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Determinants of prolonged viral RNA shedding in hospitalized patients with SARS-CoV-2 infection

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ABSTRACT

Objective: To evaluate determinants of prolonged viral RNA shedding in hospitalized patients with SARS-CoV-2 infection. *Materials and methods:* Hospitalized patients with SARS-CoV-2 positive nasopharyngeal RT-PCR were included in a single-center, retrospective study. Patients were divided in 2 groups according to the timing of viral clearance [≤14 days, "early clearance (EC)" and >14 days, "late clearance (LC)"]. *Results:* 179 patients were included in the study (101 EC, 78 LC), with median age 62 years. Median time of viral shedding was 14 days (EC/LC 10 and 19 days, respectively, $P < 0.0001$). Univariate analyses showed that age, male gender, receiving corticosteroids, receiving tocilizumab, ICU admission, low albumin and NLR ratio were associated with late viral clearance. In the multivariable analysis, older age ($P = 0.016$), albumin level ($P = 0.048$), corticosteroids ($P = 0.021$), and tocilizumab ($P = 0.015$) were significantly associated with late viral clearance. *Conclusions:* Age, albumin, tocilizumab and corticosteroid treatment were independently associated with a prolonged SARS-CoV-2 RNA shedding.

1. Introduction

The novel β-coronavirus emerged in Wuhan, China, in December 2019 and it has rapidly spread through the world. This novel virus was named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) due to the similarity with the virus that caused the SARS outbreak in the 2002–2004. Coronavirus Virus Disease 2019 (COVID-19) was then declared a pandemic by the World Health Organization (WHO) on March 11, 2020 (World Health Organization, 2020) and up to October 9, it has infected over 36 million people globally and resulted in more than 1 million deaths (Johns Hopkins University, 2020). At the time of writing, there is still no effective antiviral treatment or vaccine for SARS-CoV-2 and therefore prevention of community transmission and disease control are the only tools to reduce the global spread. Detection of viral RNA by reverse transcriptase-polymerase chain reaction (RT-PCR) is the gold standard for COVID-19 diagnosis and this technique is used in the test-based strategy to release patients.

Understanding the kinetics of infectious viral shedding in relation to possible transmission risk is crucial to guide infection prevention and control strategies (Anderson et al., 2004), even if the correlation between infectivity and viral persistence is still unclear (Widders et al., 2020). Long-term shedding of viral RNA has been reported in COVID-19 patients putting serious problems on timely discharge from hospital or home isolation (Wölfel et al., 2020, Zhang et al., 2020) in order to mitigate the risk of secondary community transmission. Although this topic is currently under consideration in SARS-CoV-2 infection (Kampen et al., 2021), only few studies investigating factors possibly influencing the viral shedding duration have been performed so far, with conflicting results. Disease severity, age, sex, and treatment have been correlated with the timing of viral persistence, but no general key determinants have been clearly demonstrated to prolong the viral shedding (Kampen et al., 2021, Carmo et al., 2020, Shi et al., 2020, Ling et al., 2020, Xu et al., 2020).

Hence, it is of urgent need to elucidate the determinants of viral shedding duration among patients with COVID-19 in order to optimize the public health policy about the discharge strategies and to achieve a rational use of laboratory resource establishing an adequate time of the viral clearance.

Based on these premises, a retrospective, single-center study was conducted with the aim of identifying clinical and/or laboratory determinants associated with prolonged viral persistence in the nasopharyngeal samples of patients with SARS-CoV-2 infection.

