Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Letters to the Editor

Quantifying the importance of the key sites on haemagglutinin in determining the selection advantage of influenza virus: Using A/H3N2 as an example

Dear Editor,

The recurrence of seasonal influenza epidemics is attributed to the continuous evolution of influenza viruses, which enables them to change pathogenicity and escape from human adaptive immunity.[1, 2] The selection advantage of influenza virus is largely contributed by a few key amino acids (AA) substitutions on the surface glycoprotein haemagglutinin (HA).[2–5] Identifying and measuring the importance of these key sites are crucial in understanding patterns of influenza activities. There is a growing need for efficient computational methods to characterize the contribution of AA sites in the evolution process. Here, we use an analytical framework to quantify the selection advantage associated with key AA sites in the HA gene, and we use influenza A/H3N2 sequence data in the USA from 2005 to 2019 for demonstration (Supplementary Material S1).

Referring to previous studies on H3N2[5, 6], the key HA AA sites were selected by identifying the sites with AA substitutions that were statistically related to the prevalence of H3N2 in the population (Supplementary Material S2), namely the effective mutations (EM) sites. The loci of 43 EM sites, out of 566 AA sites on HA glycoproteins are shown in Fig. 1. We employed information entropy to measure the stochasticity of the information stored in each AA site (Supplementary Material S3). As shown in Table 1, the site-wise information entropy of the EM site was higher than that of the nonEM sites (Supplementary Material S5), and the discriminability of entropy on distinguishing EM or nonEM sites was high in terms of the AUC > 0.97.

The dissimilarity tree was constructed by using the neighbour-joining method with the pairwise distances matrix calculated by Jones-Taylor-Thornton AA modelling framework.[7] The contribution of all EM sites as a whole, in comparing to all nonEM sites, was measured by the optimal weight (or proportion, ranged from 0 to 1) of EM sites, namely the optimal EM weight, associated with the most stable and likely phylogenetic relationships (Supplementary Material S4). The changing dynamics of the dissimilarity tree against the EM weight are presented in Fig. 2. Then, we quantified the relative contribution of EM sites by using the odds ratio (OR) of the optimal weight against the null hypothesis of baseline proportion, i.e., $43 / 566 = 0.076$. Hence, the OR was interpreted as the ratio of contribution to positive selection by the EM sites versus the nonEM sites, which also infers the association between the EM sites and observed phylogeny. As shown in Table 1, we found that EM sites had a high determination on the selection advantage of the HA glycoproteins with OR estimates ranging from 8.9 to 22.4 (Supplementary Material S6 and S7).

Since the selection of EM sites considers both mutation dynamics and population immune response[5, 6], the high OR indicates that the AA changes in the EM sites are more likely associated with generating long-term selection advantage. Our finding reaffirms that the selection advantage of the influenza virus is likely driven by a few key HA AA sites. This data-driven computational framework can be extended to quantify the importance of the key sites in other pathogens.

Authors' contributions

SZ and MHW conceived the study. SZ carried out the analysis, and drafted the first manuscript. SZ and MHW discussed the results. All authors read, revised the manuscript, and gave final approval for publication.

Declaration of Competing Interest

MHW is a shareholder of Beth Bioinformatics Co., Ltd, and BCYZ is a shareholder of Beth Bioinformatics Co., Ltd and Health View Bioanalytics Ltd.

Declarations

Ethics approval and consent to participate

The ethical approval or individual consent was not applicable.

Availability of data and materials

All influenza viruses sequence data were collected via the influenza virus database (IVD) of the National center for Biotechnology Information (NCBI). Please see the online supporting information for details.

Consent for publication

Not applicable.

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Table 1
The summary table of the estimated odds ratio (OR), statistics of information entropy and AUC of using entropy as a classifier of EM sites.

Region	Num. of strains	Information entropy				OR based on		
		nonEM site (nat)	EM site (nat)	AUC	OR (per 0.1 nat)	rank in tree	num. of ancestors in tree	log-likelihood of tree
California	383	0.02 ± 0.04, (0.00–0.16), [0.00–0.31]	0.48 ± 0.24, (0.08–0.99), [0.08–1.05]	0.993	8.99 (4.94–16.33)	17.08 (11.25–28.38)	15.64 (7.85–34.55)	8.92 (8.07–12.56)
New York	335	0.02 ± 0.04, (0.00–0.13), [0.00–0.30]	0.54 ± 0.23, (0.14–0.95), [0.11–1.07]	0.997	11.04 (5.36–22.74)	17.46 (12.11–26.86)	21.41 (12.78–33.22)	10.28 (9.40–13.61)
Texas	303	0.02 ± 0.05, (0.00–0.16), [0.00–0.48]	0.49 ± 0.26, (0.05–1.07), [0.00–1.15]	0.975	4.33 (3.11–6.03)	17.07 (12.61–26.95)	22.37 (10.05–59.93)	13.02 (13.01–14.87)

Note: The site-specific entropies are summarised as in 'mean ± SD, (IQR), [range]' form. The ORs are summarised as in 'estimate (95%CI)' form.

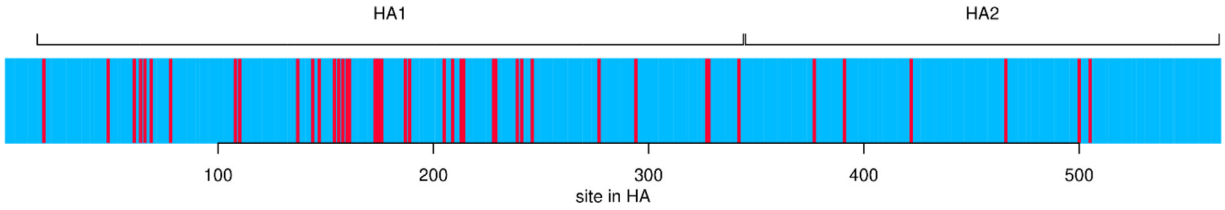

Fig. 1. The effective mutations (EM) sites (in red) and nonEM sites (in blue) on the HA glycoprotein of A/H3N2 influenza virus.

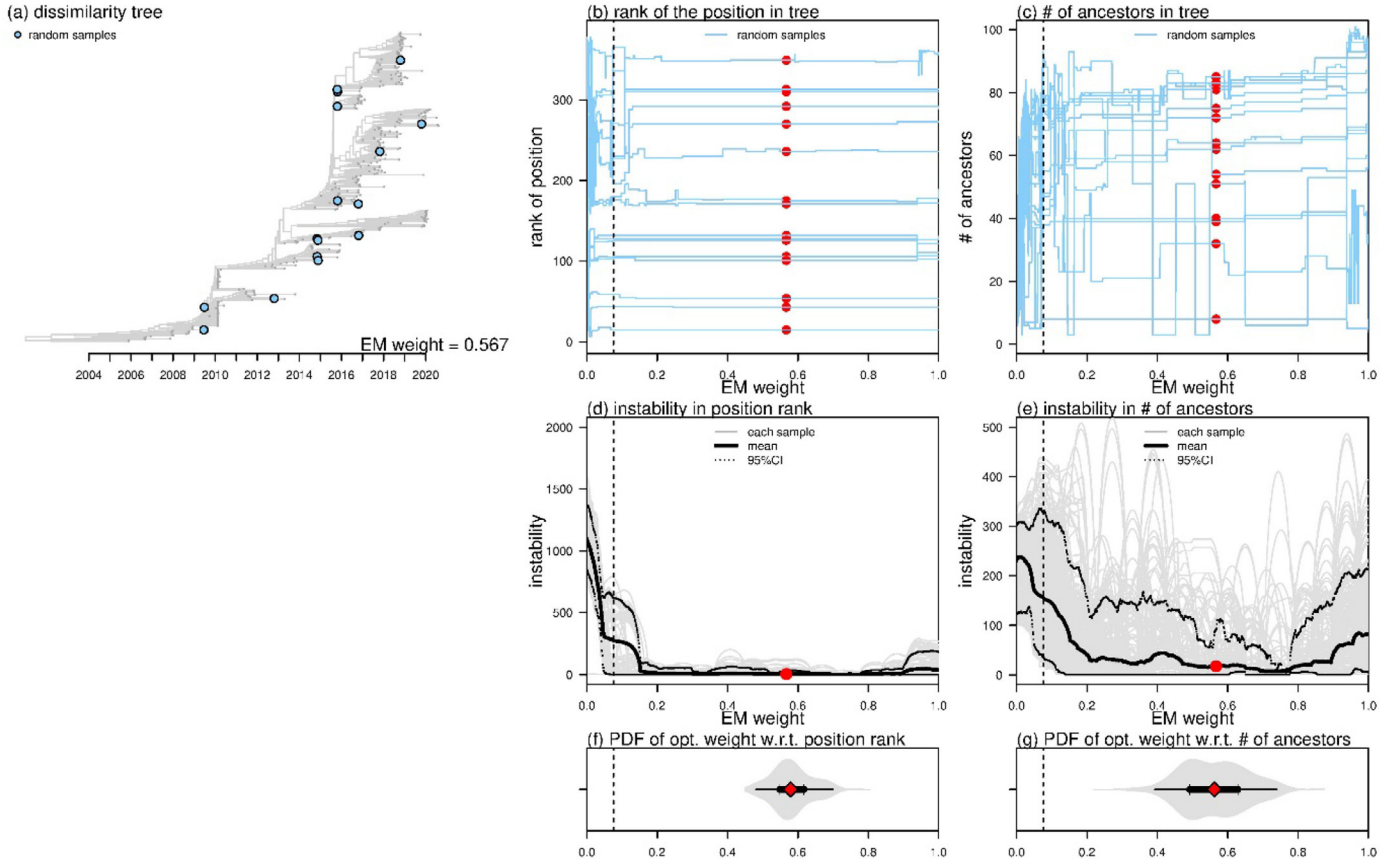

Fig. 2. The dissimilarity tree of sequences in California (CA) state, the USA and the generated instability metrics. Panel (a) show the constructed dissimilarity tree. The tree is horizontally rescaled in calendar date scale. The blue dots are the randomly selected samples of AA sequences for illustration purpose only. Panels (b) and (c) show the trajectories of the randomly selected samples by using rank in a ladderized dissimilarity tree and number of ancestors respectively. Panels (d) and (e) show the levels of instability of all AA sequences by using rank in dissimilarity tree and number of ancestors respectively. The grey curves represent the instabilities of all AA sequences. The black bold curve is the mean of the instabilities of all AA sequences, and the black dotted curves are the 95% centile. The red dot indicates the lowest instability given by the optimal weight averaged from all sequences for EM sites. Panels (f) and (g) show the optimal EM weight by using rank in dissimilarity tree and number of ancestors respectively.

Disclaimer

The funding agencies had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; or decision to submit the manuscript for publication.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2020.05.066.

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High SARS-CoV-2 infection rates in respiratory staff nurses and correlation of COVID-19 symptom patterns with PCR positivity and relative viral loads

We read with interest the study in the Journal by Chen and colleagues from Nanjing, China that demonstrated a high positivity rate (17%) in healthcare workers (HCWs),[1] but did not attempt to breakdown which type of HCWs working in which specialties had the highest infection rates. Similarly, another study from Leicester, UK compared hospitalised and community patient SARS-CoV-2 PCR (polymerase chain reaction) positivity rates with that of staff,[2] but again, did not assess which staff groups or clinical specialties were at most risk of acquiring COVID-19. Finally, one other study from Wuhan, China described the clinical features of HCWs infected with COVID-19, but again did not analyze the staff most infected by role or specialty.[3]

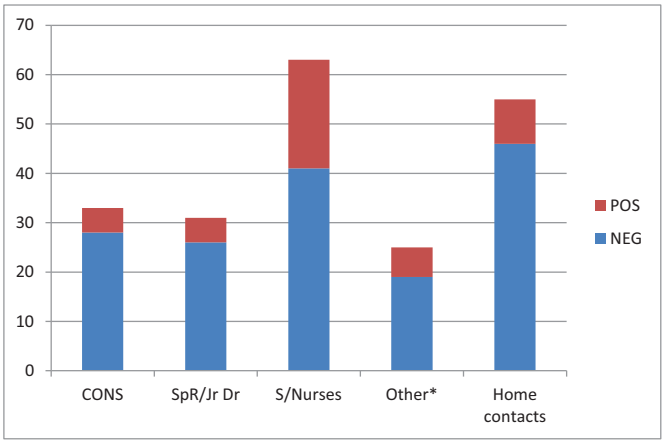
Here we present an analysis by role and specialty of symptomatic HCWs and their household contacts (total $n = 207$) that presented for SARS-CoV-2 PCR testing, during the early part of the UK COVID-19 epidemic, between 17 March and 4 May 2020. During this period, the recommendations from Public Health England (PHE) were for any symptomatic HCW to self-isolate for at least 7 days, or for any individual (including HCWs) with symptomatic household contacts to self-quarantine for 14 days.[4] To give some additional context, the UK went into lockdown on 23 March 2020,[5] and all HCWs were required to wear some form of surgical mask or better in clinical areas on 26 March 2020.[6]

Healthcare workers (mean age: 38.2 years, s.d. 9.2, range 17–60 years) also presented with symptomatic household family members (mean age: 23.8 years, s.d. 16.5, range 2–45 years) for swabbing. The rationale for this at the time was that if neither the family contacts, nor the HCW were SARS-CoV-2 PCR positive, then the HCW could return to work earlier without being a SARS-CoV-2 risk to other HCWs or patients.

During this 7 week period, a total of 152 symptomatic and/or self-isolating HCWs (54 male: 98 female) with 55 home contacts (including spouses and children) presented for swabbing (a single combined nasal/throat swab). Of the 152 HCWs, 6 were Black, 99 were Asian, and 47 were of White ethnicity. The Ausdiagnostics SARS-CoV-2 PCR assay was used for this testing. This kit has a manufacturer's reported sensitivity of 97–98% and a specificity of 99–100%, which has been confirmed elsewhere.[7]

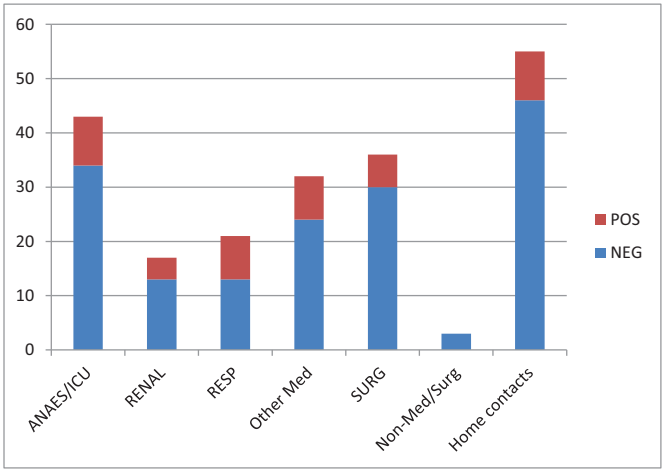
The results (Fig. 1A) showed that the highest SARS-CoV-2 PCR positive rate (34.9%) was found in staff nurses compared to those (15–16%) for junior doctors, consultants and other support staff

A



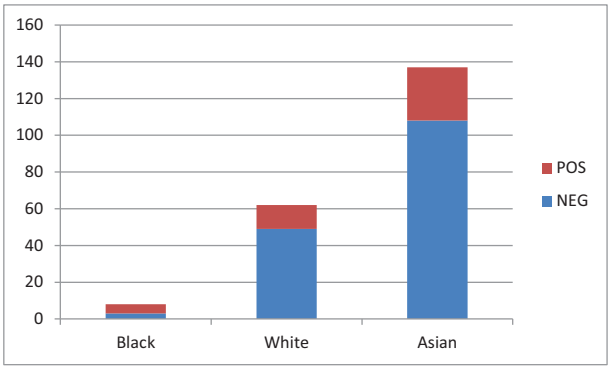
	Total	NEG	NEG (%)	POS	POS(%)
STAFF					
CONS	33	28	84.9	5	15.1
SpR/Jr Dr	31	26	83.9	5	16.1
S/Nurses	63	41	65.1	22	34.9
Other*	25	19	76	6	24
Home contacts	55	46	83.6	9	16.4
Totals	207	160		47	

B



	Total	NEG	NEG (%)	POS	POS(%)
STAFF					
ANAES/ICU	43	34	79.1	9	20.9
RENAL	17	13	76.5	4	23.5
RESP	21	13	61.9	8	38.1
Other Med	38	27	71.1	11	28.9
SURG	30	24	80	6	20
Non-Med/Surg	3	3	100	0	0
Home contacts	55	46	83.6	9	16.4
Totals	207	160		47	

C



Ethnicity	Total	NEG	NEG%	POS	POS%
Black	8	3	37.50	5	62.50
White	62	49	79.03	13	20.97
Asian	137	108	78.83	29	21.17
Totals	207	160		47	

Fig. 1. **(A)** Comparing positive and negative SARS-CoV-2 PCR results in frontline staff by role. **(B)** Comparing positive and negative SARS-CoV-2 PCR results in frontline staff by clinical specialty. **(C)** Comparing positive and negative SARS-CoV-2 PCR results in HCWs and household contacts by ethnicity.

