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Highly Sensitive Quantification of Plasma Severe Acute Respiratory Syndrome Coronavirus 2 RNA Sheds Light on its Potential Clinical Value

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(See the Editorial Commentary by Jacobs and Mellors on pages e2898–900.)

Background. Coronavirus disease 2019 (COVID-19) is a global public health problem that has already caused more than 662 000 deaths worldwide. Although the clinical manifestations of COVID-19 are dominated by respiratory symptoms, some patients present other severe damage such as cardiovascular, renal and liver injury, and/or multiple organ failure, suggesting a spread of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in blood. Recent ultrasensitive polymerase chain reaction (PCR) technology now allows absolute quantification of nucleic acids in plasma. We intend to use the droplet-based digital PCR technology to obtain sensitive detection and precise quantification of plasma SARS-CoV-2 viral load (SARS-CoV-2 RNAemia) in hospitalized COVID-19 patients.

Methods. Fifty-eight consecutive COVID-19 patients with pneumonia 8 to 12 days after onset of symptoms and 12 healthy controls were analyzed. Disease severity was categorized as mild to moderate in 17 patients, severe in 16, and critical in 26. Plasma SARS-CoV-2 RNAemia was quantified by droplet digital Crystal Digital PCR next-generation technology (Stilla Technologies, Villejuif, France).

Results. Overall, SARS-CoV-2 RNAemia was detected in 43 (74.1%) patients. Prevalence of positive SARS-CoV-2 RNAemia correlated with disease severity, ranging from 53% in mild-to-moderate patients to 88% in critically ill patients ($P = .036$). Levels of SARS-CoV-2 RNAemia were associated with severity ($P = .035$). Among 9 patients who experienced clinical deterioration during follow-up, 8 had positive SARS-CoV-2 RNAemia at baseline, whereas only 1 critical patient with undetectable SARS-CoV-2 RNAemia at the time of analysis died at day 27.

Conclusion. SARS-CoV-2 RNAemia measured by droplet-based digital PCR constitutes a promising prognosis biomarker in COVID-19 patients.

Keywords. coronavirus disease 19 (COVID-19); clinical severity; SARS-CoV-2 RNAemia; droplet-based digital PCR; biomarker.

Coronavirus disease 2019 (COVID-19) [1] is a global public health problem that has already caused more than 662 000 deaths worldwide. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [2], responsible for the development of COVID-19, was first isolated and sequenced in early January 2020 [3]. Although most patients present mild-to-moderate disease, 5%–10% progress to severe or critical disease [4], including pneumonia and acute respiratory failure. Based on data from patients with

laboratory-confirmed COVID-19 from mainland China, admission to an intensive care unit (ICU), invasive mechanical ventilation, or death occurred in 5.0%, 2.3%, and 1.4% of cases, respectively [1]. In severe cases, clinical observations typically describe a 2-step disease progression, starting with a mild-to-moderate presentation followed by a secondary respiratory worsening 9–12 days after onset of first symptoms [4–6]. Clinical deterioration is dominated by worsening of respiratory symptoms, potentially concomitant with severe systemic damage including cardiovascular, renal and liver injury, and/or multiple organ failure [4, 7]. In addition, coagulopathy has been reported in severe COVID-19 cases [8, 9]. These systemic clinical features strongly suggest the spread of SARS-CoV-2 within extrapulmonary sites via blood flow.

Detection of circulating SARS-CoV-2 RNA by conventional real-time reverse transcription-polymerase chain reaction assay