2. Materials and method

2.1. Study population

Over a 2-month period (March-May 2020), a retrospective single-center study including all adult patients with diagnosis of SARS-CoV-2

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infection and hospitalized at Azienda Policlinico Umberto I, Sapienza University of Rome was performed. Nasopharyngeal swab samples were collected and SARS-CoV-2 RNA was detected by using real time RT-PCR assay (RealStar SARS-CoV2 RT-PCR, Altona Diagnostics). Following logistical and clinical needs, other molecular methods were used (GeneFinder COVID-19 Plus RealAmp Kit, Elitech; DiaSorin Molecular Simplexa COVID-19 Direct EUA assay, DiaSorin Molecular; Xpert Xpress SARS-CoV-2 assay, Cepheid). All tests and procedures were performed following the manufacturers' protocols. Definition of pneumonia or severe pneumonia was based on the WHO interim guidance and included clinical signs of pneumonia (fever, cough, dyspnea, fast breathing) with or without signs of severe pneumonia such as respiratory rate >30 breaths/min, severe respiratory distress, or SpO2 <90% on room air (World Health Organization, 2020). For each subject, laboratory, clinical and radiological data at hospital admission, as well as treatment data and SARS-CoV-2 RNA time of detection were collected and recorded anonymously in an electronic database. As for treatment data, antiviral therapy as well as the choice of using tocilizumab or steroids was based on the national, regional and local guidelines available at the time (Focà et al., 2020, Codeluppi et al., 2020) and on clinical judgement. In particular, corticosteroids were used in patients with moderate or severe Acute Respiratory Distress Syndrome (respiratory failure with PaO2/FiO2 <200 mm Hg or worsening of respiratory conditions during hospitalization or at ICU admission or during ICU stay). Tocilizumab was used in patients with COVID-19 pneumonia and PaO2/FiO2 <300 mm Hg. Specific contraindications for the use of tocilizumab were hypersensitivity to tocilizumab, concomitant treatment with anti-rejection drugs, concomitant active infections, neutrophils level <500 cell/mL, platelets <50.000 cell/mL, alanine/aspartate aminotransferases >5-fold the normal value, intestinal perforation. The study was approved by the local Ethics Committee (ID Prot. 109/2020).

Time of viral shedding was defined as the number of days from the first viral detection by RT-PCR on nasopharyngeal specimen until the first of 2 consecutive negative results within 24 hours. Patients with a first negative sample followed by a positive nasopharyngeal specimen within 24 hours were excluded from the study. According to the duration of viral shedding, the study population was further divided in 2 groups: "early clearance (EC)" group (median ≤14 days) and "late clearance (LC)" group (median >14 days).

2.2. Statistical analysis

The data, unless otherwise stated, were given as medians with interquartile ranges (IQR, 25th−75th percentile) for continuous variables and as simple frequencies, proportions, and percentages for categorical variables. Mann−Whitney test was used for unpaired samples. Dichotomous variables were compared using Fisher's exact tests or χ^2 test statistics, as appropriate. Log-rank test and univariate Cox regression were used for categorical or continuous variables, respectively. Multivariable Cox regression model was performed to tease out the independent predictors of prolonged viral persistence. *P*-value analyses were two-sided and a *P*-value of less than 0.05 was considered statistically significant. All statistical analyses were performed with SATA/IC software (StataCorp) version 15.

3. Results

3.1. Study population

Demographic and clinical characteristics of the patients are shown in Table 1. Overall, 179 patients (103, 57.5% males; 76, 42.5%, females) were included in the study, 101 and 78 patients belonging to EC and LC groups, respectively. Median age was 62 years (interquartile range [IQR] 54−73), with LC group showing an elder population and a prevalence of male gender compared to EC group (65.5 [56.7−78] vs 59 [50−71] years and 48 [61.5%] vs 55 [54.4%] males, $P = 0.011$ and $P = 0.363$, respectively). The median time from symptoms onset to first viral detection was 7 days (4−9.9), whereas the median time of viral shedding was 14 days (9−19, range 2−45 days), with a median time of 10 days (7.75−12) and 19 days (17−24) in the EC and LC groups, respectively ($P < 0.0001$). Overall, 124 (69.3%) patients presented with at least one underlying disease, with no significant difference between the two groups. Among laboratory findings, albumin values were significantly lower in the LC group [3.7 g/L (3.4−4) vs 3.9 (3.5−4.2), $P = 0.023$]. All patients received antiviral therapy with hydroxychloroquine and/or protease inhibitor (162/179, 90.5%, and 67/179, 37.4%, respectively) whereas azithromycin and teicoplanin were given in 81 of 179 (45.2%) and 23 of 179 (12.8%) subjects, respectively. Again, no significant differences as for the antiviral treatment between the 2 groups were observed, with the exception of teicoplanin use which was more frequent in the LC group (17/78, 21.7%, vs 6/101, 5.94%, $P = 0.0027$) (Ceccarelli et al., 2020).