(24%, including healthcare assistants and those based in operating theatres, administration and estates). Of note, in this cohort, the home contact SARS-CoV-2 PCR positivity rate (16.4%) was very similar to those in the non-staff nurse HCWs, at this stage of the COVID-19 epidemic in this cohort.

Fig. 1B illustrates the SARS-CoV-2 positivity rate in HCWs working in different clinical specialties, with those working in respiratory wards showing the highest positive rate (38.1%), followed by other medical specialties (28.9%), particularly the renal dialysis wards (23.5%), the adult intensive care unit (ICU) and anaesthetics (20.9%). The latter two specialties had a lot of overlap, with many anaesthetists also covering ICU, so these HCW totals were combined and plotted together.

These findings may not be entirely surprising as most suspected COVID-19 patients would be referred initially to the respiratory teams for assessment, and staff nurses are likely to spend more time with the patients on a more frequent basis, taking and recording bedside observations, administering drugs, and being the first HCWs on-site for any patient complications.

In addition, we compared the SARS-CoV-2 PCR positivity rate against ethnicity for both the HCW and household contacts combined (Fig. 1C). This showed a high 62.5% ($n = 8$) positivity rate for

Table 1
Characteristics of 207 subjects stratified by SARS-CoV-2 PCR test results.

	Number of PCR POS cases (%) $N=47$	Number of PCR NEG cases (%) $N=160$	p-value[c]
Age			
0–18	2 (4.3)	27 (16.9)	0.07
19–34	14 (29.8)	56 (35.0)	
35–44	18 (38.3)	49 (30.7)	
45 or above	13 (27.7)	28 (17.5)	
Sex			
Female	29 (61.7)	92 (57.5)	0.73
Male	18 (38.3)	68 (42.5)	
Ethnicity			
White	13 (27.7)	49 (30.7)	0.02
Asian	29 (61.7)	108 (67.5)	
Black	5 (10.6)	3 (1.9)	
Number of systemic symptoms [a]			
0	10 (21.3)	65 (40.6)	0.36
1	13 (27.7)	43 (26.9)	
2	6 (12.8)	36 (22.5)	
3–4	18 (38.3)	16 (10.0)	
Number of respiratory symptoms [b]			
0	6 (12.8)	45 (28.1)	<0.001
1	21 (44.7)	59 (36.9)	
2	13 (27.6)	39 (24.4)	
3–5	7 (14.9)	17 (10.6)	
Duration from symptom onset to specimen collection (days)			
Mean (SD)	6.13 (5.28)	NA	NA

[a] Systemic symptoms refer to any of: fever, headache, myalgia or fatigue.
[b] Respiratory symptoms refer to any of: cough, sore throat, shortness of breath or chest tightness/pain.
[c] Chi-squared test to test the null hypothesis of independence between two categorical variables.

Black individuals, though there were very few cases; and a similar 21.2% ($n=137$) and 21.0% ($n=62$) SARS-CoV-2 PCR positivity rate for Asian and White individuals, respectively. These numbers are small and were obtained from the early part of the COVID-19 epidemic in the UK so it is not possible to recognize any specifically higher incidence of SARS-COV-2 infection in any of these BAME (Black, Asian, Minority Ethnic) groups.[8]

We then compared the SARS-CoV-2 PCR positivity rates against various demographic parameters, ethnicity and symptom patterns in both the HCWs and household contacts ($n=47$, Table 1). This showed that most of these positive cases were female ($n=29$, 61.7%), Asian ($n=29$, 61.7%), and aged 35 years or above ($n=31$, 66.0%), with at least 1 systemic symptom (i.e. any of: fever, headache, myalgia or fatigue; $n=37$, 78.7%); and at least 1 respiratory symptom (i.e. any of: cough, sore throat, shortness of breath or chest tightness/pain, $n=41$, 87.2%). The mean duration from illness onset to specimen collection in these 47 SARS-CoV-2 PCR positive cases was 6.13 (s.d. 5.28) days.

An additional analysis was performed on the PCR positive cases where their sample Ct (cycle threshold) values were available ($n=39$), using the same parameter categories as Table 1. This compared the Ct value (a relative indicator of viral load) against presenting symptom patterns. After adjusting for age, ethnicity, sex and time from symptom onset to specimen collection, it was found that the number of respiratory symptoms were positively associated with greater Ct values (i.e. lower viral loads, $p< 0.01$), while an increasing number of systemic symptoms was associated significantly with smaller Ct values (i.e. higher viral loads, $p< 0.01$) (**Table S1**). The mean duration from illness onset to specimen collection in these 39 SARS-CoV-2 PCR positive cases was 5.67 (s.d. 4.58) days.

This suggests that in this cohort, systemic symptoms may be more closely associated with the presence of higher viral loads, whereas respiratory symptoms may be more immune-mediated and can continue to persist during viral clearance. Other studies have found varying associations between symptom patterns, illness severity and viral loads,[9,10] so additional studies are needed to understand better these host-virus interactions.

In summary, we have compared the SARS-CoV-2 PCR positivity rates in this HCW and household contact cohort, across different clinical roles and specialties, ethnic groups, and explored the correlation between their symptom patterns and swab viral loads. Additional work is required to clarify further the relationships between these various parameters, such that dose-response monitoring can be applied in a rational manner when proven antiviral therapies for COVID-19 eventually become available.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2020.06.035.

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Low transmission risk of 9 asymptomatic carriers tested positive for both SARS-CoV-2 nucleic acid and serum IgG

Dear Editor,

We read with interest a recent prospective contact-tracing study in this Journal by Huang et al.[1], who reported a rapid asymptomatic transmission of COVID-19 during the incubation period demonstrating strong infectivity in a cluster of youngsters aged 16–23 years outside Wuhan. The COVID-19 pandemic has affected millions of lives worldwide partly due to its high contagiousness.[2,3] Thus far, the growing amount of evidence suggests that asymptomatic carriers are capable of transmitting SARS-CoV-2.[4,5] Here we report clinical characteristics of nine confirmed COVID-19 asymptomatic carriers, who were tested positive both for SARS-CoV-2-specific nucleic acid and IgG, but appeared to be unable to transmit the virus.

Of 4973 SARS-CoV-2 nucleic acid positive results in Union hospital, Wuhan throughout the COVID-19 outbreak (from January 25 to May 14, 2020), we found nine asymptomatic carriers who were tested positive both for SARS-CoV-2-specific nucleic acid and IgG between Mar 28 and May 12, 2020. The SARS-CoV-2 nucleic acid was tested using throat swab specimens through real-time PCR (RT-PCR) to amplify the ORF1ab gene and N gene of SARS-CoV-2 (BioGerm, Shanghai, China). The primers for ORF1ab gene and corresponding detecting products: forward primer 5′-CCCTGTGGGTTTTACACTTAA-3′, reverse primer 5′-ACGATTGTGCATCAGCTGA-3′, fluorescence probe 5′-FAM-CCGTCTGCGGTATGTGGAAAGGTTATGC-BHQ1-3′; for N gene and corresponding detecting products, forward primer 5′-GGGGAACTTCTCCTGCTAGAAT-3′, reverse primer 5′-CAGACATTTTGCTCTCAAGCTG-3′, fluorescence probe 5′-FAM-TTGCTGCTGCTTGACAGATT-TAMRA-3′. If the cycle threshold (Ct) value $<$ 35 (or $>$ 35 but less than 40 for two times), the specimens are defined as positive. Specific IgG against SARS-CoV-2 was detected using commercially available kit (#C86095G, YHLO biotechnology Co., LTD, Shenzhen, China) in an iFlash 3000 chemiluminescent immune analyzer (YHLO biotechnology Co., LTD, Shenzhen, China) through chemiluminescent microparticle immunoassay. This study was reviewed and approved by the Medical Ethical Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Nine confirmed asymptomatic carriers who were tested positive for both SARS-CoV-2-specific nucleic acid and IgG (Patient 1–3 positive for IgG and IgM, and the others positive for IgG but negative for IgM) were retrospectively analyzed (Fig. 1). These patients were enrolled from outpatient population or general population requesting regular physical check-up. They have an average age of 47y (range18–77y), five of which were male and four were female. Only one of them had comorbidity (Patient 8 with hypertension and diabetes). None of them received chest CT probably because of their lack of COVID-19 associated symptoms (Table 1). Two patients (Patient 1 and 3) were subject to in-hospital quarantine for 29 days, and then discharged because SARS-CoV-2 nucleic acid testing turned negative; the others remained quarantined in hospitals at the time of follow-up on May 12, 2020. The average Ct value of SARS-CoV-2nucleic acid testing in nine patients were 36.42 ± 2.06 (ORF1ab gene, range 31.27–38.89) and 34.87 ± 3.73 (N gene, range 27.30–39.47). From the moment of Wuhan City lockdown (January 23, 2020) to the time of being admitted for quarantine, all these patients had lived together with their family members for an average of 85 days (range 65–104 days). All these patients developed no symptoms associated with COVID-19 during their quarantine. Of note, none of the patients' close contacts (including all their family members who stayed with them) were RT-PCR tested positive for SARS-CoV-2, indicating they are not infected by these asymptomatic carriers despite of close contact.

We observed a small group of asymptomatic carriers (positive for both SARS-CoV-2-specific nucleic acid and IgG) with very low transmission risk. Our observation is consistent with a recent report suggesting that low transmission risk in asymptomatic patients.[6] Such a low transmission risk might be partly associated with relatively high Ct values. This is in accordance with the observation that compared with severe COVID-19 cases, significantly lower viral loads were found in mild COVID-19 patients.[7] The other possible reasons for low transmission risk: (1) the infectivity of the virus might be decreased in these confirmed asymptomatic carriers; (2) viral shedding might be ineffective without relevant symptoms, such as sneezing and coughing, thus reducing the risk of infections for close contacts.[8]

The analysis of this group of patients suggests that some asymptomatic carriers can be tested positive for SARS-CoV-2-specific nucleic acid and serum IgG, but they are of low transmission risk, which is different from previously-reported asymptomatic carriers[5], suggesting a new pattern may have emerged in SARS-CoV-2 infected patients and changes associated with viral load and virus infectivity. These have not been reported thus far.

However, close monitoring of the asymptomatic patients is still required. This work was limited in several aspects: (1) the sample size is limited ($n = 9$); (2) further investigation such as virus isolation and culture would strengthen the study, but we are unable to provide the results at this point, given current limited medical resources and urgent situations. The findings of this work may provide useful information for helping improve understanding and management of asymptomatic carriers and facilitating restoration of normal medical services after COVID-19 pandemic.

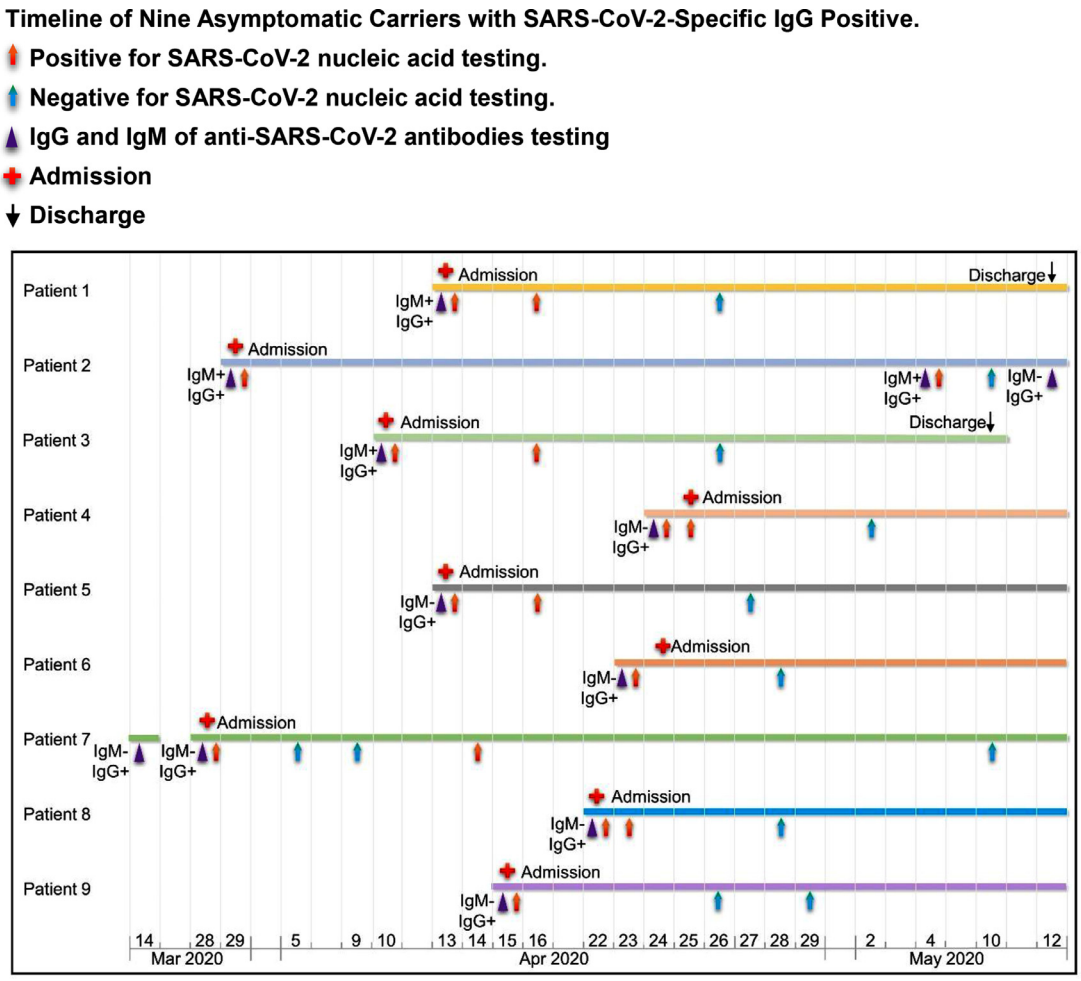

Fig. 1. Timeline of nine asymptomatic carriers with SARS-CoV-2-specific IgG positive.

Table 1
Characteristics of nine asymptomatic carriers with SARS-CoV-2-specific IgG positive.

Sex	Patient 1 Male	Patient 2 Female	Patient 3 Female	Patient 4 Male	Patient 5 Male	Patient 6 Female	Patient 7 Female	Patient 8 Male	Patient 9 Male
Age	58y	56y	52y	42y	30y	18y	55y	77y	38y
Underlying diseases	No	No	No	No	No	No	No	Hypertension, diabetes	No
Initial symptoms associated with COVID-19	No	No	No	No	No	No	No	No	No
CT evidence of pneumonia	NA	NA	NA	NA	NA	NA	NA	NA	NA
Date of positive for initial SARS-CoV-2 nucleic acid testing	Apr 13	Mar 29	Apr 10	Apr 24	Apr 13	Apr 23	Mar 28	Apr 22	Apr 15
Ct value of ORF1ab gene for initial SARS-CoV-2 nucleic acid testing	37.8	36.6	36.7	36.5	37.9	35.3	36.8	31.3	38.9
Ct value of N gene for initial SARS-CoV-2 nucleic acid testing	37.5	37.7	27.3	36.4	39.5	34.0	35.0	29.9	36.7
Date of initial testing for SARS-CoV-2-specific IgG and IgM	Apr 13	Mar 29	Apr 10	Apr 24	Apr 13	Apr 23	Mar 14	Apr 22	Apr 15
Result of initial testing for SARS-CoV-2-specific IgG and IgM	IgG (+), IgM (+)	IgG (+), IgM (+)	IgG (+), IgM (+)	IgG (+), IgM (−)	IgG (+), IgM (−)	IgG (+), IgM (−)	IgG (+), IgM (−)	IgG (+), IgM (−)	IgG (+), IgM (−)
Date of admission	Apr 13	Mar 29	Apr 10	Apr 25	Apr 13	Apr 24	Mar 28	Apr 22	Apr 15
Date of negative for initial SARS-CoV-2 nucleic acid testing	Apr 26	May 10	Apr 26	May 2	Apr 27	Apr 28	Apr 5*	Apr 28	Apr 26
Date of discharge	May 12	NA**	May 9	NA**	NA**	NA**	NA**	NA**	NA**
Symptoms associated with COVID-19 during quarantine	No	No	No	No	No	No	No	No	No
Days for patients living with their family members since Jan. 23, 2020 (Wuhan lockdown)	81	104	78	93	81	92	65	90	83
The status of SARS-CoV-2 nucleic acid testing for all close contacts	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative

Author contributions

All authors participated in design, collected and analyzed data, drafted the manuscript and approved the final submitted version. Chen, Fu, and Yang contributed equally to the work.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Should qualitative RT-PCR be used to determine release from isolation of COVID-19 patients?