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(RT-PCR), which is used for routine SARS-CoV-2 molecular diagnosis from respiratory tract samples, was previously reported in COVID-19 patients in a few studies [4, 10, 11]. Because there is no certainty whether this RNA belongs to entire infectious viral particles or comes from infected-cell lysis, “SARS-CoV-2 RNAemia” was preferred to “viremia” when referring to this circulating SARS-CoV-2 RNA. In their retrospective series of 57 COVID-19 patients, Chen et al found only 6 patients with positive SARS-CoV-2 RNAemia by RT-PCR and suggested an association between detectable serum SARS-CoV-2 RNA with disease severity [11]. However, conventional RT-PCR for SARS-CoV-2 RNA may lack sensitivity in patients showing low viremia. Furthermore, in the absence of adequate quantitative standards availability, RT-PCR does not allow precise quantification of low SARS-CoV-2 viral loads. Ultrasensitive PCR technology is now available for absolute quantification of nucleic acids in plasma, such as droplet-based digital PCR (ddPCR), a more sensitive technique for virus detection than RT-PCR [12–14]. ddPCR was primarily developed for cancer research to detect and quantify mutated circulating tumoral DNA among cell-free DNA in liquid biopsies by optimizing sensitivity and absolute quantification compared with classical quantitative PCR (qPCR). This innovative method is based on the realization of thousands of single-molecule PCRs in parallel in independent compartment, here droplets of an induced emulsion with nucleic acid extracts. Therefore, every PCR is performed separately, consequently avoiding the bias seen in conventional PCR. Every droplet containing targeted DNA or RNA will become fluorescent after RT-PCR and is detected as a positive droplet by laser excitation. In that way, ddPCR allows the generation of quantitative and accurate data without standard curves. Because the raw data fit the Poisson distribution, they allow absolute quantification of the amplified target. Besides higher sensitivity over qPCR, ddPCR also presents many advantages for analyzing complex biological samples. Indeed, performing every PCR in an isolated droplet provides higher resistance to a variety of inhibitors that could be contained within biological samples and consequently significantly increases reproducibility of the obtained data and comparability of the results between different laboratories [15]. The increased tolerance of ddPCR to PCR inhibitory molecules makes it an attractive alternative to qPCR for medical applications, including viral diagnostics [16]. Recently, ddPCR was used to quantify SARS-CoV-2 RNA in lower respiratory tract samples of COVID-19 patients and showed higher sensitivity than RT-PCR, especially with low viral loads [17].

In the present study, ddPCR was used to precisely quantify circulating SARS-CoV-2 RNA in the blood from COVID-19 patients hospitalized with different clinical severity.

METHODS

Study Design and Patients

COVID-19 patients admitted to the Cochin Hôpital, Paris, France, at the time of respiratory deterioration and between

days 8 and 12, were included between March 19, 2020, and April 3, 2020, in the setting of the local RADIPEM biological samples collection derived from samples collected in routine care. The present work, focused on SARS-CoV-2 RNAemia, is part of another study primarily designed to explore innate inflammatory responses in hospitalized COVID-19 patients [18] and used the same inclusion criteria. Our study is an ancillary study focused on circulating SARS-CoV-2 RNA from another, more global study regarding inclusion criteria for COVID-19 inpatients: age between 18 and 80 years, diagnosis of COVID-19 according to World Health Organization interim guidance (<https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/infection-prevention-and-control>), and positive SARS-CoV-2 RT-PCR testing on a respiratory sample (nasopharyngeal swab or invasive respiratory sample). Inpatients with preexisting unstable chronic disorders (such as uncontrolled diabetes mellitus, severe obesity defined as body mass index greater than 30, unstable chronic respiratory disease, or chronic heart disease) were excluded. SARS-CoV-2 RNAemia was performed on samples collected between 8 and 12 days after onset of first symptoms. The choice to sample patients 9–12 days after disease onset was related to early Chinese clinical observations reporting a 2-step disease progression, starting with a mild-to-moderate presentation followed by a secondary respiratory worsening a few days after onset of first symptoms in severe cases. The mean interval between the onset of illness and the admission to ICU was of 9–12 days in these early reports [4–6, 18].

Healthy controls were RT-PCR SARS-CoV-2 negative asymptomatic adults, matched with cases by age and sex.