A total of 39 patients (21.8%) received corticosteroids treatment [30 (16.7%) methylprednisolone, 9 (5%) dexamethasone], with the LC group significantly associated with the use of corticosteroid [24/78, (30.8%) vs 15/101, (14.85%), $P = 0.017$]. Tocilizumab was given in 77 patients (43.0%), with LC showing higher percentage of use than EC [45/78 (57.7%) vs 32/101 (31.7%) respectively, $P = 0.008$]. There were no differences on time to initiate tocilizumab or corticosteroids between the EC and LC patients, with therapy started within 10 days from hospital admission in both groups. Median duration of therapy was 7 days (IQR 7−8), with no differences between EC and LC groups. No significant correlation was found between duration of therapy and duration of viral positivity ($P = 0.12$, $r = 0.26$). Patients with a prolonged viral RNA detection showed a greater disease severity, with a total of 21 subjects transferred to the ICU (EC 7 [6.9%] vs LC 14 [17.9%], $P = 0.033$). Overall mortality was 2.8% (5/179), with no differences among EC and LC groups (2 [1.9%] vs 3 [3.8%], $P = 0.65$). Mortality rates were similar in patients receiving or not tocilizumab or corticosteroids (3/77 (3.9%) vs 2/102 [1.9%], $P = 0.65$ and 1/39 [2.5%] vs 4/140 [2.8%], $P = 0.9$, respectively).

3.2. Predictors of prolonged viral shedding

Severity of disease (expressed by ICU transfer), receiving tocilizumab or steroids as a part of infection treatment, male sex, age, albumin level, and NLR ratio were associated with persistence of viral positivity at nasopharyngeal specimen (Fig. 1). In the multivariable analysis, age ($P = 0.016$), albumin level ($P = 0.048$), receiving corticosteroids ($P = 0.021$), and tocilizumab ($P = 0.015$) were significantly associated with late viral clearance (Table 2).

4. Discussion

Despite the multitude of studies published during the ongoing COVID-19 pandemic, knowledge about factors possibly influencing prolonged viral shedding is still limited. In this study, it was found that age, albumin levels and receiving corticosteroids or tocilizumab were independently associated with SARS-CoV-2 RNA shedding.

Consistent with previous findings, we observed that median SARS-CoV-2 RNA shedding in upper respiratory specimens was 14 days (IQR 9-19) (Hu et al., 2020), although a wide variability among different studies was observed, reflecting the heterogeneity of populations (Gombar et al., 2020, Cevik et al., 2021, Agarwal et al., 2020).

The main finding of the present research is the association between tocilizumab therapy and the duration of viral shedding. To our knowledge, no studies focusing on this aspect have been published so far. Tocilizumab, an interleukin-6 (IL-6) inhibitor with anti-inflammatory properties, has been used for the treatment of SARS-CoV-2 infection with the aim of reducing and controlling the cytokine storm present during the infection. In fact, during severe SARS-CoV-2 infection pathogenic T-cells and inflammatory monocytes incite

Table 1
Demographic and clinical characteristics of the patients.