Dear Editor,

COVID-19 patients can continue to shed viral RNA well beyond clinical recovery.[1-4] Problematically, while qualitative RT-PCR, the most commonly used diagnostic test for COVID-19, identifies SARS-CoV-2 virus genome, it fails to distinguish between viable infectious virus and noninfectious viral particles. Persistently positive RT-PCR following clinical recovery does not necessarily indicate infectiousness, yet such testing is still being used as a surrogate marker of infectivity.

Healthcare providers and public health officials are asked to provide guidance for the discontinuation of isolation precautions in persons with suspected or confirmed COVID-19. For symptomatic persons with COVID-19, the current guidance from the United States Centers for Disease Control and Prevention suggests either a symptom-based or a test-based strategy. The symptom-based strategy—in which isolation can be discontinued after 3 days have passed since clinical recovery and 10 days since symptoms first appeared—can be used in non-immunocompromised patients and leads to most cases being released within two weeks.[5] However, the test-based strategy—in which isolation can be discontinued when two consecutive samples of respiratory specimens, collected ≥24 h apart, are negative by RT-PCR—is being used "out of an abundance of caution" for recovered persons for whom there is low tolerance for infectious risk, and this is presenting a dilemma.[6]

In fact, in the absence of legal guidance to the contrary, the administrators of some healthcare and congregate living facilities are requiring the test-based strategy as a condition for an employee to return to the workplace, for a patient to be transferred to another healthcare facility, or for isolation to be discontinued in the recovered patient.[7] Consequently, many ostensibly well COVID-19 patients, including mothers of newborns, have been in prolonged isolation for six or more weeks beyond recovery.[7] Such persons also include healthcare providers who may pose a risk of transmitting infection to patients who are at high risk for complications and to other healthcare workers. Another high-stakes scenario is congregate living facilities (e.g., correctional/detention facilities, homeless shelters, retirement communities, ships) where there might be increased risk of transmission, morbidity and mortality. Not surpris-

Abbreviations: COVID-19, coronavirus disease 2019; ELISA, enzyme-linked immunosorbent assay; mL, milliliter; RNA, ribonucleic acid; RT-PCR, reverse transcriptase polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

ingly, this results in undue hardship for affected persons and their families, as well as for employers.

The concern in relying solely on non-test-based criteria is that they may not prevent all instances of secondary transmission—including in immunocompromised patients.[5] By contrast, a *qualitative* PCR test-based strategy may unnecessarily prolong the need for isolation. This is suggested by an emerging understanding of the clinical, microbiological, and serological aspects of the natural history of COVID-19, as well as by the results of a recent epidemiological study. At around 18 days after onset of illness, 50% of patients are still positive by RT-PCR of nasopharyngeal swab, 5% may still be positive by day 33, and some cases have been reported to be positive more than eight weeks after illness onset.[3, 6] Yet the median time from illness onset to clinical recovery for mild cases is only 14–15 days. Using total antibody enzyme-linked immunosorbent assays (ELISAs) that detect antibodies to the receptor-binding domain of the spike protein, the median seroconversion time of 173 patients with SARS-CoV-2 infection was 11 days after illness onset, and by day 14 almost 90% of patients had seroconverted, and by day 39 all had detectable antibodies.[8]

The detection of SARS-CoV-2 using cell culture may not have utility in clinical practice given its low sensitivity and long turnaround time. However, it is a useful proxy for infectious virus shedding that can serve to evaluate other potential surrogate markers such as viral RNA load (using quantitative RT-PCR) and serology testing (using serum neutralization antibody titers). The longest time from symptom onset to isolating SARS-CoV-2 has been 18 days, and the lowest viral RNA load at the time that SARS-CoV-2 could no longer be isolated has ranged from ≥33–35 cycle threshold (<6.51 Log10 RNA copies/mL).[1, 5]

Perhaps the most persuasive data, published to date, of the lack of association between post-recovery viral RNA shedding and infectiousness are from the contact investigations that the Korea Centers for Disease Control and Prevention carried out on 285 COVID-19 patients who had met symptom-based and test-based criteria for release from isolation.[2] Of these, 107 (37.5%) were re-tested because of symptom onset, and 170 (59.6%) were re-tested for screening purposes—fregardless of symptoms. Of the 284 persons who were checked for symptoms, 126 (44.7%) were symptomatic. Contact tracing of these 285 "re-positive" cases identified 790 contacts. After a minimum 14-day monitoring of these contacts, 27 of the contacts were found to be positive, of which 24 were previously confirmed, and the remaining 3 were newly-confirmed cases with a history of an exposure to another confirmed case in their family or religious community. The virus could not be isolated from cell culture in two of these cases, and was not possible in the remaining one because the PCR result was indeterminate. Furthermore, neutralizing antibody production, suggestive of acquired protective immunity, was detected in the first serum sample of all "re-positive" cases.

For those with prolonged shedding of viral RNA, requiring conversion of qualitative RT-PCR for release from isolation has potential economic, physical, psychological, and social detriments. For many, there is a loss of income which disproportionately affects the poorest and most vulnerable whose employment often does not include sick leave. Psychosocially, a prolonged period of physical isolation can lead to loss of connection and perceived social isolation ("loneliness") which is associated with suicidal behavior and psychotic disorders among persons with severe mental illness, and is linked to depression in those without a preexisting psychiatric disorder.[9] Furthermore, social isolation has been associated with higher mortality in general ("all-cause mortality"), including cancer and cardiovascular disease.[10] In COVID-19 patients with prolonged isolation, these issues are even more compounded by their reduced access to medical care. Further research into more accurate test-based criteria for determining release from isolation is much needed—potentially ones using *quantitative* RT-PCR and/or *quantitative* immunoassays, with cut-off values that have been validated to correlate with lack of infectivity.

Authors' contributions

CMPV conceived the manuscript; all authors performed literature search; KK wrote the first draft of the manuscript; CMPV revised it; all authors reviewed it.

Declaration of Competing Interest

None

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Outcome of universal screening of neonates for COVID-19 from asymptomatic mothers

Dear Editor,

Pandemic SARS-CoV-2 is the third Coronavirus to cause severe respiratory illness in humans. Little is known about the effects on pregnant women and the question of whether vertical transmission can occur remains unanswered and controversial.

Universal screening of pregnant women has been described in the literature however to date universal screening of newborns has not. Here we report our unusual findings of the results of a universal screening programme of asymptomatic and healthy mothers together with their newborns .

Our Trust, with a delivery rate of 7300 births per year, introduced universal nasopharyngeal screening using RT-PCR of all inpatients, including newborn babies on 27th April 2020.

Between 27th April and 21st May, 481 infants were delivered and 418 were screened with maternal consent. Nine (2.2%) infants born to asymptomatic mothers screened positive for SARS-CoV-2 all within the first 24 h, three within the first three hours. Of these nine, eight mothers tested negative (Table 1). Only one infant (Case 5) was symptomatic - requiring oxygen for two hours and high flow humidified nasal cannulae for 22 hours. Chest X-ray showed streaky hila bilaterally and hazy consolidation in both lower lobes.

This is the first description of a universal screening programme for term and near term neonates. The finding of eight positive infants whose mothers tested negative for SARS-CoV-2 infection has never been described. Four possible explanations for this finding exist.

Firstly all positive tests were contaminated. However all samples were taken on different days by different staff, all of whom were wearing appropriate PPE and none of whom became ill which makes this explanation very unlikely.

Secondly the mothers' results were false negatives. RT-PCR results are influenced by test technique and by clinical severity and duration of symptoms. All the mothers in our cohort were asymptomatic and the sensitivity of the test may have been insufficient to detect the small quantity of viral RNA that these mothers may have had at the time of the test. However, three of the infants were tested within three hours, two following elective Caesarean birth. If these mothers were false negatives these positive neonatal results raise the possibility of vertical transmission due to the short time interval for possible postnatal transmission.

Thirdly all eight could be false positive results. The quoted specificity of the test is 100% with a cycle threshold of 35 cycles. All eight infants had cycle thresholds of 29–31 which, although within the manufacturer's quoted specificity, are towards the higher end. Internal validation of the test gave a 100% positive predictive value to 35 cycles suggesting that all eight are valid results.

Fourthly these asymptomatic mothers could have been positive previously and, while no longer shedding viral RNA in the nasopharynx, had shed RNA or RNA fragments into the amniotic fluids which were still within the infant nasopharynx on the first day of life but cleared by the second test. This would explain the high cycle thresholds on the neonatal swabs and could be addressed by maternal/infant serology testing but this was not available at the time.

Vertical transmission of SARS-CoV-2 has not been proven. To date case series and reports have focussed on infants born to symptomatic positive mothers. Rose et al.[1] reviewed eight case series with a total of 69 pregnant women delivering 70 infants. Only four of 63 tested infants had positive throat swabs. The largest series to date is the UK Obstetric Surveillance System[2] national cohort study. In this series of 427 pregnant women hospitalised with SARS-CoV-2, 247 gave birth or suffered pregnancy loss. 12 infants tested positive; six of those within the first twelve hours. No description of infection control techniques practised by the mothers is given.

There are several reports of placental infection in symptomatic COVID-19 positive women.[3-6] Two mid-trimester cases[3,4] are described with pre-viable foetuses where viral RNA was positive from the foetal surface of the placenta but not from the fetuses. Penfield et al.[5] describe placental and membrane swabs sent on eleven

Table 1
Characteristics of infants swabbing positive for SARS-CoV-2 under the universal screening programme.

Number	Sex	Gestational age at birth (Weeks)	Birthweight (gms)	Mode of delivery	Age at 1st test (hh:mm)	Result	CT	Age at 2nd test (days)	Result	Maternal 1st test	Maternal 2nd test
Case 1	M	42+2	4420	EMCS	09:21	+ve	29	N/D	–	+ve	N/D
Case 2	F	41+5	2950	SVD	06:15	+ve	30	3	-ve	-ve	-ve
Case 3	M	38+4	3185	ELCS	02:15	+ve	31	7	-ve	-ve	-ve
Case 4	F	41+6	3020	EMCS	09:39	+ve	31	4	-ve	-ve	-ve
Case 5	M	39+1	3460	ELCS	04:39	+ve	29	2	-ve	-ve	-ve
Case 6	M	3240	3240	SVD	02:31	+ve	31	5	-ve	-ve	-ve
Case 7	M	39+1	3685	ELCS	02:09	+ve	29	N/D	–	-ve	N/D
Case 8	F	36+4	2815	EMCS not in labour	10.20	+ve	30	2	-ve	-ve	N/D
Case 9	F	37+4	2635	SVD	5.14	+ve	30	1	-ve	-ve	N/D

CT – Cycle Threshold, SVD-Spontaneous Vaginal Delivery, ELCS-Elective Caesarean Section, EMCS: Emergency Caesarean Section N/D- not done.

COVID-19 positive patients of which three were positive although no infant tested positive on days one to five of life. Kirtsman et al.[6] describe a single case of a 35 week gestation infant born to a COVID-19 positive mother with positive placental and membrane swabs and positive nasopharyngeal swabs immediately after birth and on days two and seven.

Although viral RNA and DNA has been found in amniotic fluid from a number of different viruses including Zika virus and Ebola, vertical transmission of human coronaviruses was not described in either the SARS or MERS outbreaks[7] and viral RNA from these related beta-coronaviruses was not found in amniotic fluid.

Universal screening of 533 pregnant women in Italy[8] found three positive for SARS-CoV-2 infection all of whom had uneventful deliveries. Neonatal swabs were not reported. Of 215 women tested under a universal swabbing programme in New York City[9] 33 were positive for SARS-CoV-2 of whom 29 (88%) were asymptomatic. Neonatal outcome was not reported. In a second New York study[10] 43 pregnant women tested positive for SARS-CoV-2. 18 well infants were delivered and all subsequently tested negative.

Universal screening of neonates for SARS-CoV-2 infection has not been described and was introduced in our institution alongside universal screening of all in-patients. We describe the unexpected finding of positive infants with negative mothers. The study is limited by its size and the fact that no confirmatory samples such as placental swabs, amniotic fluid or serology in either mother or baby were obtained due to the nature of the universal screening programme. Nevertheless, the positive swabs in 2% of newborns, all within the first twenty four hours and three within the first three hours of life represent a very interesting finding. This study demonstrates that universal swabbing of all newborns is easy and could add substantially to our understanding

Author's contributions

All authors made equal contribution to the conception of the work. KM and SP collected the data. KM drafted the work and performed the original literature search. NG and SP performed additional literature searches and reviewed and edited the manuscript. All authors reviewed and approved the final version of the manuscript

Disclosure statement

We declare no competing interests.

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SARS-CoV-2 RNA detection in tears and conjunctival secretions of COVID-19 patients with conjunctivitis

Dear Editor,

We read with great interest the recent study of Azzi et al.[1] regarding the possible role of saliva in the detection of SARS-CoV-2. We congratulate the authors for their excellent study and their results highlighting that saliva represents a promising tool in COVID-19 diagnosis.

Similarly to saliva, there is evidence regarding the presence of SARS-CoV-2 RNA in tears and conjunctival secretions in patients with COVID-19.[2,3] However, collecting ocular fluids or secretions for SARS-CoV-2 detection appears to have limited diagnostic value.[4]

Conjunctivitis has been also described as an ocular manifestation related to SARS-CoV-2 infection, with prevalence of conjunctivitis ranging from 0.8% to 31.6%.[5,6] Some studies suggest that SARS-CoV-2 detection rate in conjunctival secretions could be higher in patients with conjunctivitis.

The main purpose of our study was to evaluate the presence of viral RNA of SARS-CoV-2 in conjunctival swab specimen of COVID-19 patients with conjunctivitis and its identification value. To the best of our knowledge, this is the first study of its kind that evaluates a large patient series with conjunctivitis related to COVID-19.

This cross-sectional study was conducted at the Hospital Clinico San Carlos (HCSC) of Madrid, Spain. The study was approved by the Clinical Research Ethics Committee of this institution and

was conducted in accordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from all patients.

From April 15 to May 15, 2020 hospitalized patients for COVID-19 with conjunctivitis were consecutively recruited. The inclusion criteria were: over 18 years of age; patient with positive reverse transcriptase–polymerase chain reaction (RT-PCR) test from nasopharyngeal swab for SARS-CoV-2, hospitalized due to COVID-19, conjunctivitis diagnosis and ability to give verbal consent. Those patients admitted to the intensive care unit, unable or unwilling to give consent were excluded.

Upon notification of a possible conjunctivitis case, 2 ophthalmologists examined the patients and if a diagnosis of conjunctivitis was confirmed, a conjunctival swab was collected. The conjunctival samples were obtained from both eyes in patients with bilateral conjunctivitis and from the affected eye in patients with unilateral conjunctivitis.

Conjunctival swab was collected with a sterile synthetic fiber swab (Flexible minitip size Nylon® flocked swab) into the lower fornix without topical anesthesia. We used the same swab to obtain a specimen from both eyes in cases of bilateral conjunctivitis. The swab was immersed into a viral transport medium (Universal transport Media-UTM®, Copan, Italy), and stored at 4°C before being tested for SARS-CoV-2. RT-PCR assays were performed at Microbiology Department of HCSC with cuantitative GeneXpert Xpert Xpress® SARS-CoV-2 (Cepheid, USA). Viral loads are inversely correlated with cycle threshold (Ct) values, Ct value of 40 indicates negative results.

Of the 543 hospitalized patients, 28 patients were notified as possible conjunctivitis and 21 of them had positive RT-PCR test from nasopharyngeal swab for SARS-CoV-2. Of those, 14 patients were finally diagnosed with conjunctivitis and conjunctival swab was collected. The mean age of the patients was 72.6 years (range 33-92 years) and the male-to-female ratio was 0,36 (5:9). Six patients (43%) had mild, 5 patients (36%) had moderate disease and 3 patients (21%) had severe disease.

SARS-CoV-2 RNA was detected in conjunctival swab of one patient (7%) among the 14 patients with conjunctivitis and laboratory-confirmed COVID-19. In this patient, the PCR Ct was 25, which means an elevated viral load.

Despite the main modes of transmission of SARS-CoV-2 are through respiratory droplets and direct contact with contaminated objects or surfaces, other routes such as ocular transmission should not be ignored.

SARS-CoV-2 RNA has been detected in tears and conjunctival secretions of patients both with and without conjunctivitis.[7] Zhou et al. found a proportion with positive results for conjunctival SARS-CoV-2 detection of 2.5% (3 patients out of 121 patients). Of the 8 patients with conjunctivitis included in the latter study, only one tested positive for SARS-CoV-2 in conjunctival swab. Another study carried out in China analyzed tear and conjunctival samples of 30 patients with COVID-19. The only one patient with conjunctivitis yielded positive real-time polymerase chain reaction (RT-PCR) results.[2] Our study was conducted on patients with conjunctivitis, finding a proportion of 7.14% (1/14) with positive RT-PCR for conjunctival specimen, higher than most other studies in COVID-19 patients without conjunctivitis. Therefor, RT-PCR could be of more value in conjunctivitis patients.