The clinical severity of COVID-19 was described according to the adaptation of the Sixth Revised Trial Version of the Novel Coronavirus Pneumonia Diagnosis and Treatment Guidance published on February 19, 2020 (http://www.kankyokansen.org/uploads/uploads/files/jsipc/protocol_V6.pdf). Mild cases were defined as patients with mild clinical symptoms (fever, myalgia, fatigue, and diarrhea) and no sign of pneumonia on thoracic computed tomography (CT) scan. Moderate cases were defined as patients with clinical symptoms associated with dyspnea, radiological findings of pneumonia on thoracic CT scan, and requiring a maximum of 3 L/min of oxygen. Severe cases were defined as respiratory distress patients requiring > 3 L/min of oxygen with no other organ failure. Critical patients were those with mechanical ventilation, shock, and/or multiple organ failure and hospitalized in an ICU. Clinical evolution was evaluated during hospital stay and clinical deterioration defined as: increased oxygen intake requiring introduction of high-flow nasal oxygen therapy or mechanical ventilation, transfer to the ICU, or death.

Biological collection and informed consent were approved by the Direction de la Recherche Clinique et Innovation and the French Ministry of Research (no. 2019-3677). The study

conforms to the principles outlined in the Declaration of Helsinki and received approval by the appropriate institutional review board (Cochin-Port Royal Hospital, Paris, France; no. AAA-2020-08018).

Quantification of SARS-CoV-2 RNAemia

Plasma SARS-CoV-2 RNA (140 µL) was extracted using QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The elution volume was 35 µL; we added 10.5 µL of the elution to the RT-PCR mix for amplification. SARS-CoV-2 RNAemia was quantified at each time point by droplet-based Crystal Digital PCR (Stilla Technologies, Villejuif, France) on the Naica System (Stilla Technologies) using the following commercial RT-PCR amplification kit (Novel Coronavirus Digital PCR Detection Kit, Apexbio, Beijing, China) following the manufacturer's instruction. The kit includes primers and FAM- and HEX-labeled probes specific to 2 distinct regions (ORF1ab and Nucleocapside [N] genes) of the SARS-CoV-2-positive strand RNA genome and primers and a Cy5-labeled probe for the detection of a human housekeeping gene detected on the third channel of the Naica system. Positivity of this housekeeping gene was necessary to validate the RT-PCR assay before any further analysis. This single-assay design permits the simultaneous detection of 2 independent SARS-CoV-2 sequences reported as conserved while concurrently monitoring PCR effectiveness using the third channel of detection. Plasma samples with 1 of the 2 ORF1 or N genes or both genes detected were considered as positive samples and results were automatically analyzed using "Crystal reader" (Stilla) and "Crystal Miner" software (Stilla) based on the most amplified gene positive droplets. SARS-CoV-2 RNA concentrations (cp/mL) were finally calculated considering the extracted volume of plasma.

Statistical Analysis

Descriptive statistics were computed for the population at baseline. Quantitative variables were described as mean ± standard deviation if normally distributed, or median and interquartile range (IQR) otherwise. Categorical variables were described as group sizes and percentages.

Bivariate comparisons between clinical classes were computed using Fisher's test for categorical variables and the Kruskal-Wallis 1-way analysis of variance for continuous variables. When comparing control and COVID-19 patients, the Mann-Whitney rank-sum test was used for quantitative variables.

An ordinal regression multivariate model was used to evaluate the association between the clinical class and circulating viral DNA quantification (log values), adjusted for clinical predictors. Patients' clinical outcomes are presented using Kaplan-Meier curves. We did not perform a formal statistical survival analysis because the study was not primarily designed

for survival analysis, the patients belonged to different clinical classes at baseline, and the outcome was a composite criterion of mechanical ventilation requirement or death.

Computations were performed using the R software, and the ordinal package for the ordinal regression model. *P* values < .05 were considered significant.

RESULTS

Patients' Characteristics

Fifty-eight COVID-19 patients and 12 healthy controls were analyzed. Demographic and clinical characteristics of the patients are shown in Table 1. Median age of the patients was 55.1 years (IQR, [48,63]) and 81% were male.