Characteristics	TOTAL n. (%) 179 (100)	EC n. (%) 101 (56.4)	LC n. (%) 78 (43.6)	*P value*
Demographics				
Age, years, median (IQR)	62 (54-73)	59 (50-71)	65.5 (57-77.7)	0.011
Gender, males/females (*n*)	103/76	55/46	48/30	0.363
Comorbidities, *n*. (%)				
Patients with at least one underlying disease	124 (69.3)	61 (60.3)	63 (80.8)	0.0035
Hypertension	88 (49.2)	42 (41.6)	46 (59)	0.147
Vascular disease	38 (21.2)	18 (17.8)	20 (25.6)	0.268
Diabetes	35 (19.6)	16 (15.8)	19 (24.4)	0.184
Coronary artery disease	24 (13.4)	12 (11.9)	12 (15.4)	0.514
Chronic obstructive pulmonary disease	23 (12.8)	10 (9.9)	13 (16.7)	0.259
Cancer [a]	22 (12.3)	11 (10.9)	11 (14.1)	0.503
Heart failure	16 (8.9)	8 (7.9)	8 (10.3)	0.607
Autoimmune disease	13 (7,5)	6 (5.9)	7 (9)	0.563
Cerebral disease	10 (5.6)	5 (5)	5 (6.4)	0.749
Asthma	9 (5)	6 (5.9)	3 (3.8)	0.734
Hepatic disease	7 (3.9)	5 (5)	2 (2.6)	0.468
Immune suppression[b]	6 (2.8)	2 (2)	4 (5.1)	0.406
Virological status, median (IQR)				
Days from symptoms onset to first positive swab	7 (4-9.8)	7 (4.2-9.7)	6.9 (3.9-10.4)	0.672
Days of viral shedding	14 (9-19)	10 (7.75-12)	19 (17-24)	<0.0001
Laboratory findings at the admission, median (IQR)				
White blood cells, × 10∧6/L	5675 (4383-7103)	5560 (4178-6663)	5865 (4543-7345)	0.177
Neutroohils, × 10∧6/L	4055 (2873-5745)	3790 (2748- 5003)	4415 (3330-6538)	0.011
Lymphocytes, × 10∧6/L	820 (630-1282)	875 (640-1193)	790 (585-1250)	0.36
Neutrophil-to-lymphocyte ratio, n	4.51 (2.90-7.28)	4.32 (2.71-6.55)	5.246 (3.27-9.19)	0.014
Albumin, g/L	3.8 (3.4-4.1)	3.9 (3.5-4.2)	3.7 (3.4-4)	0.023
D-dimer, μg/L	918 (501-1640)	759 (466-1269)	1217 (527-2838)	0.003
Lactate dehydrogenase, UI/L	291 (236-378)	288 (236-370)	301 (240-402)	0.560
C-Reactive Protein, mg/dl	3,66 (1.19-10.05)	3.13 (0.83-9.03)	5.63 (1.62-14.03)	0.023
Clinical features, *n*. (%)				
Fever	166 (92.7)	95 (94.1)	71 (91)	0.401
Cough	103 (57.5)	57 (56.4)	46 (59)	0.880
Dyspnea	92 (51.4)	52 (51.5)	40 (51.3)	0.466
Fatigue	39 (21.8)	29 (28.7)	10 (12.8)	0.011
Dysgeusia/anosmia	27 (15.1)	16 (15.8)	11 (14.1)	0.834
Diarrhea	24 (13.4)	12 (11.9)	12 (15.4)	0.518
Headache	4 (2,2)	2 (2)	2 (2.6)	0.999
Treatment, *n*. (%)				
Corticosteroids	39 (21.8)	15 (14.8)	24 (30.8)	0.017
Tocilizumab	77 (43)	32 (31.7)	45 (57.7)	0.008
Hydroxychloroquine	162 (90.5)	92 (91.1)	70 (89.7)	0.800
Azithromycin	81 (45.2)	42 (41.6)	39 (50)	0.100
Protease inhibitors	67 (37.4)	40 (39.6)	27 (34.6)	0.53
Teicoplanin	23 (12.8)	6 (5.9)	17 (21.8)	0.0027
Duration of therapy, days, median (IQR)	7 (7-8)	7 (6-7)	7 (7-8)	0.872
Outcomes, n.(%)				
Transfer to Intensive Care Unit	21 (11.7)	7 (6.9)	14 (17.9)	0.033
Mortality	5 (2.8)	2 (2)	3 (3.8)	0.65