The low positive rate of SARS-CoV-2 RNA test by RT-PCR in tears and conjunctival secretions exhibits a relatively low likelihood of detecting the virus in ocular samples of COVID-19 patients. Therefore, the development of conjunctivitis could be a consequence of an inflammatory response, rather than due to viral replication. Also, time of sampling could explain negative tests for SARS-CoV-2 RNA. In the current study, most samples were taken on the second day of conjunctivitis symptoms. It would be interesting to know the amount of time that the virus is detectable in ocular secretions.

The presence of SARS-CoV-2 RNA in ocular secretions may be explained by hand-eye inoculation. Transmission may occur through accidental inoculation of viral particles from the patient's hands or by direct eye contact with infected upper respiratory droplets or contaminated fomites, as it happens in other types of viral conjunctivitis such as adenoviral conjunctivitis.[8]

Although a high COVID-19 cohort was followed, a small percentage developed conjunctivitis during the hospital admission. Also, RT-PCR does not have 100% sensitivity, so negative test results may be false negative and do not exclude the presence of the virus. Sensitivity may be increased if multiple specimens are collected. However, due to the limited RT-PCR reagents and kits during the pandemic situation, we were only able to collect one sample for both eyes.

The detection of SARS-CoV-2 RNA in tears and conjunctival swabs highlights the role of the eye as a possible route of transmission of the disease, since the ocular surface might represent both a potential site of virus replication and a transmission route of the infection. Further large sample and more comprehensive studies are warranted to evaluate the presence of SARS-CoV-2 in tears and conjunctival swabs and its diagnostic value, especially in patients with conjunctivitis.

Declaration of Competing Interest

None.

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Tumor biomarkers predict clinical outcome of COVID-19 patients

Dear Editor,

A recent article in *Journal of Infection* by Fu and colleagues have summarized the clinical characteristics of coronavirus disease 2019 (COVID-19) in China, and described that those with medical comorbidities tend to have more severe clinical symptoms and higher case-fatality rate, according to data of 43 studies involving 3600 patients.[1] Of the data from China, 81% cases were mild, 14% were severe, and 5% were critical, and the case-fatality rate was 2.3% in all cases and 49.0% in critical cases.[2] Older age and comorbidities, such as cardiovascular disease, confer a higher risk for severe disease, and young and otherwise healthy patients are also at risk for complications.[3] ARDS (Acute respiratory distress syndrome) and respiratory failure, sepsis, acute cardiac injury and heart failure were the most common critical complications during exacerbation of COVID-19. Several laboratory outcomes indicated the severity and the clinical outcome of COVID-19 patients. Previous studies reported that tumor biomarkers, such as carcino embryonic antigen (CEA), cytokeratin 19 fragment (CYFRA21-1), neuron-specific enolase (NSE), squamous cell carcinoma antigen (SCCA) and Pro-Gastrin Releasing Peptide (ProGRP), were elevated in the patients with benign lung disorders, such as pneumonia and pulmonary fibrosis.[4,5,6] We would like to share our findings that the role of tumor markers related lung cancer in COVID-19 patients as predictive indicators for clinical outcome.

A total of 129 patients diagnosed as COVID-19, with 20 moderate (15.50%), 73 severe (56.59%) and 36 critical severe cases (27.91%) on admission, were included in this study. In addition, a total of 80 age-and gender- matched health individuals were enrolled as controls. The patients self-reported medical history of comorbidities were recorded on admission and were classified as hypertension, cardiovascular disease, diabetes (type 2), chronic obstructive pulmonary disease (COPD) and others. Of 129 cases, 114 cases (88.37%) were discharged from hospital for their recovery from COVID-19, and 15 cases (11.63%) were deceased during the treatment, shown in Supplementary Table 1. For the characteristics of patients, we observed that the mean age of patients was significantly different among the subgroup of severity, and the mean age of patients with critical severe was significant higher than those who with severe or moderate. The distribution of patients with diabetes, chronic kidney disease and others comorbidities have significant differences among the sub-groups of disease severity. Most deceased cases (14/15) were with the critical severe COVID-19 and one with severe COVID-19. Patients who deceased have significant higher ration of comorbidities of chronic kidney disease (p=0.001), shown in Table 1.

The plasma concentration of all the five biomarkers were significantly elevated in cases than those in controls ($p_{all}<0.01$). In addition, the significant differences of plasma level of CEA, CYFRA21-1 and SCCA were observed among the subgroups of severity of disease and clinical outcome (Table 1) and plasma level of CEA, CYFRA21-1, SCCA were significantly increased with the advance serverity of disease. Whereas, there were no significant differences of NSE and proGRP contration amonge the different severity subgroups, shown in Supplementary Figure 1.

To further analyze prognostic role of tumor biomarker in COVID-19 patients, a logistic regression was applied to measure the associations of tumor biomarkers level to risk of death. Crude OR, OR adjusted for age and gender (model 1), and OR adjusted for molel1 plus comordities (model 2) were used to assess the relative risk, respectively. The results revealed that increased level of CEA (OR=1.13, 95%CI:1.03-1.23, p=0.010; adjust model 1 OR=1.12, 95%CI: 1.02-1.23, p=0.016; adjust model 2 OR=1.12, 95%CI: 1.01-1.26, p=0.029), CYFRA21-1 (OR=1.73, 95%CI:1.35-2.21, p=0.000; adjust model 1 OR=1.673, 95%CI: 1.34-2.36, p=0.000; adjust model 2 OR=1.73, 95%CI: 1.32-2.28, p=0.000), NSE (OR=1.09, 95%CI: 1.02-1.17, p=0.016; adjust model 1 OR=1.11, 95%CI: 1.04-1.19, p=0.003; adjust model 2 OR=1.15, 95%CI: 1.05-1.27, p=0.004) and SCCA (OR=1.28, 95%CI: 1.11-1.48, p=0.016; adjust model 1 OR=1.22, 95%CI: 1.06-1.41, p= 0.007; adjust model 2 OR=1.24, 95%CI: 1.07-1.45, p= 0.006) were associated with increased risk of death, respectively, shown in Table 2.

The ROC curve revealed that SCCA (AUC: 0.937, p=0.000, cut-off: 2.57 ng/ml), CYFRA21-1(AUC: 0.882, p=0.000, cut-off: 7.29 ng/ml) and CEA (AUC: 0.737, p=0.003, cut-off: 8.55 ng/ml) could predicte the clinical outcome of COVID-19 patients, shown in Figure 2. The correlation of biomarkers dynamics and patient outcome was also evaluated with 22 discharged and 11 deceased patients, and the result revealed that the increased concentration of CYFRA21-1, SCCA and NSE were the risk of death (Supplementary Table 2), indicating that dynamic monitor for the three biomarkers could predict the clinical outcome of COVID-19 patients.

This study revealed that age, diabetes, chronic kidney disease and other diseases were associated with the severity of COVID-19 patients. In which, chronic kidney disease was also regarded as a risk of death of COVID-19 patients, which was consistent the result of publised data.[7] Acutally, the most common cause of death in COVID-19 patients is viral pneumonia leading to inflammatory response results in the progression to multi-organ failure. Therefore, those patients have history of diabetes, chronic kidney disease were more susceptiable to develop multi-organ failure and lead to death. Tumor biomarkers related lung cancer that CEA, CYFRA21-1, NSE, SCCA, ProGRP were previously reported to be elevated in the pneumonia patients[5] or benign lung diseases.[6,8] In this study, we observed that all five tumor biomarkers were significantly increased in the plasma of COVID-19 patients than those in health controls, that CEA, CYFRA21-1 and SCCA were significantly different among the subgroups of severity of disease and clinical

Table 1
Clinical characteristics of the patients according to disease severity and the clinical outcomes

Clinical characteristics	Severity of Disease			P-value	Clinical outcome		P-value
	Moderate	Severe	Critical severe		Discharged	Deceased	
No	20	73	36		114	15	
Age, Mean(SD),y	61.90(12.89)	66.59(11.49)	70.92(12.32)	0.026	66.40±12.24	72.13±11.86	0.090
Gender, n(%)							
Male	11(14.10)	41(52.56)	26(33.33)	0.235	67(85.90)	11(14.10)	0.278
Female	9(17.65)	32(62.75)	10(19.61)		47(92.16)	4(7.84)	
Hypertension, n(%)							
Yes	5(9.09)	31(56.36)	19(34.55)	0.131	47(85.45)	8(14.55)	0.373
No	15(20.27)	42(56.76)	17(22.97)		67(90.54)	7(9.46)	
Cardiovascular disease, n(%)							
Yes	4(17.39)	9(39.13)	10(43.48)	0.135	19(82.61)	4(17.39)	0.341
No	16(15.09)	64(60.38)	26(24.53)		95(89.62)	11(10.38)	
Diabetes, type 2, n(%)							
Yes	5 (17.86)	10 (35.71)	13 (46.43)	0.026	23(82.14)	5(17.86)	0.245
No	15(14.85)	63(62.38)	23(22.77)		91(90.09)	10(9.90)	
COPD, n(%)							
Yes	0(0)	9(81.82)	2(18.18)	0.163	11(100)	0(0)	0.208
No	20(16.95)	64(54.24)	34(28.81)		103(87.29)	15(12.71)	
Chronic kidney disease, n(%)							
Yes	1(11.11)	2(22.22)	6(66.67)	0.025	5(55.56)	4(44.44)	0.001
No	19(15.83)	71(59.17)	30(25.00)		109(90.83)	11(9.17)	
Others, n(%)							
Yes	1(2.78)	21(58.33)	14(38.89)	0.025	30(83.33)	6(16.67)	0.267
No	19(20.43)	52(55.91)	22(23.66)		84 (90.32)	9(9.68)	
Clinical outcome, n(%)							
Discharge	20(17.54)	72(63.16)	22(19.30)	0.000			
Die	0(0)	1(6.67)	14(93.33)				
Tumor biomarkers, IQR							
CEA (ng/mL)	2.39(1.20,3.82)	3.48(2.26,4.95)	5.03(3.09,8.58)	0.000	3.39(2.14,5.05)	5.43(3.55,11.42)	0.003
CYFRA21-1(ng/mL)	2.24(1.79,3.03)	3.30(2.43,4.09)	5.06(2.78,9.83)	0.000	3.14(2.35,4.05)	9.72(7.32,12.08)	0.000
NSE (ng/mL)	12.76(11.85,15.11)	13.01(10.67,16.28)	12.56(10.74,18.28)	0.970	12.40(10.86, 15.34)	15.92(12.77,28.41)	0.022
SCC (ng/mL)	0.99(0.64,1.68)	1.03(0.73,1.39)	2.62(1.18,4.10)	0.000	1.07(0.75,1.59)	3.59(2.75,8.77)	0.000
proGRP(pg/mL)	42.13(36.56,46.41)	44.06(35.67,57.15)	56.27(32.55,88.89)	0.219	44.61(36.38,61.35)	34.60 (16.57,102.55)	0.476

Table 2
The relative risk of tumor biomarkers to death

Patients	CEA		CYFRA21-1		NSE		SCCA		proGRP	
	OR(95%CI)	P value	OR(95%CI)	P value	OR(95%CI)	P value	OR(95%CI)	P value	OR(95%CI)	P value
Discharged	reference		reference		reference		reference		reference	
Deceased	1.13(1.03,1.23)	0.010	1.73(1.35,2.21)	0.000	1.09(1.02,1.17)	0.016	1.28(1.11,1.48)	0.001	1.01(0.99,1.02)	0.417
Model1	1.12(1.02,1.23)	0.016	1.73(1.34,2.36)	0.000	1.11(1.04,1.19)	0.003	1.27(1.09,1.48)	0.002	1.00(0.99,1.02)	0.832
Model2	1.12(1.01,1.26)	0.029	1.73(1.32,2.28)	0.000	1.15(1.05,1.27)	0.004	1.24(1.07,1.45)	0.006	1.00(0.98,1.01)	0.601

Model1, adjusted for age and gender; Model2, adjusted for model1 plus comorbidities.

outcome, and that CEA, CYFRA21-1, SCCA could predicte the clinical outcome of COVID-19 patients. This study firstly reported the role of tumor biomarkers in COVID-19 patients.

In short, we concluded that the concentrations of tumor biomarkers of CEA, CYFRA21-1, NSE, SCCA, ProGRP were elevated in COVID-19 patients, and that CEA, CYFRA21-1, SCCA could predicte the clinical outcome of COVID-19 patients.

Declaration of Competing Interest

The authors declare no competing interests

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Supplementary materials

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Interleukin-6 as prognosticator in patients with COVID-19

Dear Editor,

Identifying risk factors for early progression toward severe disease and/or mortality is fundamental for the practical management of COVID-19 patients. Evidence shows that pro-inflammatory cytokines play a pivotal role in the pathophysiology of lung damage in patients affected by coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Therefore we read with much interest the recent article published in Your Journal by Ye Q. et al. who describe the “cytokine storm” in COVID patients.[1] A lot of patients affected by COVID-19 develop a fulminant and damaging immune reaction sustained by cytokines leading to alveolar infiltration by macrophages and monocytes.[1] Interleukin-6 (IL-6) is one of the main mediators of inflammatory and immune response initiated by infection or injury and increased levels of IL-6 are found in more than one half of patients with COVID-19.[2] Levels of IL-6 seem to be associated with inflammatory response, respiratory failure, needing for mechanical ventilation and/or intubation and mortality in COVID-19 patients.[3,4] In a meta-analysis including nine studies (total 1426 patients) reporting on IL-6 and outcome in COVID-19, mean IL-6 levels were more than three times higher in patients with complicated COVID-19 compared with those with non complicated disease, and IL-6 levels were associated with mortality risk.[4] However, whether IL-6 could be a better prognosticator than clinical and laboratory variables remains unclear. Therefore, we tested the role of IL-6 as risk factor for negative outcome compared with other demographic and clinical variables or biomarkers collected at hospital admission. Age over 60 years, presence of at least one co-morbidity among arterial hypertension, diabetes, cardiovascular disease, asthma, chronic lung disease, chronic kidney disease, liver disease, HIV infections, and malignancy for at least 6 months, lymphocyte count under 1.0×10^9/L, lactate dehydrogenase (LDH) over 500 U/L, CALL score $>$ 9 points (*C*=presence of co-morbidity, *A*=age over 60 years, *L*=lymphocyte count under 1.0×10^9/L, *L*=LDH over 250 U/L or 500 U/L)[5], D-Dimer over 500 microg/L, and IL-6 over 25 pg/mL were the analyzed variables. Quantitative determination of IL-6 levels was performed by using an immunoenzymatic chemiluminescent assay (Access Immunoassay System, Beckman Coulter, USA, lowest limit of detection 0.5 pg/mL). After exclusion of patients requiring immediate intensive

Table 1
Risk factors for the combined endpoint progression to severe COVID-19 and/or in-hospital mortality. Logistic regression analysis.

Variable	Odds ratio	95% CI
Age over 60 years	1,4882	0,3663–6,0466
CALL score > 9 points	4,5577	0,7383–28,1352
Co-morbidity	0,3150	0,0634–1,1561
D-Dimer > 500 microg/L	0,9882	0,2638–3,7009
IL-6 > 25 pg/mL	11,6460	2,8123–48,2277
LDH > 500 U/L	0,5033	0,1061–2,3888
Lymphocyte count< 1.0 x 10^9	0,6145	0,1473–2,5638

CI: confidence interval; CALL score: *C*=presence of co-morbidity, *A*=age over 60 years, *L*=lymphocyte count under 1.0 x 10^9/L, *L*=LDH over 250 U/L or 500 U/L; IL-6: Interleukin-6; LDH: lactate dehydrogenase.