At inclusion, the degree of severity of COVID-19 was categorized as mild to moderate in 17 patients (median oxygen requirement 2 L/min), severe in 15 patients (median oxygen requirement 5 L/min), and critical in 26 patients. The median time from symptoms onset to inclusion was of 9.5 days

Table 1. Clinical Characteristics of Hospitalized COVID-19 Patients

	N	%	Med	IQR
Sex	58			
Female	11	19		
Male	47	81		
Age, y	58		55, 14	15
Clinical classes	58			
Mild/moderate	17	29		
Severe	15	26		
Critical	26	45		
Clinical deterioration	58			
Death	3	5.2		
Mechanical ventilation	3	5.2		
Optiflow	3	5.2		
Absence	49	84		
Monitoring delay	58		7	11
Pulmonary radiography showing ground-glass opacities	55			
No	0	0		
Yes	55	100		
Pulmonary radiography showing bilateral patchy distribution	55			
No	3	5.5		
Yes	52	95		
Controlled comorbidities	58			
No	32	55		
Yes	26	45		
Oxygen requirement	58			
No	3	5		
Yes	29	50		
Mechanical ventilation	26	45		
Antiviral treatment	58			
No	43	74		
Yes	15	26		

Abbreviations: COVID-19, coronavirus disease 2019; IQR, interquartile range; med, median.

(IQR: 7.0–12.5): respectively 9 days (IQR: 9–11), 10 days (IQR: 9–11), and 10 days (IQR: 9–11) in the mild to moderate, severe, and critical categories (analysis of variance test = 0.43). All 55 CT scans available at the time of admission were abnormal, showing ground-glass opacities (100%) with bilateral patchy distribution (95%). During patients' clinical monitoring (median time of follow-up of 7 days; IQR: 3.0–14.0), no patient in the mild-to-moderate group required admission to the ICU or use of mechanical ventilation, compared with 5 of 15 in the severe group. None of the healthy controls had any comorbidity, and neither did 55% of the COVID-19 patients. In the remaining COVID-19 patients (45%), comorbidities were: non-insulin-dependent diabetes mellitus (n = 7), solid cancer (n = 6), hematological cancer (n = 3), asthma (n = 3), autoimmune diseases (n = 3), and chronic obstructive pulmonary disease (n = 1). Fourteen COVID-19 patients received an antiviral treatment at the time of their hospitalization for disease

progression by hydroxychloroquine ± azithromycin (n = 5), lopinavir/ritonavir (n = 8), and remdesivir (n = 1).

SARS-CoV-2 RNAemia Results by ddPCR

At inclusion, 43 (74.1%) of COVID-19 patients were positive for SARS-CoV-2 RNA in plasma, with at least the N gene detected. Only 28% were positive for ORF1 gene, systematically associated with N gene positivity.

Proportion of positive SARS-CoV-2 RNAemia was significantly different between patients from different clinical classes, from 53% in mild-to-moderate patients to 88% in critically ill patients ($P = .036$) (Table 1).

Overall, SARS-CoV-2 RNAemia was associated with COVID-19 severity ($P = .035$, Kruskal-Wallis), as depicted in Figure 1, with median SARS-CoV-2 RNAemia of 89, 279, and 301 cp/mL, in mild-to-moderate, severe, and critically ill patient groups, respectively. Raw plasma SARS-CoV-2 RNA value