EC = Early-clearance; LC = Late-clearance;
[a] cancer in the last 5 years;
[b] Immune suppression was defined as the presence of congenital or acquired immunodeficiency or the receipt of chronic immunosuppressant drugs for organ transplantation.

inflammatory response with large amount of IL-6 production (Fu et al., 2020). Furthermore, in rheumatoid arthritis patients tocilizumab was shown to induce a significant reduction in the peripheral memory B cell, with a decline of serum immunoglobulin levels (Roll et al., 2011), which might have a role in slowering viral clearance during the course of COVID-19. Therefore, the interference of the drug with the immune system might possibly explain this phenomenon. Nevertheless, further studies are needed to understand the immunological mechanisms involved in the SARS-CoV-2 viral clearance and to reveal how blocking IL-6 receptor is associated to a late viral shedding.

Similarly, receiving corticosteroids resulted to be an independent factor associated with viral persistence. This finding is consistent with other recent observational studies of patients with COVID-19 (Ling et al., 2020, Wang et al., 2020), confirming past reports that showed a link between corticosteroids therapy and a prolonged viral shedding in different viral infections, such as seasonal influenza, SARS and MERS (Lee et al., 2009, Arabi et al., 2018, Lee et al., 2004). The corticosteroid immune modulatory action and the impairment of T-cell immunity might be the reasons explaining this finding. However, the effect of corticosteroids on duration of viral shedding is still controversial, with few studies no confirming this association (Shi et al., 2020, Zha et al., 2020, Yan et al., 2020). These conflicting data could be explained by the heterogeneity of study populations and, possibly, by the different types and dosages of corticosteroids, suggesting the need of additional investigations. This is especially true considering that recent clinical trials clearly demonstrated the advantage of using corticosteroids during SARS-CoV-2 infection (Horby et al., 2020). Therefore, with the expectation of an increasing use of steroids for COVID-19, understanding the relation between steroids and viral shedding appears to be crucial.

Although the aim of the study was to find factors associated with a prolonged viral shedding and not to assess whether tocilizumab or corticosteroids were beneficial for patients, we also analysed mortality rates in patients receiving or not tocilizumab or corticosteroids and we found no statistical differences.