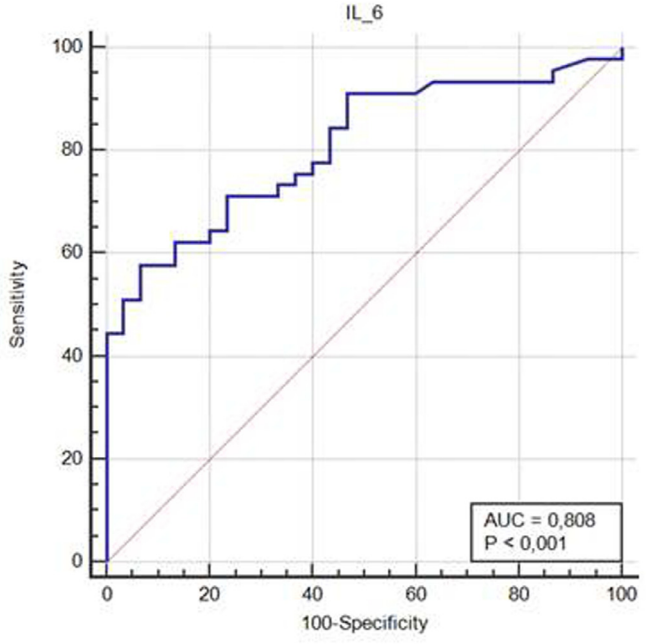

Fig. 1. Receiver operating characteristic (ROC) curve showing the predictive power of IL-6 for predicting progression to severe COVID-19 and/or in-hospital mortality.

care unit (ICU) admission, we analyzed risk factors for the combined endpoint progression to severe COVID-19 syndrome and/or in-hospital mortality in an Italian COVID-19 population admitted to a non intensive ward from March 12 to April 20, 2020. Progression toward clinical worsening was defined as respiratory rate ≥ 30 breaths/min, resting $SatO_2$ ≤ 93%, paO2/FiO2 ratio ≤ 300 or requiring of mechanical ventilation, such as in previous studies.[5] The study population consisted of 77 patients, 44 males (57.1%), with mean age 64 ± 17 years. Of them, 45 patients (58.4%) met criteria for the combined endpoint. Six patients (7.8%) died. CALL score > 9 points (55.3% vs 26.6%, $p = 0.0099$) and IL-6 > 25 pg/mL (65.9% vs 23.3%, $p = 0.0004$) were significantly more frequent in patients with the combined endpoint. At logistic regression analysis IL-6 over 25 pg/mL (OR 11.6, 95% CI 2.8–48,2) was found independent risk factor for the combined endpoint (Table 1). Mean levels of IL-6 in patients who met criteria for the combined endpoint were significantly higher compared with those of patients who did not (134.3 ± 19.5 vs 15.6 ± 14.8 pg/mL, $p < 0.001$). The area under the receiver operating characteristic (ROC) curve (AUC) for IL-6 as predictor of the combined endpoint was 0.80 (95% CI 0.70–0.89) (Fig. 1). The AUC for IL-6 as predictor of in-hospital mortality was 0.90 (95% CI 0.81–0.95), while it was 0.75 (95% CI 0.64–0.84) for IL-6 as predictor of progression to severe COVID-19.

In conclusion, in our COVID-19 population, IL-6 levels at hospital admission seem to be a good prognosticator for the combined endpoint progression to severe disease and/or in-hospital mortality, and it seems to be the best prognosticator for negative outcome. Therefore, our study supports the hypothesis that targeting the cytokine storm induced by SARS-CoV-2 by using anti-IL-6 drugs could be a valid therapeutic option, together with supportive care strategies, for improving outcomes in COVID-19 patients.[6]

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Etoposide treatment adjunctive to immunosuppressants for critically ill COVID-19 patients

Dear Editor,

In a recent article in the *Journal*, Cantini and colleagues[1] present favorable outcomes in a small cohort of moderate COVID-19 pneumonia patients treated with lopinavir-ritonavir in addition to baricitinib, a Janus kinase inhibitor (anti-JAK). Baricitinib is a cytokine release inhibitor and is active against SARS-CoV-2 endocytosis.

Current evidence suggests that systemic hyperinflammation and immune dysregulation play a key role in the development of severe lung and multiorgan damage found in critically ill COVID-19 patients.[2,3,4] This massive cytokine release closely resembles that of macrophage activation syndrome or secondary hemophagocytic lymphohistiocytosis, hematological conditions in which acute respiratory distress syndrome (ARDS) is also common.[2,5] Changes in lymphocyte sub-populations, cytokines dysregulation, presence of highly cytotoxic CD8+ *T* cells, and the accumulation of pro-inflammatory monocytes/macrophages in the lungs, seem to participate in the immune-mediated tissue damage.[3,4,6]

Etoposide is a WHO Essential Medicine and powerful selective suppressor of activated T-cells and monocytes that reduces the production of inflammatory cytokines. Given its effectiveness against hyperinflammation,[5,7] essentially by targeting monocytes and activated T cells and by moderating the cytokine storm, we propose a rationale for its use in critically ill COVID-19 patients. In this report we review the clinical course and outcome of 11 severe COVID-19 patients treated with etoposide as salvage therapy following prior immunosuppressants.

Patients eligible for etoposide treatment were older than 18 years, presented biochemical alterations suggestive of severe hyperinflammation (ferritin levels >1000 ng/ml and/or IL-6 values >50 pg/ml), ARDS (defined by PaO_2/FiO_2 < 300) and were not under mechanical ventilation. Prior treatment consisted on combinations of oxygen support, lopinavir-ritonavir, antimicrobials, methylprednisolone, and interleukin inhibitors. Patients not responding to a 3-day course of methylprednisolone plus Tocilizumab (IL-6 inhibitor) or Anakinra (IL-1 inhibitor) were offered etoposide. Orotracheal intubation, mechanical ventilation and prone positioning were applied when necessary according to the course of respiratory function. Prophylactic enoxaparin (40 mg per day) gas given regularly, and therapeutic anticoagulation was prescribed if thrombotic complications appeared. The study was conducted at the University Hospital of Burgos, Spain, and approved by the Local Institutional Ethics Committee (CEIm reference number, 2307) for off-label use of the drug. Informed consent from every participant (or relative) was obtained.

Thirteen patients received etoposide (50–150 mg/m^2) out of 709 COVID-19 patients admitted to our center during the study period (March 2 to April 10, 2020). Overall, 412/709 developed ARDS (58.1%), of which 169 received methylprednisolone plus Tocilizumab (23.8%). Two out of 13 patients receiving etoposide were excluded because they were already intubated. A total of 11 patients (1.8%), 9 males and 2 females, with a median age of 58 (range, 41 to 79) were included. Clinical characteristics are shown in Table 1. Median PaO_2/FiO_2 at admission was 98 (range, 52 to 174). Following etoposide treatment, the PaO_2/FiO_2 ratio improved an average of 195% (Fig. 1). Three patients needed mechanical ventilation. Nine patients fully recovered and were finally discharged home. Two patients died as a consequence of thrombotic complications. Patient #4 markedly improved her respiratory function allowing extubation but developed massive cerebral ischemic stroke (cardiac ultrasound detected a large thrombus in the right atrium),

Table 1

	Patient1	Patient2	Patient3	Patient4	Patient5	Patient6	Patient7	Patient8	Patient9	Patient10	Patient11	Patient12	Patient13
Characteristic													
Age	56	41	63	79	60	70	55	57	64	58	79	73	42
Sex	male	male	male	male	female	male	male	female	male	male	male	male	male
Hypertension/obesity	No	No	No	Yes	Yes	No	No	Yes	No	Yes	Yes	Yes	Yes
pO2/FIO2 ratio	150	94	150	73	98	96	112	63	52	174	63	118	154
pCO2 (mmHg)	29	32	44	36	36	39	38	46	45	48	28	34	33
Ferritin (ng/mL)(**)	2020	1492	4014	4543	1316	1835	3232	3054	3359	2029	1697	3770	2665
CRP (ng/mL)	345	181	94	204	93	185	243	89	123	103	244	126	93
D-dimers (μg/mL)	3.4	5,5	1,8	7,8	16,5	1,1	1,2	1,9	2,3	0,3	2,8	10,3	0,6
Lymphocytes abs (x10^3/μL) (***)	0.3	0,4	0,4	0,1	0,3	0,6	0,6	1,8	0,5	0,5	0,2	0,4	0,1
Dose of etoposide (mg/m2)*	80	80	100	50	100	100	150	150	150	150	50	50	174
Total number of doses	1	1	2	1	2	1	2	2	2	2	2	1	1
Post etoposide pO2/FIO2	430	452	435	–	200	445	287	120	160	321	180	120	340
Etoposide administrated in ICU	YES	YES	NO	YES	YES	NO	NO	YES	YES	NO	YES	NO	NO
Mechanical ventilation	noninvasive	noninvasive	noninvasive	invasive	invasive	noninvasive	spontaneous	noninvasive	invasive	spontaneous	invasive	noninvasive	spontaneous
Outcome	Discharged	Discharged	Discharged	DNR	Death	Discharged	Discharged	Discharged	Discharged	Discharged	Hospitalized	Death	Discharged
Hospital stay (days)	15	15	13	11	13	14	7	20	5	5	15	14	32
Cytopenia >2 lines	NO	NO	NO	NO	YES	NO	NO	NO	YES	NO	NO	YES	NO
Infections	NO	NO	NO	NO	Enterococcus	NO	NO	NO	HSV-1	NO	NO	NO	NO

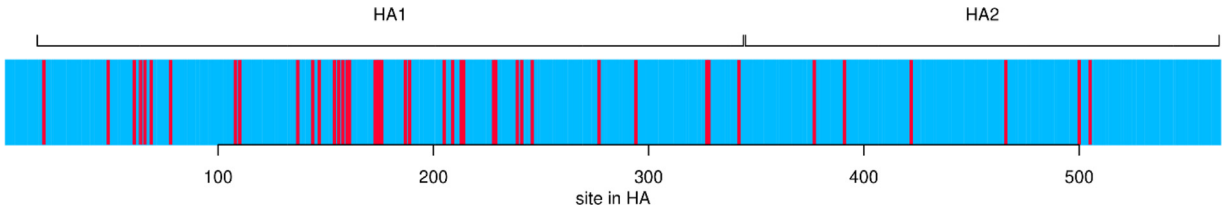

Fig. 1.

2 days after ventilation withdrawal, and died 16 days after admission. Patient #5 recovered from severe ARDS with profound leukopenia, was discharged from the ICU, and died suddenly at day 24, presumably due to massive pulmonary thromboembolism, although autopsy was not performed. Apart from hematological toxicity and infection in patient #5, no other adverse effects attributable to etoposide were observed and the tolerance was good.

Noticeably, in our experience, only 1–2 doses of etoposide were enough to observe clinical improvement among severely ill COVID-19 patients, in terms of inflammatory serum markers (ferritin, CRP, D-dimer), vasopressor therapy requirement and respiratory support. In fact, only 3 patients ultimately required intubation, yet 2 of which died. These preliminary results on 11 patients confirmed the safety and efficacy of etoposide as adjunctive salvage treatment for critically ill COVID-19 ARDS patients, exhibiting systemic hyperinflammation and previously treated with corticosteroids and interleukin inhibitors.

A growing evidence suggests that COVID-19 disease is a biphasic disease.[3,4,8] The initial stage, at which pre-symptomatic or pauci-symptomatic patients exhibit a preliminary and reversible state of immune-suppression associated to the viral load, ideally benefits from antivirals. Later on, patients may develop more severe leucopenia (mainly lymphopenia) along with increased inflammatory markers (CRP, ferritin, IL-6) that may end in a systemic hyperinflammatory state with accompanying cytokine discharge, accumulation of activated cells responsible for the lung damage, need for mechanical ventilation, thrombotic complications, and eventual death.[2,3,4,8]

Although corticosteroid therapy in COVID-19 remains controversial, recent studies suggest a clinical benefit for severely ill COVID-19 ARDS patients in terms of mortality rate, need for mechanical ventilation, and hospital stay.[9,10] Regarding oxygenation parameters, we observed that many severe COVID-19 patients presented with alarming PaO_2/FiO_2 ratios (commonly under 150, see Table-1) that, according to Berlin ARDS criteria, were immediate candidates for orotracheal intubation and mechanical ventilation. However, many of them exhibited a relatively preserved pulmonary function (mild dyspnea with or without tachypnea), showed preserved oxygen extraction and adequate organ perfusion without lactic acidosis, and ultimately avoided intubation. We hypothesize that SARS-CoV-2 related ARDS distinct pathophysiologic features permit management of many critically ill patients with non-invasive ventilatory support, waiting for the anti-inflammatory reversal effect of etoposide plus adjunctive immunomodulators.

The lack of comparison group and the low number of participants are obvious limitations of this study. Due to the severity of patients included in the study, with remarkable hyperinflammation data, all patients had received methylprednisolone and Tocilizumab or Anakinra prior to etoposide, so both drugs can be potential confounders in the interpretation of the results. However, etoposide was administered whenever patients did not respond to prior anti-inflammatory treatment, and at the stage of progressive organ dysfunction.

In this preliminary experience, salvage treatment with etoposide in adjunction to immunosuppressants resulted in overall favorable outcome of a small cohort of severely ill COVID-19 ARDS patients presenting with systemic hyperinflammation. The currently ongoing clinical trial NCT04356690, started on May 8, 2020, will likely contribute to evaluate the safety and efficacy of etoposide in COVID-19 patients.

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Breastfeeding Risk from Detectable Severe Acute Respiratory Syndrome Coronavirus 2 in Breastmilk

Dear editor,

An emerging severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), causing coronavirus disease 2019 (COVID-19) pandemic, imposes a great threat to global public health.[1] The transmission and pathophysiology of SARS-CoV-2 gradually known among various populations, but public health effects of COVID-19 on women and their outcomes should not be ignored.[1,2] In pregnant and perinatal women, vertical transmission of SARS-CoV-2 from an infected mother to her newborn is a controversial issue.[2-4] SARS-CoV-2 was not detected in vaginal fluid from 10 women with COVID-19[5], however, no clear evidence regarding optimal delivery timing and safety of vaginal or cesarean delivery preventing SARS-CoV-2 vertical transmission has been reported.[6] Thus, the management of COVID-19 in pregnancy based on obstetrical indications and maternal–fetal status is highly concerned. Here, we report clinical characteristics of COVID-19 pneumonia in puerperal women and evidence of SARS-CoV-2 shedding in her breastmilk.

Five hospitalized pregnant women clinically diagnosed with COVID-19 (according to the "pneumonia diagnosis protocol for novel coronavirus infection (trial version 5)", gave birth to their babies. Of the five women, four were admitted to the Renmin Hospital of Wuhan University, Wuhan, China, while 1 was admitted to the Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China from February 1 to March 25, 2020. The maternal information including clinical symptoms, epidemiological survey, puerperal data, radiological, and laboratory results, was obtained through electronic medical records or direct communication with patients and their families. SARS-CoV-2 infection of puerperal women was confirmed by series of investigations, such as clinical examination, laboratory tests, chest X-rays, and two independent RT-PCR tests. We used SARS-CoV-2 ORF1ab/N PCR detection kit (GeneoDx Biotech, Shanghai, China) for viral nucleic acid from nasopharyngeal swabs, vaginal secretion, and breastmilk, and SARS-CoV-2 antibody detection kit (YHLO Biotech, Shenzhen, China) for IgM-IgG antibody from blood serum, as previously reported.[2]

Between February 1 and March 25, 2020, five pregnant patients with COVID-19 were included to analyze this study (Table 1). The mean age of five mothers was 32 years (range 27 to 34 years), with the mean gestational age of 38 weeks plus 1 week (range 35 weeks to 40 weeks plus 1 week). All mothers' main onset symptoms were fever (40%), cough (20%), nasal congestion (20%), rhinorrhea (20%), poor appetite (20%), chest distress (40%), dyspnea (40%), and diarrhea (20%), that is consistent with clinical signs and symptoms, as previously described.[7] Chest CT scan of all patients (except Patient 4) before delivery showed typical viral pneumonia, such as patchy and scattered ground-glass opacities, and blurred borders. Four patients (80%) had cesarean section delivery, while one patient (Patient 4) (20%) delivered her infant in vaginal mode. During hospitalization (range 6 to 41 days), the outcomes of puerperal women patients and their neonates were good, and patients underwent laboratory tests, recorded in detailed information (Fig. 1A). Patient 3 with COVID-19 pneumonia had lymphopenia ($<1 \times 10^9$ cells per L), while the other four patients (80%) had low lymphocyte ratio except one case (Patient 1). All patients (100%) had elevated concentrations of C-reactive protein (CRP) (>10 mg/L) with below the normal range concentrations of Procalcitonin (PCT). Two (40%) had slightly increased concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). In addition, four patients (80%) had normal white blood cell (WBC) count except Patient 4, who had mild increased WBC count (Table 1). None of the patients had co-infection with other common respiratory viruses (enlisted in Table 1).

Five (100%) nasopharyngeal swab samples from patients were tested positive for SARS-CoV-2 RT-PCR. All the available vaginal secretion samples were negative for SARS-CoV-2 RT-PCR test, which is similar as previously reported.[5] During follow-up, three of four (75%) available serum samples from patients had significantly elevated concentrations of SARS-CoV-2 IgM and IgG (Table 1). More importantly, four out of five (80%) patient's breastmilk samples were negative for SARS-CoV-2 RT-PCR, which is similar to previous observations,[2,8] while one (20%) patient's (Patient 3) breastmilk showed SARS-CoV-2 RNA test positive (Table 1). Additionally, the breastmilk samples from Patient 3 after delivery for two and three days, remained positive for SARS-CoV-2 (Fig. 1B). Of note, the Ct value of RT-PCR test results was relatively high as 38.2 and 38.5 (Fig. 1B, a and b), suggesting the persistent presence of SARS-CoV-2 in human breastmilk from a patient with COVID-19.