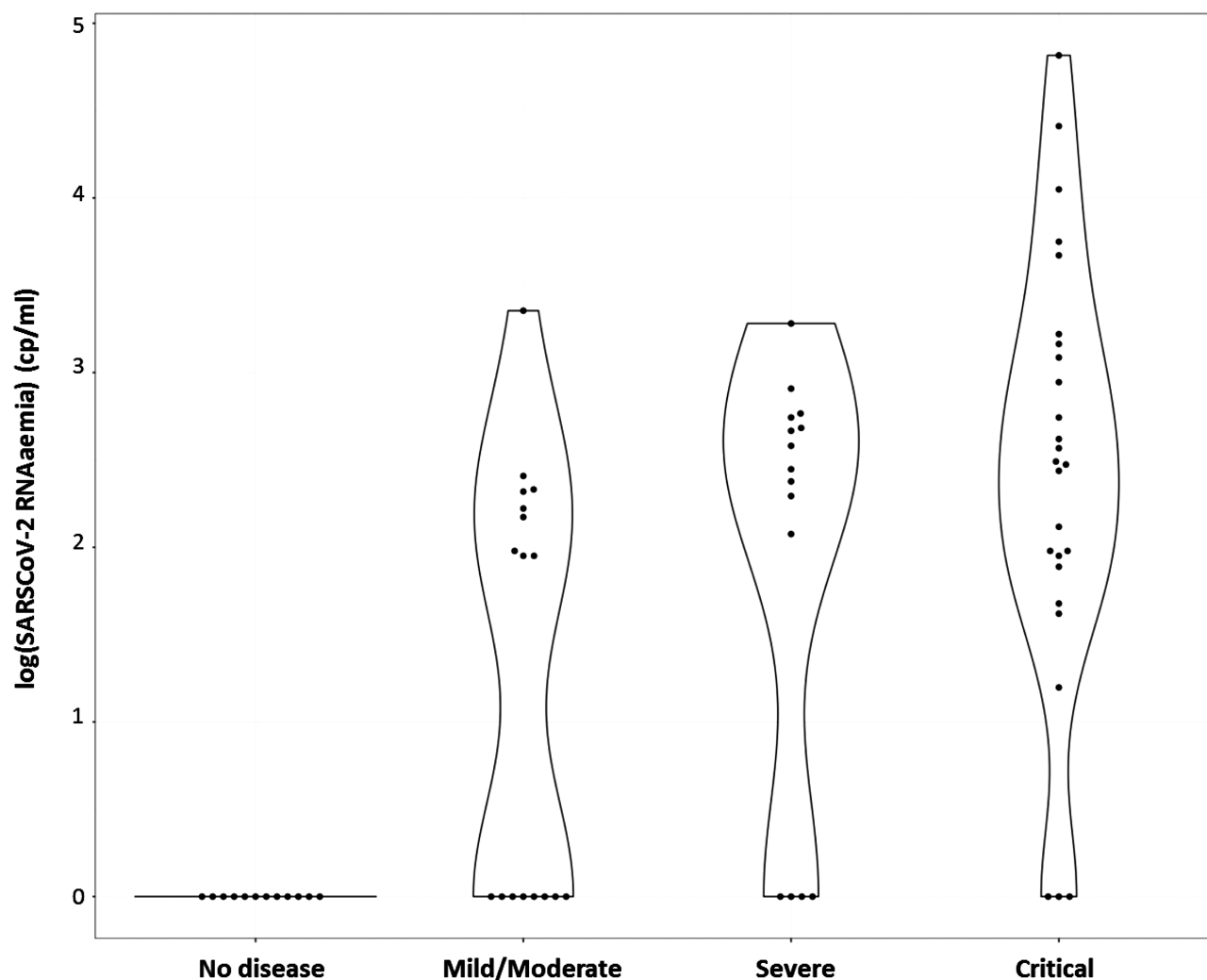


Figure 1. Plasmatic SARS-CoV-2 RNAemia (cp/mL) measured by droplet-based digital PCR according to clinical classes. Abbreviations: PCR, polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

for each patient in the 3 different clinical classes are detailed in [Supplementary Table 1](#).

In multivariate analysis adjusted on age, sex, and comorbidities, SARS-CoV-2 RNAemia was strongly associated with the clinical class (adjusted hazard ratio, 2.2; 95% confidence interval, 1.39–3.9; $P = .0018$) ([Table 2](#)).

All healthy controls showed negative SARS-CoV-2 RNAemia with no detection of both specific SARS-CoV-2 N and ORF-1 genes.

Clinical Monitoring and Correlation With Baseline SARS-CoV-2 RNAemia

Clinical deterioration was observed in 9 COVID-19 patients during follow-up: introduction of high-flow nasal oxygen therapy ($n = 3$), introduction of mechanical ventilation ($n = 3$), or death ($n = 3$).

Eight of the 9 COVID-19 patients with clinical deterioration had positive SARS-CoV-2 RNAemia at baseline, whereas only 1 critical patient with undetectable SARS-CoV-2 RNAemia at the time of analysis died at day 27 ([Figure 2](#)). Of note, the patient with the highest SARS-CoV-2 RNAemia (65 476 cp/mL) died from COVID-19 1 day after plasma sampling.