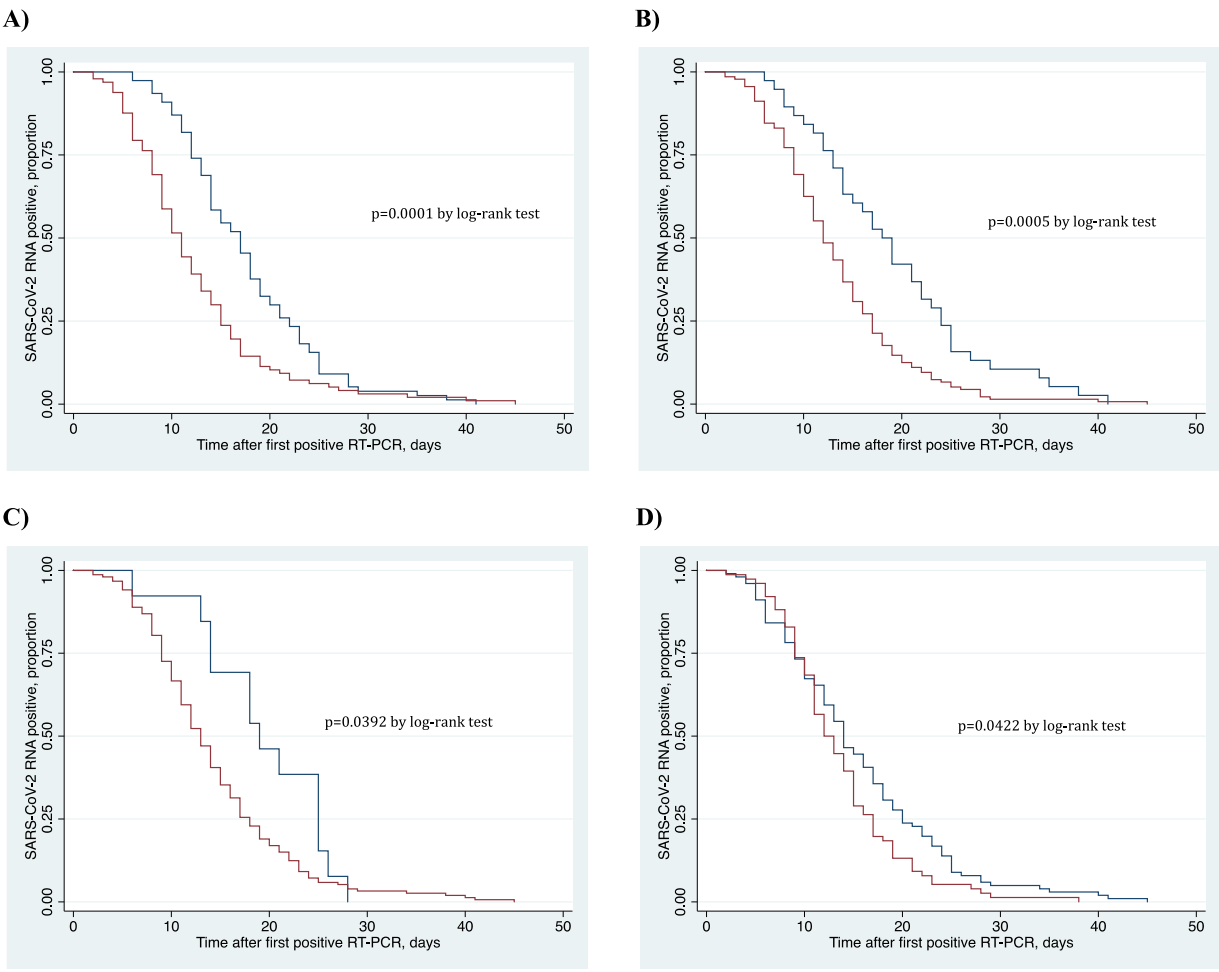

Fig. 1. Cumulative proportion of patients with detectable SARS-CoV-2 RNA by day after first positive RT-PCR between patients who received tocilizumab (blue line) and patients who did not received tocilizumab (red line) (A), between patients who received steroids (blue line) and patients who did not received steroids (red line) (B), between patients who were transferred to ICU (blue line) and patients who were not transferred to ICU (red line) (C) and between male (blue line) and female patients (red line) (D). ICU = Intensive Care Unit; RT-PCR = Reverse Transcriptase-Polymerase Chain Reaction. (Colored version of figure is available online.)

Another interesting result of the present research is that albumin might possess a role in determining the duration of viral shedding, further highlighting the increasing attention toward this molecule in the setting of SARS-CoV-2 infection. In fact, not only hypoalbuminemia was associated with worse outcome and coagulopathy (Violi et al., 2020, Violi et al., 2020), but also a recent report showed that patients with decreased albumin levels experienced prolonged viral RNA shedding, confirming what observed by our group (Fu et al., 2020). The antioxidant and anti-inflammatory properties of albumin as well as its ability to down-regulate the expression in the cells of the human angiotensin-converting enzyme 2 receptors (Taverna et al., 2013, Liu et al., 2009) might explain this finding.

Table 2
Factors associated with time of viral clearance at multivariable Cox regression analysis.

Determinants	Multivariable analysis		
	HR	CI95%	*P-value*
Age	0.98	0.97-0.99	**0.016**
Sex	1.33	0.94-1.89	0.104
Tocilizumab	1.51	1.08-2.12	**0.015**
Steroids	1.59	1.07-2.36	**0.021**
ICU transfer	1.46	0.75-2.83	0.261
Albumin	1.02	1.00-1.05	**0.048**
NLR	0.99	0.95-1.03	0.940

ICU = Intensive Care Unit; NLR = Neutrophils/Lymphocytes Ratio; HR = Hazard ratio.