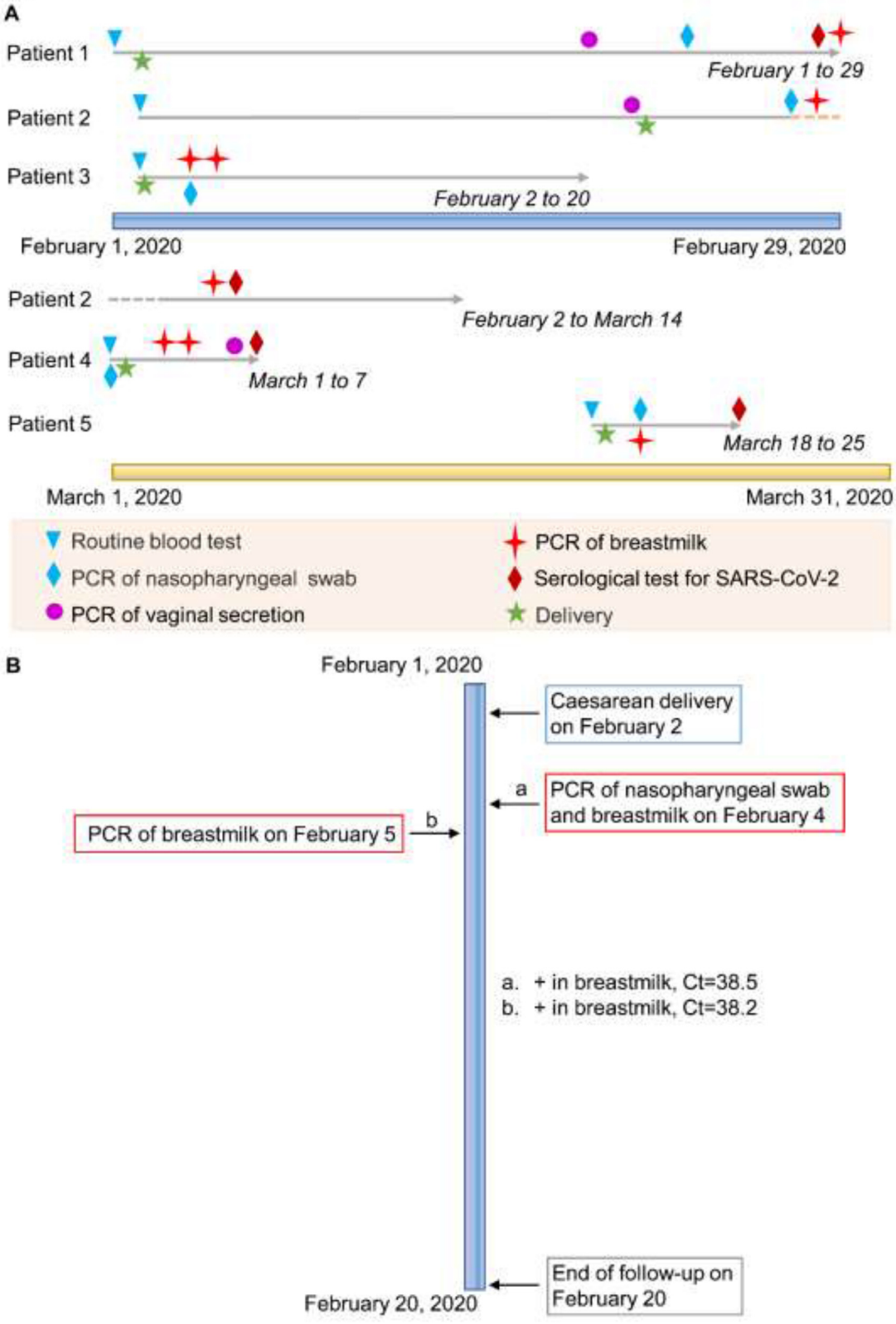

Fig. 1. Timeline of puerperal women with COVID-19 in hospital after onset of illness. **(A)** During hospitalization after onset of illness, the recorded events of all patients undergoing laboratory tests and delivery were marked with different diagrams on the indicated date. **(B)** Main records of Patient 3 during hospitalization. Real-time PCR against SARS-CoV-2 nucleic acid (shortened to PCR) was tested for breastmilk from Patient 3 after delivery for two (a) and three (b) days, respectively. +, positive result for SARS-CoV-2 nucleic acid test; -, negative result for SARS-CoV-2 nucleic acid test; Ct, Curve threshold value of SARS-CoV-2 N gene.

Table 1
Summary of clinical features and laboratory results of five puerperal patients with COVID-19

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Characteristics	..	..	..	..	..
Age (years)	29	29	34	27	32
Interval between admission to hospital and symptom onset	9 days	6 days	8 hours	8 days	1 day
Interval between delivery and admission to hospital	1 day	20 days	3 hours	10 hours	6 hours
Gestation age (weeks)	35^{+5}	35	40	38^{+2}	40^{+1}
Delivery mode	cesarean	cesarean	cesarean	vaginal	cesarean
CT findings	Patchy ground-glass opacities in both lungs	Scattered ground-glass opacities in both lungs	Blurred borders in left lung	Normal	Blurred borders in upper lobe and lower lobe of right lung
Symptoms and signs	..	..	..	..	..
Fever	-	+	+	-	-
Cough	+	-	-	-	-
Nasal congestion	-	+	-	-	-
Rhinorrhoea	-	+	-	-	-
Poor appetite	+	-	-	-	-
Chest distress	+	+	-	-	-
Dyspnea	+	+	-	-	-
Diarrhoea	+	-	-	-	-
Body temperature (°C)	36.0	37.9	37.8	37.2	36.8
Clinical course	..	..	..	..	..
Duration of fever	0	6 days	8 hours	0	0
Duration of hospitalization (days)	28	41	18	6	6
Laboratory test	..	..	..	..	..
White blood cell count, $\times 10^9$/L (normal range: 3.5-9.5)	4.28	8.03	6.72	10.06	7.95
Neutrophil count, $\times 10^9$/L (normal range: 1.8-6.3)	2.68	6.57	5.37	7.71	6.44
Neutrophil ratio, % (normal range: 40-75)	68.30	81.9	80	76.60	80.90
Lymphocyte count, $\times 10^9$/L (normal range: 1.1-3.2)	1.01	1.08	0.97	1.64	1.08
Lymphocyte ratio, % (normal range: 20-50)	23.60	13.4	14.4	16.30	13.6
CRP, mg/L (normal range: 0-10)	53.2	57	11.5	74.8	43
PCT, ng/mL (normal range: <0.1)	0.075	0.086	0.03	0.004	0.003
ALT, U/L (normal range: 7-40)	13.0	40	50	13.0	15
AST, U/L (normal range: 13-35)	26.0	38	37	17.0	20
PCR of nasopharyngeal swab	+ Ct=36.8	+ Ct=33.3	+ Ct=37.2	+ Ct=36.1	+ Ct=34.3
PCR of vaginal secretion	-	-	NA	-	NA
PCR of breastmilk	-	-	+	-	-
SARS-CoV-2 IgG, AU/mL (normal range: <10)	128.79	107.89	NA	7.59	63.85
SARS-CoV-2 IgM, AU/mL (normal range: <10)	77.42	279.72	NA	0.62	20.96
ADV DNA	-	-	-	-	-
Boca DNA	-	-	-	-	-
H1N1 RNA	-	-	-	-	-
H3N2 RNA	-	-	-	-	-
HCOV RNA	-	-	-	-	-
HMPV RNA	-	-	-	-	-
HPIV RNA	-	-	-	-	-
HRSV RNA	-	-	-	-	-
HRV RNA	-	-	-	-	-

NA=not available; +=positive; -=negative; CRP=C-reactive protein; PCT=Procalcitonin; ALT=Alanine aminotransferase; AST=Aspartate aminotransferase; PCR, short for Real-time PCR against SARS-CoV-2 nucleic acid; Ct=Curve threshold value of SARS-CoV-2 N gene; ADV=Adenovirus; H1N1=Influenza virus A, H1N1; H3N2=Influenza virus A, H3N2; HCOV=Human seasonal coronavirus; HMPV=Human metapeumovirus; HPIV=Human parainfluenza virus; HRSV=Human respiratory syncytial virus; HRV=Human rhinovirus

In brief, SARS-CoV-2 causes milder COVID-19 in children as compared to adults,[9] while newborns are still vulnerable to SARS-CoV-2 infection through the maternal–fetal transmission. The breastmilk (containing antibodies and other antimicrobial factors) feeding to infants safely is highly concerned in puerperal women with COVID-19[7]. it's hard to ignore SARS-CoV-2 infection risk factors in breastfeeding.

Although some human milk samples from SARS-CoV-2 infected mothers in China resulted negative in puerperal stage,[2,8] safe breastfeeding should be encouraged according to standard infant feeding guidelines and necessary precautions for IPC (infection protection and control),[10] as breastmilk is rich in essential antibodies and nutrients to increase infant's immunity against infectious diseases. The existence of SARS-CoV-2 in breastmilk from COVID-19 puerperal patients highlights the risk of virus transmission through breastfeeding.

Based on our observations, the clinical characteristics of puerperal women (Patient 3) with breastmilk positive results for SARS-CoV-2 on two and three days post-delivery, were similar to those puerperal women having breastmilk negative results for SARS-CoV-2, which provides focused evidence of SARS-CoV-2 persistently presence in breastmilk from COVID-19 women. In fact, this study is limited by small sample size and retrospective method. Some considerations should be taken into account when interpreting the findings, such as the dynamic presence of SARS-CoV-2 in breastmilk or confirmation of live SARS-CoV-2 in breastmilk.

Collectively, we reported detectable SARS-CoV-2 nucleic acid in human breastmilk from a puerperal woman with COVID-19. Although our conclusions are limited by the small sample size, we believe our findings are important for the concern of SARS-CoV-2 infection risk in breastfeeding of mother with COVID-19 to her neonate.

Authors' Contributions

Conception and design: C Zhu, W Liu, Z Luo, and Y Xia, Obtaining written consent from patients and ethical approval, collecting

samples: C Zhu, W Liu, Z Luo, and Y Xia, Acquisition, analysis, or interpretation of data: H Su, S Li, M Shereen, Z Lv, Z Niu, D Li, and F Liu, Confirming data accuracy: S Li, M Shereen, Z Lv, Z Niu, D Li, F Liu, Z Luo, and Y Xia, Drafting the manuscript: C Zhu, W Liu, and H Su, Revising the manuscript: Z Luo, and Y Xia, All authors had approved the final version of manuscript to be published, and agreed to be accountable for all aspects of the work.

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Disclosure statement

We declare no competing interests.

Ethics

The study was approved by the Ethics Committee and Institutional Review Board of the Renmin Hospital of Wuhan University (file no. WDRY2020-K066), and the Tongji Hospital of Huazhong University of Science and Technology (file no. TJ-IRB20200201). Written informed consent was obtained from each enrolled patient.

The data in this study can be provided after the Article is published with the permission of the corresponding authors. We can provide participant data without names and identifiers, but not the study protocol, statistical analysis plan, or informed consent form through an appointed email address for communication. The corresponding authors have the right to decide whether to share the data or not regarding to the research objectives and plan provided.

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Temperature-dependent surface stability of SARS-CoV-2 ☆

Dear Editor,

The ongoing severe respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic puts a large strain on public healthcare systems worldwide. Given that effective treatments and vaccines are not yet available, it is of utmost importance to elucidate potential routes of SARS-CoV-2 transmission to device effective transmission-based precautions. Ye et al. recently described SARS-CoV-2 contaminated surfaces in COVID-19 patient care areas in a hospital environment.[1] Moreover, infectious virus has been shown to persist on surfaces for several hours to days at room temperature (RT).[2] Despite the current consideration of respiratory droplets as the main route of SARS-CoV-2 transmission,[3] contaminated surfaces could indicate the possibility of surface contact transmission. Importantly, temperature variation has been shown to influence the surface stability of SARS-CoV[4] and moreover, recently differences on temperature-dependent SARS-CoV-2 stability in solution were reported.[5] This raises the question whether seasonal changes which are accompanied by temperature fluctuations might actively influence virus stability. We examined the stability of SARS-CoV-2 on inanimate surfaces at 4°C, RT and 30°C in order to understand seasonal temperature variation of possible surface transmission. Surface stability over time was assessed with a carrier test on metal discs for 4h, 8h, 24h and subsequently every 24h up to 9 days at a humidity of 30-40%. Virus suspensions were mixed with 0.3 % bovine serum albumin (BSA) as interfering substance

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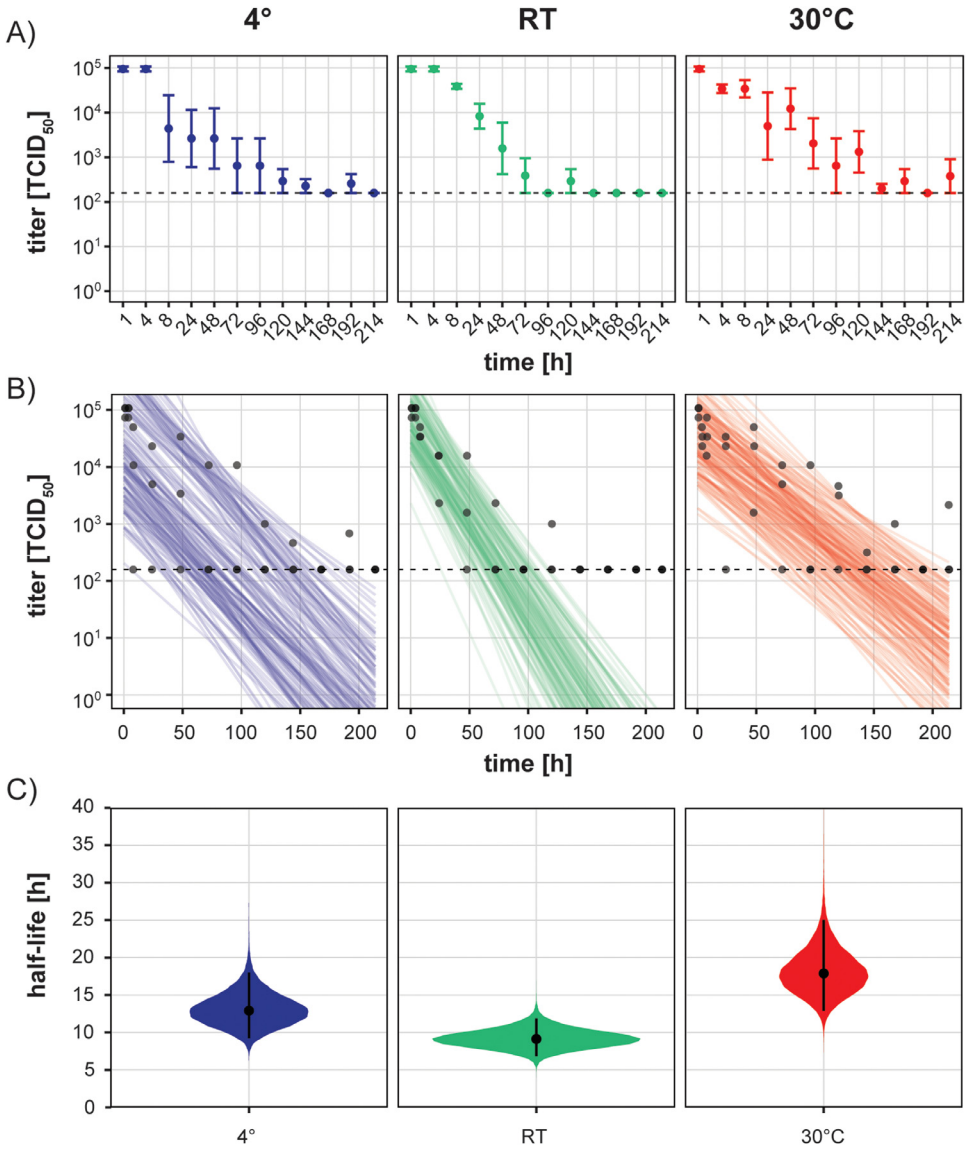

Fig. 1. Temperature-dependent Infectivity of SARS-CoV-2 on inanimate surfaces. A) Measured $TCID_{50}$/mL values of recovered virus at indicated timepoints in hours at 4°C (blue panel), room temperature (green panel) and 30°C (red panel). Dots indicate mean of three independent experiments along with the standard error shown as bars. Dashed lines mark the lower limit of quantification. B) Regression plots indicating the predicted decay of virus over time. Dots (partially off-set along the x-axes to reduce overlap) show individual $TCID_{50}$/mL values of single experiments. Fifty lines per replicate represent possible decay patterns for each experimental condition[2]. C) Violin plots of estimated half-life ranges of the virus at indicated temperatures. The dots indicate the posterior median estimates with 95% confidence intervals.