DISCUSSION

In the present study, SARS-CoV-2 RNAemia was measured by ddPCR in a large cohort of hospitalized COVID-19 patients at the time of disease worsening. SARS-CoV-2 RNAemia was detectable in the majority of patients, confirming that the SARS-CoV-2 may invade the systemic compartment beyond the lungs. Circulating SARS-CoV-2 viral load showed a large amplitude spanning almost 4 log, with an overall significant difference in viral load levels between clinical classes, showing higher viral loads in severe/critical patients, compared with the mild to moderate ones. The median SARS-CoV-2 RNAemia was very similar between severe and critical patients and such a small difference in SARS-CoV-2 RNAemia cannot be used to distinguish them. The clinical classification was made according to the Sixth Revised Trial Version of the Novel Coronavirus Pneumonia Diagnosis and Treatment Guidance; similar viral loads reflect a strong continuum between these 2 groups of patients. The proportion of patients with detectable SARS-CoV-2 RNAemia was also strongly correlated with disease severity. Furthermore, viremic COVID-19 patients showed a tendency to have a higher risk of poor outcome, unlike nonviremic patients. Taken together, these observations strongly suggest that SARS-CoV-2 RNAemia is associated with disease severity, making it a potential biomarker of COVID-19 worsening.

Few studies have previously reported the detection of SARS-CoV-2 viral RNA within COVID-19 patients' blood [4, 10, 11]. In these studies, SARS-CoV-2 RNAemia was assessed using classical RT-PCR, considered the gold standard for SARS-CoV-2 RNA molecular diagnosis in nasopharyngeal swabs;

Table 2. Univariate and Multivariate Analysis of Demographic, Clinical, and Virological Data

	Clinical Classes						Univariate Analyses			Multivariate Analyses			
	Mild/Moderate			Severe			Critical	P	OR	P	OR	P	
	N	%	Median	N	%	Median							N
Sex	17			15			26			.52		2.6 (0.63-11)	.19
Female	5	29		2	13		4	15					
Male	12	71		13	87		22	85					
Age	17		51	24		57.54	12		55.16	.24		1 (0.98-1.1)	.23
Clinical deterioration	17			15			26			.00016			
Death	0	0		0	0		3	12					
Mechanical ventilation	0	0		3	20		0	0					
Optiflow	0	0		3	20		0	0					
Absence	17	100		9	60		23	88					
Controlled comorbidities	17			15			26			.0021		7.3 (2.2-29)	.0023
No	15	88		8	53		9	35					
Yes	2	12		7	47		17	65					
Positive SARS-CoV-2 RNAemia	17			15			26			.036			
No	8	47		4	27		3	12					
Yes	9	53		11	73		23	88					
SARS-CoV-2 RNAemia (cp/mL)	17		89	167		279.76	458		303.57	.015		2.2 (1.4-3.9)	.0018
Log(SARS-CoV-2 RNAemia) (cp/mL)	17		2	2.2		2.45	1.7		2.48			1.2	

Abbreviations: IQR, interquartile range; OR, odds ratio; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2. Figures in bold indicate significant results in uni- and multivariate analysis.

