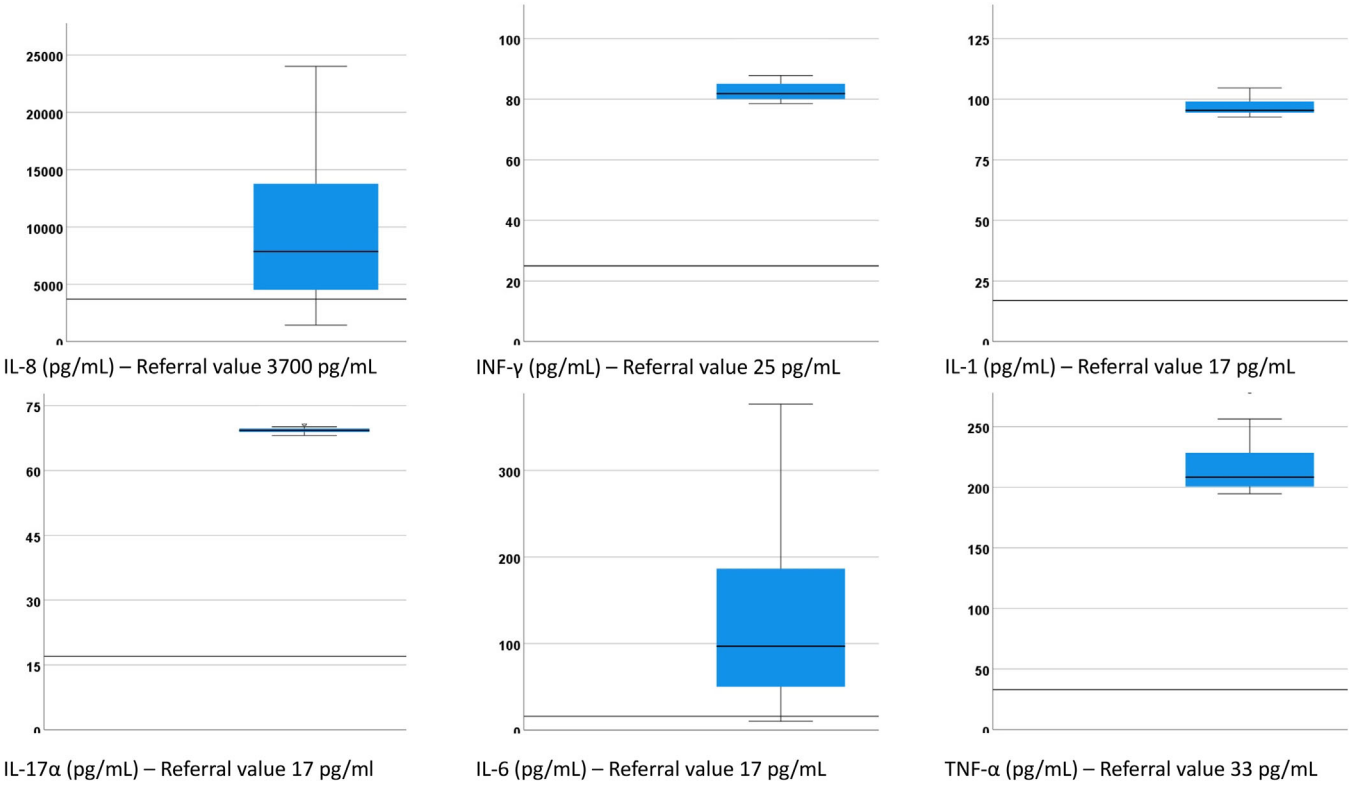

FIGURE 2 The level of interleukin (IL)-8, IL-4, IL-6, IL-17, tumor necrosis factor α (TNF-α), and IL-1β in the seminal fluid of all patients

TABLE 3 Seminal chemokines globally and comparison according to semen analysis at enrollment

	Total ($n = 31$)	Normozoospermic ($n = 21$, 67.7%)	Oligozoospermic ($n = 3$, 9.7%)	Crypto-azoospermic ($n = 7$, 22.6%)	p
IL-8 (pg/ml)	7860 (4412–14,400)	7670 (3757–10,942)	6720 (4680–19,190)	13123 (7046–32,806)	0.21
IL-1 (pg/ml)	95 (94–99)	95 (94–97)	96 (96–96)	116 (103–160)	0.01
IL-4 (pg/ml)	18 (17–18)	18 (17–18)	18 (17–18)	18 (18–19)	0.76
IL-6 (pg/ml)	97 (50–186)	88 (45–186)	111 (90–132)	164 (141–204)	0.49
IL-17 (pg/ml)	69 (69–70)	69 (69–70)	69 (69–69)	70 (70–70)	0.35
TNF-α (pg/ml)	208 (201–228)	206 (200–219)	245 (211–279)	246 (208–329)	0.09
INF-γ (pg/ml)	82 (80–85)	82 (80–86)	82 (81–83)	82 (79–84)	0.86

Abbreviations: IL, interleukin; INF-γ, interferon-γ; TNF-α, tumor necrosis factor-α .
All values are expressed as median (interquartile range) or n (%). Statistical analyses are Kruskal-Wallis H test and Fisher's Exact test accordingly.