and dried on metal discs for 1h at RT. Initial virus (SARS-CoV-2/München-1.1/2020/929) concentration was 1.58×10^7 50% tissue culture infectious dose per milliliter [$TCID_{50}$/mL] and declined to 9.63×10^4 $TCID_{50}$/mL after 1h drying. At each individual time point after drying, the inoculated area was incubated for 1 min with sterile water and subsequently mixed with cold Dulbecco's modified minimal essential medium. The resulting suspension was serially diluted, and the $TCID_{50}$/mL values were determined by crystal violet staining. Half-lives and decay rates of viable virus were calculated using a previously published Bayesian regression model[2] (https://github.com/dylanhmorris/sars-cov-2-stability). Since the results of the model are strongly depended on the values assumed for the initial inoculum, we used the titers of the dried virus at t=1h as initial values.[2] In contrast to the high stability of SARS-CoV-2 in solution[5] the infectivity of the virus was strongly reduced upon drying. After 1h of drying on a metal disc, the measured viral titers were reduced up to 100-fold. However, after the initial loss of infectivity, the recovered virus titers remained stable over the next 4h to 8h with only minimal decline at 30°C and a larger variability at 4°C (Fig. 1A). Beyond 8h we observed a stable, slow decline of viral titers at all temperatures over several days (Fig. 1A). We were able to recover detectable amounts of infectious virus even after 180h on metal surface. At all temperatures tested, we observed an exponential decay rate, which prompted us to use a previously developed algorithm to model possible decay rates and estimates for viral half-lives under the tested circumstances (Fig. 1B). Due to a higher variance in actually measured titers at 4°C and 30°C, the modelled regression lines follow a rather broad spectrum also mirrored in the greater confidence intervals for predicted half-lives (Fig. 1C). We estimated the half-lives of the three different ambient temperatures (Fig. 1C). At room temperature the median half-life is predicted to be 9.1h and thereby slightly higher than previous reports.[2] These differences most originate from different initial titers of the inoculum and different experimental setups .The decay rate at 4°C was slower with an estimated median half-life of 12.9h. Surprisingly, virus incubation at 30°C after drying showed the highest predicted half-life with 17.9h. Overall, our results demonstrate that SARS-CoV-2 infectivity is strongly reduced during the initial drying process; however, afterwards the virus remains infectious in a dried state for several days regardless of ambient temperature changes. Of note, one caveat of this study is the constant humidity. Previous studies have shown that CoV survival on inanimate surfaces was dependent on the humidity with high (80%) and low (20%) humidity increasing survival compared to medium (50%) humidity.[6]

We found that the surface stability of SARS-CoV-2 does not display major differences at 4°C, RT and 30°C. Our results challenge the previously suspected temperature-dependent virus surface stability, especially with regard to seasonality of the SARS-CoV-2 transmission. Our data, as well as other models implicate that higher temperatures (up to 30°C) do not necessarily inactivate SARS-CoV-2.[2] Nevertheless, other human and environmental factors such as viral load, humidity, and solar radiation which were not considered in our controlled laboratory settings might further influence SARS-CoV-2 surface stability and thus cause variations in seasonal SARS-CoV-2 surface transmission.

Contributor's Statement

A. Kratzel, S. Steiner: study design, data collection, data interpretation, writing; D. Todt: data analysis, data interpretation, figure, writing; P. V'kovski: study design, data collection; Y. Brueggemann: study design, writing; J. Steinmann: study design, writing; E. Steinmann: study design, writing; V. Thiel: study design, writing; S. Pfaender: study design, data interpretation, writing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Retrospective screening for SARS-CoV-2 in Greater Glasgow and Clyde ICUs between December 2019 and February 2020

Dear Editor,

On 31st December 2019, the World Health Organization was alerted to cases of pneumonia of an unknown aetiology in Wuhan City, China [1]. The novel virus responsible, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has subsequently caused a pandemic.

Global health surveillance was quickly implemented to identify cases of COVID-19. Although effective, a study by Deslandes et al. [2] indicated potential virus circulation prior to cases first detected via surveillance. The study detailed a Parisian patient with no recent travel history admitted to ICU, who retrospectively tested positive for SARS-CoV-2 [2]. The respiratory sample in question was taken approximately one month before it is believed the epidemic began in France [2].

The first Scottish positive case was confirmed on 1st March, in a patient who had travelled to Italy [3]. At this time sampling was recommended only for individuals who had travelled to an epidemic region and displayed symptoms. This screening criteria may have missed community cases. To investigate further, we retrospectively tested respiratory samples from Greater Glasgow and Clyde (GGC) which had been sent to the West of Scotland Specialist Virology Centre (WoSSVC).

Respiratory samples were selected which had been submitted from adult ICUs, HDUs and CCUs, across three hospital sites in NHS GGC between 1st December 2019 and 28th February 2020 (none appropriate for 29th February). 206 samples were accepted for extended respiratory screening from 164 patients. 160 had not received a SARS-CoV-2 test as part of the initial respiratory screen. Four were negative. Following routine testing these samples had been stored at −80 °C.

For each adult patient ($n = 160$), we tested the initial sample, unless a superior sample type was received within 48 h e.g. samples from the lower respiratory tract (LRT) rather than upper respiratory tract (URT) [4], or the sample was previously inhibited or insufficient. If all samples from a patient were insufficient, previously inhibited or unavailable then the patient was excluded ($n = 12$). In two instances, two samples were tested from the same patients following admission to two different wards several weeks apart. This resulted in our testing 150 samples from 148 adult patients (Fig. 1).

The initial extended respiratory screen was negative for 96 samples and positive for 54 (Table 1). A range of respiratory samples were included – 99 from URT (gargle $n = 43$, nasopharyngeal aspirate $n = 3$, and nose/nose and throat/throat swab $n = 53$),

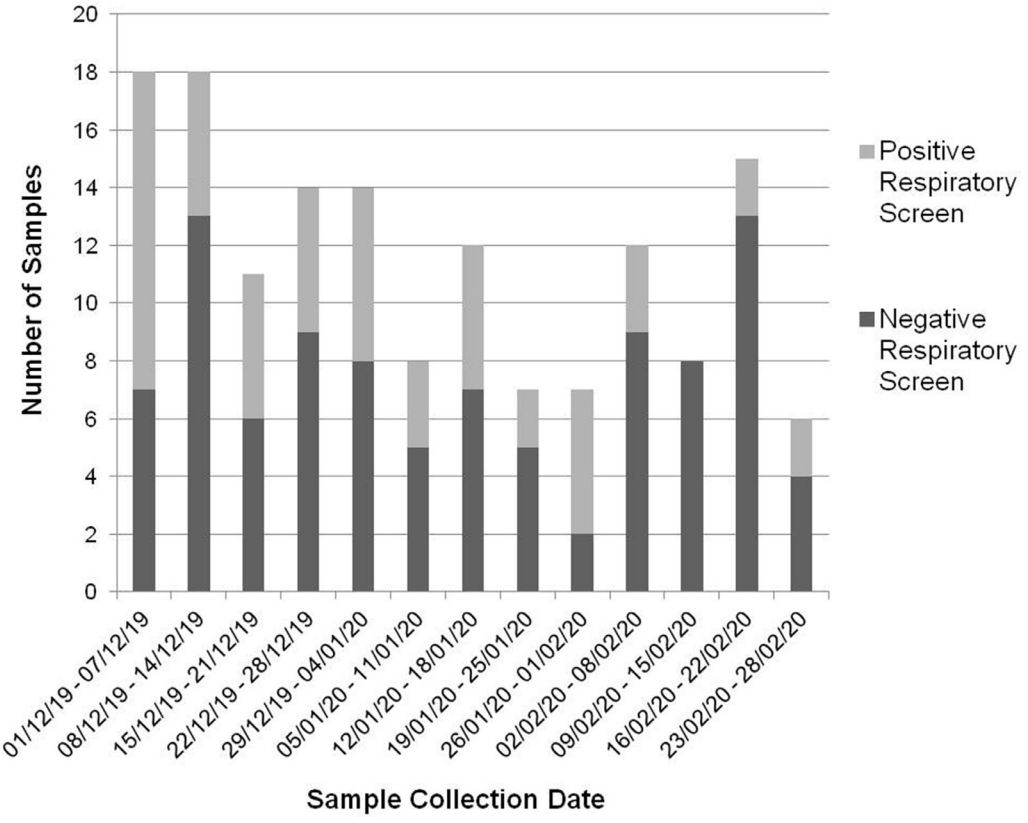

Fig. 1. Summary of the 150 respiratory samples from 148 adult ICU patients, with positive and negative respiratory screening results, which were then retrospectively tested for SARS-CoV-2.

Table 1
Summary of respiratory screening results for the 150 adult samples included in the retrospective study. URT: Upper respiratory tract sample. LRT: lower respiratory tract sample.

Extended respiratory screen target	Number of samples (% total samples)	URT sample	LRT sample
rhinovirus	12 (8.00)	8	4
respiratory syncytial virus	10 (6.67)	3	7
influenza A or B	9 (6.00)	6	3
seasonal coronavirus	6 (4.00)	5	1
parainfluenza 1,2,3,or 4	4 (2.67)	1	3
human metapneumovirus	3 (2.00)	3	0
adenovirus	2 (1.33)	1	1
Mycoplasma pneumonia	1 (0.67)	1	0
2 respiratory pathogens	6 (4.00)	3	3
3 respiratory pathogens	1 (0.67)	0	1
Total number of samples with positive respiratory screen	54 (36)	31	23
Total number of samples with negative respiratory screen	96 (64)	68	28
Total number of samples tested	150 (100)	99	51

and 51 from LRT (bronchial aspirate $n = 11$, bronchoalveolar lavage $n = 5$, and sputum $n = 35$).

A sub-set of samples from paediatric ICU patients were included, which had no significant virological or microbiological results two weeks prior to sample collection. 24 samples, from 23 patients were collected between 4th December 2019 and 28th February 2020, 13 being URT and 11 being LRT samples.

Samples were extracted using the Abbott *M*2000*sp* instrument (Abbott Laboratories, Illinois) or the NUCLISENS easyMAG (Biomérieux, Marcy-l'Étoile). Sample extracts and appropriate controls underwent real-time RT-PCR, using primers and probes for RNA-dependant RNA polymerase (RdRp) and envelope (E) genes [5]. In-house verification demonstrated a limit of detection of 1000 copies/mL for RdRp gene and 200 copies/mL for E gene. Samples requiring confirmation were re-tested using the Roche cobas SARS-CoV-2 Test to detect open reading frame 1a (ORF 1a) and E genes (Roche Diagnostics, Basel) as it had similar sensitivity to that of the E gene assay.

Of the 174 samples tested, 166 were negative for SARS-COV-2 using real-time RT-PCR to detect RdRp and E genes. The remaining eight samples which were RdRp gene negative but had spurious traces on the E gene assay were re-tested using the cobas and also deemed negative.

SARS-CoV-2 was not detected in >90% of the relevant adult ICU population prior to March 2020. It is unlikely, therefore, that patients were hospitalised before March 2020 with clinically significant respiratory symptoms due to SARS-CoV-2. The use of two different PCR gene targets reduced the likelihood of false-negatives through primer/probe mismatches. Additionally, SARS-CoV-2 was absent in our sub-set of paediatric ICU samples. This is unsurprising given the sample-set size and as severe COVID-19 occurs in a low percentage of children [6].

These findings differ from those of Deslandes et al. [2] who tested 14 nasopharyngeal samples taken between 2nd December 2019 and 16th January 2020. The authors detected one positive from late December [2]. Additionally, Hogen et al. [7] retrospectively tested 2888 nasopharyngeal and bronchoalveolar lavage samples taken between January and February in San Francisco. Two were positive from late February, which overlapped with the first reported cases in the nearby area [7]. However, Hogan et al. [8] demonstrated no positives in 1700 samples retrospectively tested from November to December 2019.

The results support the initial Scottish SARS-CoV-2 testing strategy. Retrospective testing of a larger set of samples is necessary to fully rule-out early community transmission. However, the benefit must be balanced with existing limitations in reagents and current testing demands. Sample pooling could overcome this, and although less sensitive, would allow economical surveillance of a larger population to inform Scottish transmission dynamics prior to March 2020 [7,8].

There are limitations in this study. Firstly, the initial storage of the samples may have impacted detection [9]. Long-term storage at −80 °C is, however, unlikely to have been detrimental. Secondly, testing was not exhaustive. Presentation of COVID-19 in GGC ICUs prior to March 2020 cannot be ruled out without blanket testing.

Additionally, only one sample was retrospectively tested for the majority of patients included. Significantly higher rates of SARS-CoV-2 RNA detection is found in LRT samples, compared with URT [4]. Thus, with only a third of tested samples being of the LRT, false-negative results could have occurred for the remaining two thirds. Specifically, gargle samples may have been less appropriate. Gargles have, however, been demonstrated to be a suitable alternative to sputum for SARS-CoV-2 detection [10]. Finally, samples were only tested from patients hospitalised in three GGC sites. The lack of SARS-CoV-2 positivity cannot, therefore, be assumed for those in ICUs in the rest of Scotland, and we cannot rule-out community transmission.

In summary this retrospective study demonstrated no SARS-CoV-2 positivity in the 174 selected respiratory samples collected from GGC ICUs prior to the first reported Scottish case.

Declaration of Competing Interest

None.

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Data Statement

Research data is confidential.

Ethics Approval

None required.

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The stress-inducible molecular chaperone GRP78 as potential therapeutic target for coronavirus infection

Dear Editor,

The current coronavirus pandemic has become the greatest threat to global public health, thus there is an urgent need for identifying therapeutic targets. A recent report in this journal by Ibrahim and colleagues describing the potential binding interaction between the SARS-CoV-2 spike protein and the host 78-kDa glucose regulated protein (GRP78) raised the possibility that GRP78 could be a facilitator for viral entry[1] and disruption of such interaction may be used to develop novel therapeutics specific against this virus.[2] Our laboratory has a longstanding interest in the regulation and function of GRP78, which is a stress-inducible, multifaceted chaperone protein serving critical functions in the endoplasmic reticulum (ER) and other cellular compartments, impacting both health and disease.[3,4]

The ER is the major site of synthesis, folding, and maturation for membrane and secretory proteins. When the folding capacity of the ER is overwhelmed due to increased protein synthesis, the cell undergoes ER-stress which activates the Unfolded Protein Response (UPR), a complex network of signaling pathways aiming to restore ER homeostasis or trigger apoptosis depending on context, duration, and intensity of the stress.[3] GRP78, also referred to as BiP/HSPA5, is a master regulator of the UPR, and is upregulated upon ER stress to alleviate proteotoxic stress. As such, GRP78 has emerged as a key target to combat diseases, like cancer, where uncontrolled cellular proliferation causes ER overload leading to UPR activation.[3] Interestingly, viral infection also creates ER stress and triggers the UPR.[5] As outlined below, GRP78 is an important host factor for viral infection and targeting GRP78 has the potential to disrupt multiple stages of the viral life cycle including entry, production and subsequent cellular infection (Fig. 1).

GRP78 has been reported to facilitate viral entry for a wide variety of viruses, including human and bat coronaviruses[6] (Table 1). The role of GRP78 in these studies was investigated through the use of siRNA targeting GRP78, antibody against GRP78, proteolytic cleavage of GRP78 by SubAB, as well as small molecule AR12 and natural product EGCG both of which inhibit the ATPase activity of GRP78.[3,6,7] How might GRP78, normally residing in the ER, facilitate viral attachment onto host cells? Upon ER stress, including coronavirus infection, a fraction of GRP78, an abundant ER luminal protein, is actively translocated from the ER to the cell surface and assume new functions, including viral entry[3,4,6,8] (Fig. 1). In the case of MERS-CoV and bCoV-HKU9 coronaviruses, their spike proteins bind to cell surface GRP78 (csGRP78) in addition to their cognate receptors.[6] Thus, csGRP78 may enhance viral entry by stabilizing the interaction between host and viral factors required for viral entry, which is consistent with our recent observations that csGRP78 can interact with and stabilize cell surface receptors such as CD44 and CD109.[8,9] Furthermore, in cell types where the primary viral receptor expression is low, csGRP78 may serve as an alternative host factor for viral entry. Future studies are required to test out these concepts, as well as to establish whether GRP78 is a critical host factor for SARS-CoV-2 entry. The notion that upregulation of GRP78 on the surface of virally infected cells can be exploited to direct antiviral and immunomodulatory drugs to cell populations infected by SARS-CoV-2 is also worthy of investigation.