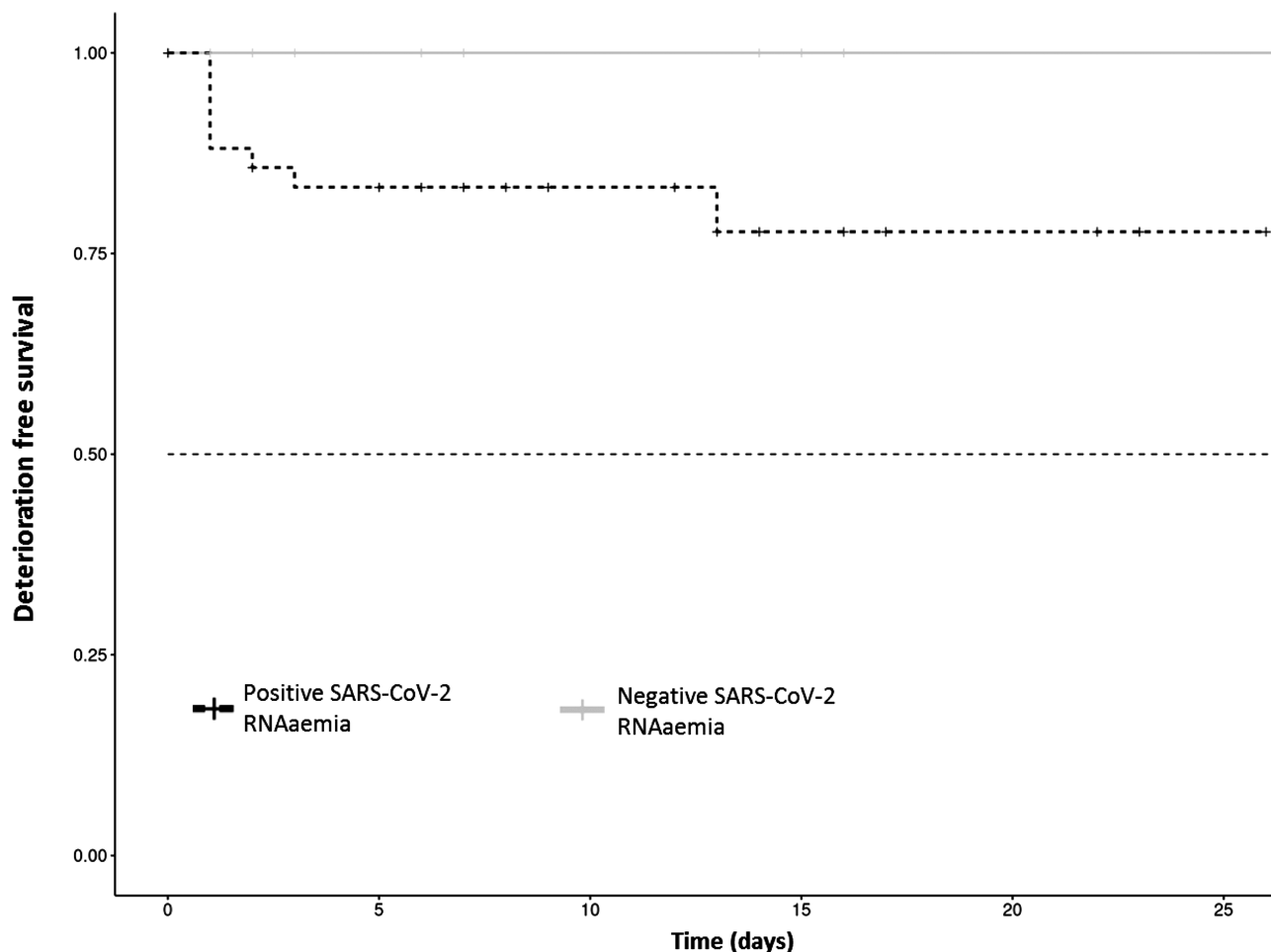


Figure 2. Kaplan-Meier curves showing the prognostic value of circulating SARS-CoV-2 RNAemia as assessed by frequency of deterioration events at time of plasma viral detection by droplet-based digital PCR. The gray and hatched black lines represent patients without and with detectable SARS-CoV-2 RNAemia, respectively. The hatched line at 0.5 represents the median survival. Abbreviations: PCR, polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

detectable SARS-CoV-2 RNA in plasma was a rare event. RT-PCR did not allow precise quantification of the level of circulating SARS-CoV-2 RNA because adequate quantitative standards were not available. Thus, only semiquantitative evaluation of SARS-CoV-2 RNAemia could have been established by reference to the cycle threshold of amplification curves in 1 limited series [11]. Furthermore, low viral loads are likely to be inaccurately quantified by classical RT-PCR as was recently shown in lower respiratory samples in which ddPCR showed significantly better performance to detect and quantify low viral load [17, 19, 20]. Therefore, SARS-CoV-2 RNAemia could have been underdetected by using conventional RT-PCR technology in early studies.