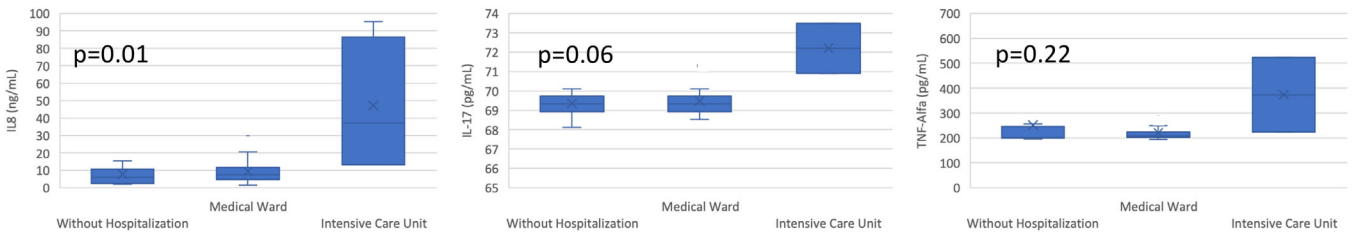

FIGURE 3 Interleukin (IL)-8, IL-17, and TNF-α values in patients divided according to the severity of coronavirus disease 2019 (COVID-19) infections. The lines and "X" represent the median and the average of the sample, respectively

or adaptive, activated to fight against the virus.[25] In addition, the testicular damage can be due to the high local temperatures reached during viral dissemination.[26,27]

C-reactive protein levels were found high in all patients during the disease and were still high after the recovery. Oligo-zoospermia and crypto-azoospermia, found in three and eight patients, respectively, after COVID-19 recovery, were significantly associated with the concentration of plasmatic C-reactive protein measured at admission time and during the peak of the disease.

Systemic inflammatory markers are usually poorly related to semen quality, as suggested by men with metabolic syndrome, which yields higher C-reactive protein blood values compared to healthy subjects without altering substantially semen quality.[28] Similarly, procalcitonin appears to be present both in seminal plasma and in blood serum in case of male urogenital tract infections, but with its seminal levels comparable to healthy men. In our cohort of COVID-19 recovered men, procalcitonin and C-reactive protein, both at admission and the peak, were statistically significantly higher in crypto-azoospermic patients compared to normozoospermic patients ($p = 0.05$; $p = 0.03$ and $p = 0.02$, respectively) suggesting a possible involvement of these markers in testicular impairment.

Although the occurrence of the SARS-CoV-2 genome in seminal fluid appears to be a rare event,[9] high levels of pro-inflammatory cytokines were detected in all semen samples from post-COVID-19 patients. Cytokine concentrations were more than 2–3-fold higher than those found in control subjects (age >18 years) in pre-COVID age (2014) from our group, using the same detection technology.[13]

The levels of seminal IL-8 and, to a lesser extent, of IL-17, were associated with disease severity and were significantly higher in ICU requiring patients compared to other groups suggesting that an inflammatory process is present in the genital tract. Fifty percent of the patients admitted to ICU due to COVID-19 complications were also crypto-azoospermic[9] and testicular damage was significantly correlated with signs of systemic inflammation. Thus, our data may suggest that the impairment of testicular function could be a consequence of systemic inflammation following viral dissemination. Whether such inflammatory status persists after healing is presently unknown and further studies are required to address such eventuality.

High inflammatory infiltration was found in the testis of patients who succumbed to COVID-19, suggesting alterations of testicular cell function, including Leydig cells leading to reduced testosterone production.[29] SARS-CoV genome is 79% homologous to that of SARS-CoV2 and the two viruses utilize the same ACE2-receptor to entry in the cells. Thus, it is conceivable to hypothesize that inflammation-mediated disruptions of testicular cells, including the Leydig cells, also occur during SARS-CoV infection.

The high concentrations of TNF-α, IL-17, and IFN-γ detected in the seminal fluid of patients recovered from COVID-19 indicate that, in addition to innate immunity, Th-1 and Th-17 responses are activated in the upper genital tract as a response to SARS-CoV-2 infection. Activated immune cells infiltrated in the male genital tract may also trigger autoimmune responses following the exposure of hidden antigens, and develop antibodies against the germ cells as reported for patients who died for SARS-CoV infection.[29]

In addition, our data confirm that IL-4 could have a protective effect on the testicular function of COVID-19 patients, as previously reported.[30]

Although novel, our results should be evaluated considering the study design and limitations. First, this is a prospective study including a small cohort of COVID-19 recovered patients with no control group. Besides, in light of the small study sample and the requested active participation, our results could be influenced by selection bias due to several issues, such as education, employment, the desire to have children, or andrological diseases. In addition, we have no data about the semen quality and semen inflammatory markers of enrolled patients before SARS-CoV-2 infection. Moreover, we analyzed data from a median follow-up time of 35 after recovery that this is a rather short time, and further studies to evaluate the long-term impact of covid-19 infection on semen quality are needed. Another limitation of the study might be found in the low number of patients with available serum procalcitonin data, which may affect the statistical power of this part of the study, and might represent a selection bias. In addition, even if TNF-α was statistically significant, the results are affected by levels out of scale (above 280 pg/ml) found in three patients. According to the previous limitations, some of the data presented should be interpreted cautiously. Finally, hormone levels were not assessed in our study. Nevertheless, although several limitations, our study represents the largest population analyzed in current literature with a full panel of seminal inflammatory markers.

5 | CONCLUSION

According to our initial results, patients with coronavirus disease 2019 infections might have high levels of pro-inflammatory cytokines in seminal plasma, in particular in patients with severe impairment of semen parameters, thus suggesting an impact of inflammatory processes on testicular function. Further studies are warranted to evaluate whether such a condition persists after a longer period from recovery as well as to clarify severe acute respiratory syndrome coronavirus 2 mechanisms of action on the male genital tract.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest related to this article.

AUTHOR CONTRIBUTIONS

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Data processing - *Taking responsibility in the execution of data management and reporting*: S. Morselli

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Critical review - *Reviewing the article before submission not only for spelling and grammar but also for its intellectual content*: M. Gacci, S. Serni, E. Baldi, F. Annunziato, M. Maggi, L. Vignozzi, A. Bartoloni, G. M. Rossolini, and A. Antonelli

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