Beyond viral entry, GRP78 can play a major role in viral protein synthesis and maturation (Table 1). Viruses are obligate intracellular parasites which depend primarily on the cellular machinery to manufacture their proteins required for virion production, assembly, and budding. Additionally, many viruses including SARS-CoV-2 are enveloped by a lipid bilayer containing viral glycoproteins on its surface to bind host cell receptors to facilitate their entry. Since these viral envelope proteins are membrane-embedded, they are synthesized and processed in the ER. Unlike cellular protein synthesis, which is tightly regulated to maintain homeostasis, viruses, such as coronavirus, can selectively shut down host protein production and usurp the host ER translational machinery to synthesize the viral proteins in massive quantities. This results in ER overload, leading to ER stress and UPR activation. Consequently, ER stress and GRP78 upregulation have been reported during infection by a wide variety of viruses.[5–7] In addition to its role in viral protein folding, GRP78 upregulation during viral replication could protect the virus-infected host cells from undergoing apoptosis since GRP78 is known to bind and maintain the ER-associated apoptotic machineries in their inactive forms and exert pro-survival effects especially under ER stress.[3] These features make the ER a particularly important cellular compartment for viral production and viruses have evolved complex mechanisms to exploit and manipulate the ER to enhance their replication. Conversely, the depen-

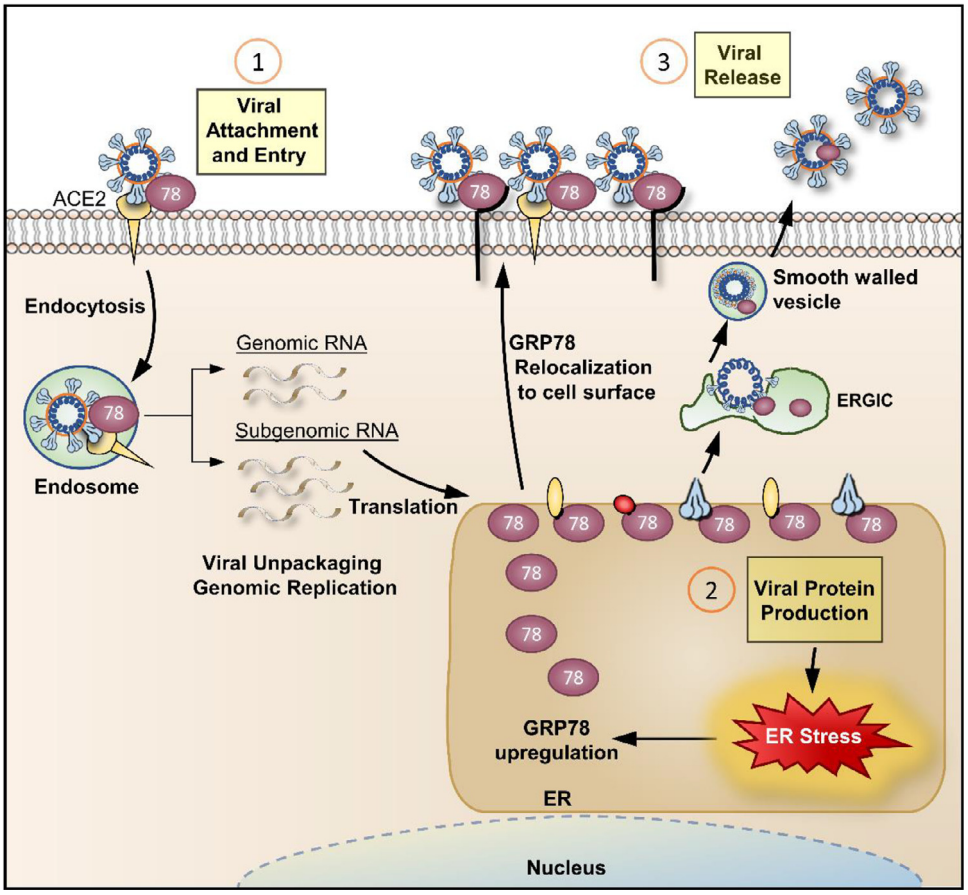

Fig. 1. *Potential roles of GRP78 in the viral infection cycle.* Virus life cycle consists of three essential stages: (1) viral attachment and entry, (2) viral protein production and (3) viral release and re-infectivity. GRP78 potentially plays important roles in all three stages. During viral attachment and entry, cell surface GRP78 may stabilize the interaction between the viral spike protein and the cellular host receptor to facilitate entry or serve as alternative host factor for viral entry. During active viral replication and protein production, ER-localized GRP78 aids in the proper folding and processing of viral proteins as well as maintaining ER homeostasis, providing a conducive cellular environment for viral assembly and maturation. ER stress induced by viral infection could also drive cell surface GRP78 translocation, further promoting viral entry. During final viral assembly and budding from the ER-Golgi intermediate compartment (ERGIC), GRP78 may be associated with the viral particles and released together with mature virions to enhance their infectivity as an accessory host factor.

Table 1
Effects of anti-GRP78 agents in the viral life cycle. Anti-GRP78 agents have been shown to interfere with entry and production of a wide range of viruses spanning many different virus families. Examples in each virus family are shown and the anti-GRP78 agents used in the published studies were as follows: (a) siRNA against GRP78; (b) antibody against GRP78; (c) proteolytic cleavage of GRP78 by subtilase cytotoxin (SubAB); (d) small molecule AR12; and (e) natural product epigallocatechin gallate (EGCG).

Family	Virus	Steps Inhibited by anti-GRP78 agents	Anti-GRP78 agents
Coronaviridae	Bat coronavirus HKU9	Entry	a,b
	Middle East respiratory syndrome coronavirus	Entry	a,b
Filoviridae	Ebola Virus	Entry, Production	a,d,e
Flaviviridae	Dengue Virus	Entry, Production	a,c,d
	Zika Virus	Production	a,d,e
	Japanese Encephalitis Virus	Entry, Production	a,b,c
Orthomyxoviridae	Influenza Virus	Production	a,d
Retroviridae	Human Immunodeficiency Virus	Production	d
Papillomaviridae	Human Papilomavirus	Production	a
Picornaviridae	Coxsackievirus	Entry, Production	a,b,d
Herpesviridae	Human Cytomegalovirus	Production	c,d
Polyomaviridae	Simian Vacuolating Virus 40	Production	a,c,d

dence of viruses on the ER and its key resident chaperone GRP78 for viral protein production and host cell survival could be the virus' Achilles heel and offers a unique opportunity for combating SARS-CoV-2 and other virus infections.

The last step in a successful viral life cycle is the release of progeny virions to infect new cells. Here, GRP78 may also be critical for viral infectivity. Firstly, GRP78 depletion during viral replication could lead to reduced synthesis or improper folding of viral proteins resulting in impaired budding or immature virions with diminished infectivity. Secondly, GRP78 could facilitate the assembly of various viral components by maintaining ER homeostasis and thus provide a conducive environment for virus maturation. Lastly, GRP78 could be captured into the viral particles and could enhance subsequent cellular infection. Indeed, it has been reported

that GRP78 was found in Japanese encephalitis virus particles and mature virions that lacked GRP78 displayed significant decrease in viral infectivity.[10] It will be interesting to determine the topology of GRP78 in these virions and the generality of this interesting and surprising observation.

In conclusion, we hope that the current scientific evidence presented here and our perspectives will stimulate further interest in GRP78 as a promising target and expand the emerging development of anti-GRP78 agents in the fight against SARS-CoV-2 and viral infection in general.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Elevated nucleoprotein-induced interferon-γ release in COVID-19 patients detected in a SARS-CoV-2 enzyme-linked immunosorbent spot assay

Dear Editor,

The mechanisms underlaying the host defense against SARS-CoV-2 remain largely unknown. The relative contribution and importance of the innate, humoral and cellular immune response have to be elucidated to improve our understanding of coronavirus disease 2019 (COVID-19) pathogenesis and to aid vaccine development. In a recent article in the *Journal*, Demey and colleagues presented data on four lateral flow assays (LFIA) for the detection of antibodies directed against SARS-CoV-2.[1] They assessed the kinetics of antibody appearance using these assays in 22 patients after they were tested positive by RT-PCR. They reported a sensitivity of 100% on day 15 post onset of symptoms.

In addition, various other studies suggest a suppressed T-cell immunity in patients with severe COVID-19 based on decreased T-cell numbers or abnormal interferon gamma (IFN-γ) expression by T-lymphocytes detected by flowcytometry.[2–4]

The objective of the present study was to determine the cellular and humoral immunity of cases with various levels of COVID-19 disease severity. The functional T-cell responses to two SARS-CoV-2 antigens (the mosaic surface protein and the nucleoprotein) were measured by using an inhouse enzyme-linked immunosorbent spot (ELISpot) interferon-γ release assay (see supplementary information for method), in 27 patients with confirmed COVID-19 and 16 healthy controls. Patienst were confirmed by using reverse transcriptase polymerase chan reaction (RT-PCR). Of the 27 COVID-19 patients, nine were included from the intensive care unit (ICU) and 18 from the pulmonary ward. The moment of sampling varied from six to 32 days post onset of symptoms. In addition, the concomitant humoral immune response was assessed by detection of SARS-CoV-2 specific IgA and IgG antibodies, directed against the structural protein (S1 domain) of SARS-CoV-2, with a commercial enzyme-linked immunosorbent assay (ELISA) (EUROIMMUN Medizinische Labordiagnostika AG, Lv̈beck, Germany).

The SARS-CoV-2-specific T-cell response measured in the ELISpot induced by the mosaic surface protein and the nucleoprotein showed different patterns. In all but one of the 27 COVID-19 cases, the T-cell response against the mosaic surface protein was absent or weak, with ELISpot results with less than 20 spot forming cells (SFC). The outlier was recruited from the pulmonary ward and exhibited 45 SFC (Fig. 1a). In contrast, the T-cell response against the nucleoprotein measured by the ELISpot assay was elevated (10–150 SFC) in 12 of 19 patients (63%) that were sampled at $\geq$14 days post onset of symptoms (Fig. 1b). A subgroup of 9 (Fig. 1b, red oval) showed a delayed or reduced T-cell response against the nucleoprotein, compared to the other patients. Five of these showed practically no response, and four showed a weak response (10–20 SFC) at 18–32 days post onset of symptoms.

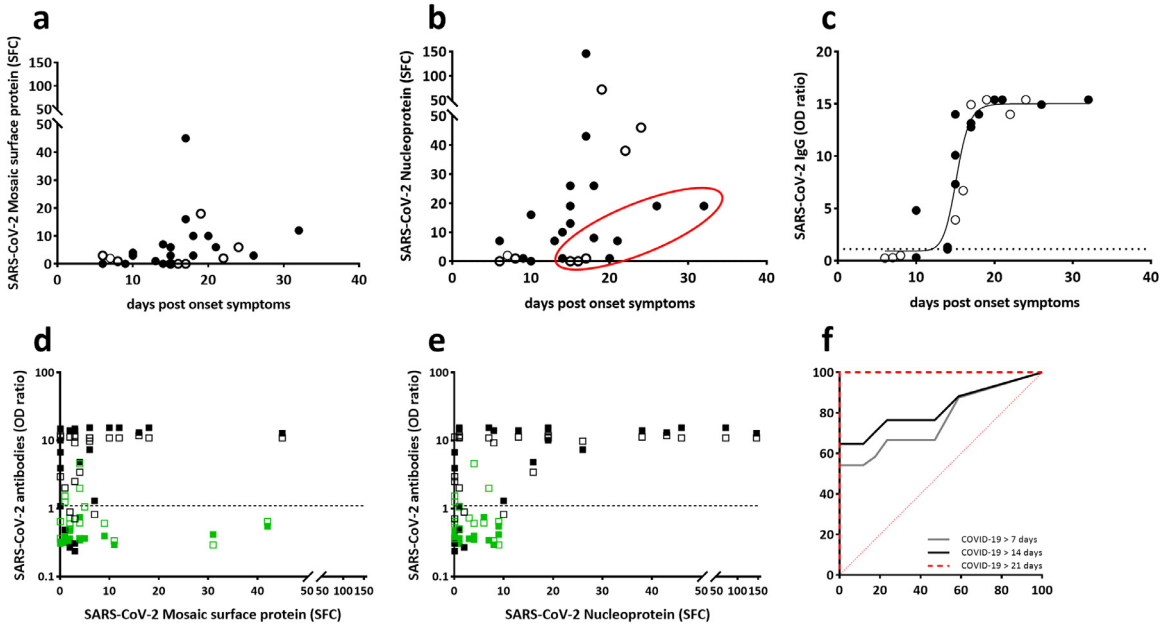

Fig. 1. SARS-CoV-2 ELISpot mosaic surface protein (a) and nucleoprotein (b) IFN-γ spot forming cells (SFC) in relation to days post onset of symptoms. SARS-CoV-2 specific IgG antibody response in COVID-19 patients versus days post onset of symptoms (c). Open and closed circles represent COVID-19 patients from the ICU and the pulmonary ward, respectively (a–c). The red oval encloses patients which seem to have a delayed or reduced T-cell response (b). Correlation between T-cell reactivity (SFC) against the SARS-CoV-2 mosaic surface protein (d) and the SARS-CoV-2 nucleoprotein (e) and concomittant SARS-CoV-2 antibody responses (IgA open symbols, IgG closed symbols) in COVID-19 patients (black symbols) and healthy controls (green symbols). The broken line represents the cut-off of the SARS-CoV-2 antibody ELISA. Fig. 1f depicts the ROC analyses of the SARS-CoV-2 nucleoprotein ELISpot results in COVID-19 patients at >7 days, >14 days and >21 days post the onset of symptoms versus healthy controls.

Absolute lymphocyte numbers loaded in the SARS-CoV-2 ELISpot were not significantly different between the normal and the delayed or reduced responders (data not shown). However, the number of spot forming cells following stimulation with the mitogen control was also significant lower in the delayed or reduced responders ($P < 0.001$) (see supplementary Figure 1). Moreover, SARS-CoV-2 IgA and IgG antibody levels did not differ between the normal and delayed or reduced responders (data not shown).

Serology showed a sigmoidal pattern, with a sharp increase in specific IgA (see supplementary Figure 2) and IgG antibodies (Fig. 1c) against the structural protein (S1 domain) of SARS-CoV-2 around 14 and 15 days post onset of symptoms, respectively. Most of the healthy controls showed antibody levels below the cut-off. Except four controls, who showed detectable anti-SARS-CoV-2 IgA antibody levels, while anti-SARS-CoV-2 IgG antibodies were negative (see supplementary Figure 3).

Figs. 1d and 1e depict the combined T- and B-cell response in COVID-19 patients and healthy controls. Thirteen (48%) of the 27 COVID-19 patients had 10 or more SFC in response to stimulation with the nucleoprotein, whereas none of the healthy controls reached that level. One COVID-19 case and two healthy controls showed a strong T-cell reactivity (of more than 30 SFC) against the mosaic surface protein as measured in the ELISpot assay. This COVID-19 case also showed the highest T-cell reactivity against the nucleoprotein (146 SFC) as measured in the ELISpot assay. SARS-CoV-2 IgA and IgG antibodies were positive in the COVID-19 case and negative in the two healthy controls.

As none of the healthy controls had more than nine SFC specific for the SARS-CoV-2 nucleoprotein in the ELISpot assay, 10 or more spots was determined to be indicative for COVID-19 disease. Prolonged illness, i.e. when sampled more days post onset of symptoms, increased the chance of finding higher numbers of spots. Receiver operating characteristc (ROC) analysis was performed for the nucleoprotein ELISpot results at >7, >14 and >21 days post onset of symptoms. All ROC analyses showed significant areas under the ROC curve, respectively 0.77 ($p = 0.004$), 0.82 ($p = 0.001$) and 1 ($p = 0.002$) for detection of COVID-19 disease (Fig. 1f).

Interestingly, in a recent study published by Grifoni et al.[5] SARS-CoV-2 epitope pools were used to probe CD4+ T-cell responses. They found that M, spike and N proteins were co-dominant, and that each protein was recognized by 100% of the 20 COVID-19 cases studied. With respect to SARS-CoV-2 CD8+ T-cell responses, the spike protein was less dominant, while significant reactivity was noted for M, N and other antigens. Similarly, in our study T-cell reactivity was detected in the SARS-CoV-2 ELISpot assay against the nucleoprotein in the majority of patients. In contrast however, the mosaic surface protein, consisting of exposed extracellular domains of the SARS-CoV-2 spike, envelope and membrane proteins generated only a very modest T-cell response in most patients. As these are all trans-membrane proteins and therefore poorly soluble in aqueous solutions, the use of native proteins was not technically feasible. It is therefore possible that the mosaic nature of the recombinant protein and the production of the protein in *E. coli* could have affected the potential of this protein to elicit spot formation in the ELISpot.

Further studies investigating the association between SARS-CoV-2 neutralizing antibodies and ELISpot reactivity might reveal whether patients develop a protective immunity after COVID-19.

Authors' contributions

ST, GL, AB,MH and RK designed and performed the study and/or analyzed data. ST and MH wrote the manuscript. HG, RK, CR, KK, GL and AB provided intellectual input and advice on study design and analysis.

Declaration of Competing Interests

none

Acknowledgement

Not applicable

Ethics committee approval

The regional Medical Research Ethics Committees United approved the study (Nieuwegein, the Netherlands; MEC-U: NL73618.100.20).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2020.06.015.

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