ddPCR, primarily developed in the cancer research field, was optimized to enhance sensitivity and obtain absolute quantification compared with classical qPCR. This new technology is based on the realization of millions of single-molecule PCRs in parallel with independent compartments (droplets of an emulsion); the resulting amplification products reflect more closely

the original composition of nucleic acid mixtures than conventional PCR [12–14] because every targeted DNA or RNA molecule will be amplified and detected separately. The use of ddPCR for the analysis of biological samples that are complex in nature presents many advantages over conventional PCR procedures. In addition to a higher sensitivity over qPCR or RT-PCR and not needing calibration curves, ddPCR also demonstrates substantially higher resistance to a variety of inhibitors that could be contained within biological samples [15]. This increased tolerance of ddPCR to PCR inhibitory molecules makes it an attractive alternative to qPCR or RT-PCR for medical applications, including viral diagnostics [16] and cancer research [13]. Finally, digital RT-PCR was described as presenting high potentiality over conventional real-time RT-qPCR [21]. In the present study, SARS-CoV-2 RNAemia assessed by ddPCR was detected in roughly 75% of hospitalized COVID-19 patients, and ddPCR was highly specific for the detection of SARS-CoV-2 RNAemia. Compared with recent studies reporting positive blood SARS-CoV-2 viral load by RT-PCR in less than

one-half of hospitalized patients for COVID-19 [11, 22], our results indicate that ddPCR is more efficient and more precise in detecting and quantifying SARS-CoV-2 RNAemia compared with classic RT-PCR.

In 1 previous study, SARS-CoV-2 RNAemia indirectly assessed with the cycle threshold of RT-PCR was correlated with COVID-19 severity [11]. We show here that precise quantification of SARS-CoV-2 RNAemia by ddPCR confirms these previous observations. In our study, detectable SARS-CoV-2 RNAemia was also more frequently detected in patients with increasing severity. These data suggest that the systemic spread of SARS-CoV-2 could be of importance in the different clinical features observed. It has been clearly shown that immunopathologic mechanisms are involved in distant clinical deterioration of COVID-19 patients [23]; however, it cannot be ruled out that an uncontrolled replication of SARS-CoV-2 could be the origin of this immune deregulation.

During follow-up, 9 patients experienced clinical deterioration, from important respiratory worsening in severe patients (n = 6) to death in critical patients (n = 3). Strikingly, 8 of these 9 patients had positive SARS-CoV-2 RNAemia. Considering this engaging finding, it could be relevant to quantify RNAemia in patients before clinical deterioration, and it would be interesting to prospectively assess the predictive power of plasmatic SARS-CoV-2 RNAemia for clinical deterioration as the second step of COVID-19 generally occurring between days 8 and 12 from the first symptoms. Therefore, longitudinal measurements starting at the onset of COVID-19 symptoms would add very interesting information on viral dynamics at different stages of the disease. However, such data were not available because of the cross-sectional design of our study but should be investigated in further protocols.

Detectable SARS-CoV-2 RNAemia observed in most hospitalized COVID-19 patients could begin to explain systemic damage and/or multiple organ failure observed in some cases. However, we cannot be certain that detected SARS-CoV-2 RNAemia corresponds to circulating infectious viral particles and is a marker for an infectious systemic spread of SARS-CoV-2. Finally, because we did not report any secondary extrapulmonary damage during the follow-up of our patients, we could not confirm the hypothesis that SARS-CoV-2 systemic spread could be at the origin of other systemic organ failure. We will address this question in further studies by quantifying SARS-CoV-2 RNAemia by ultrasensitive ddPCR in COVID-19 patients with systemic damage. It will be also necessary to determine how this SARS-CoV-2 RNAemia could represent an easy-to-use and useful biomarker in monitoring COVID-19 patients.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. D. V., S. K., B. T., and H. P. conceived and designed the research; G. P., N. R., F. R., A. S. H., and J. H. performed the experiments; M. W. performed statistical analyses; D. V., S. K., M. W., V. T., P. L. P., and H. P. analyzed the results; D. V., S. K., M. W., V. T., P. L. P., L. B., J. H., B. T., and H. P. drafted the manuscript. All authors validated the final version of the manuscript.

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