Age was another independent factor associated with RNA persistence in nasopharyngeal samples. Apart from being a well-known determinant of poor prognosis in COVID-19 (Zhou et al., 2020), previous studies have also demonstrated that elderly is associated with late SARS and SARS-CoV-2 negativity (Hu et al., 2020, Cevik et al., 2021, Wang et al., 2020, Liu et al., 2004). One possible explanation could be the age-dependent dysfunction of innate and adaptive immune response that could lead older patients to a more difficult pathogen eradication, thus predisposing to a prolonged viral shedding (Opal et al., 2005). Although age is commonly associated with comorbidities, in our study the presence of comorbidity did not have an influence on RNA persistence, suggesting that age per se might influence the viral shedding (Xu et al., 2020, Wang et al., 2020, Liu et al., 2020).

In contrast to recent studies, in the present report neither sex nor disease severity, expressed as ICU transfer, were factors independently associated with viral persistence. Male gender has been linked with more severe symptoms, higher mortality rate and a more prolonged shedding than female in SARS-CoV-2 infection (Shi et al., 2020, Xu et al., 2020, Zhou et al., 2020). The specific mechanism of sex-related difference in SARS-CoV-2 infection is unclear; however, the underlying mechanisms may be related to sex-hormones, which affect different components of the immune system (Bouman et al., 2005), and to the effect of gender on the expression of ACE-2 (Clotet-Freixas et al., 2018, Gupte et al., 2012). Therefore, being aware that sex might have an important role in influencing SARS-CoV-2 RNA shedding, we

strongly believe that additional studies with higher number of patients are warranted.

Similar conflicting data could be found in the literature regarding the impact of infection severity on duration of viral positivity. In fact, a significant difference of viral shedding time in ICU and non-ICU patients has been described (Kampen van et al., 2021, Shi et al., 2020, Fang et al., 2020), whereas a recent paper (Carmo et al., 2020) surprisingly showed that mild nonhospitalized cases presented a longer duration of viral shedding than inpatients.

Although the use of antiviral drugs might hypothetically lead to an early viral clearance, as it was shown for lopinavir/ritonavir (Yan et al., 2020, Fu et al., 2020, Zuo et al., 2020), we did not find a relation between antivirals and duration of viral shedding, in agreement with Shi et al (2020) and Hraiech et al (2020).

This study has some limitations. In fact, RT-PCR for SARS-CoV-2 RNA on nasopharyngeal swab in the absence of viral cultures does not discriminate between viable and nonviable viruses. Therefore, detection by RT-PCR represents only a surrogate marker for infectivity, making the great dilemma, "shedding vs infectivity" more and more actual (Widders et al., 2020). Unfortunately, we were not able to perform viral load quantification during the first phase of pandemic. We acknowledge that this might represent an additional limit of the present investigation and that further studies are needed to test whether viral load at admission and thereafter could be responsible for the prolonged viral shedding. Then, having data on RT-PCR only at the time of hospitalization rather than immediately from symptoms onset might have underestimated the time needed for viral clearance.

5. Conclusion

Age, albumin levels, and receiving corticosteroids or tocilizumab represent key determinants of prolonged SARS-CoV-2 RNA shedding. A better understanding of viral kinetics might be useful in the hospital clinical management in order to early identify patients at risk of prolonged viral shedding and to provide crucial insights into a rational use of laboratory resource.

Declarations of interest

The authors report no conflicts of interest relevant to this article.

Contribution of authors

CDF, OA: conceptualization; CDF, OA, CF, SG, VS, MV, RG: data collection; CM, TO: diagnostic analyses; OA, CDF: writing; MCM, VM: critical review of the manuscript.

Author statement

All authors revised and approved the revised version of the manuscript.

Data availability statement

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

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