Circulating ubiquitous RNA, a highly predictive and prognostic biomarker in hospitalized COVID-19 patients

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Brief summary : Blood biomarkers for the clinical monitoring and the management of hospitalized COVID-19 patients are clearly needed. Herein, we report the particular usefulness of ubiquitous circulating RNA monitoring by ddPCR as a predictive and prognosis biomarker for hospitalized COVID19 patients.

ABSTRACT

Background: Approximately 15-30% of hospitalized COVID-19 patients develop acute respiratory distress syndrome, systemic tissue injury, and/or multi-organ failure leading to death in around 45% of cases. There is a clear need for biomarkers which quantify tissue injury, predict clinical outcomes and guide the clinical management of hospitalized COVID-19 patients.

Methods: We herein report the quantification by droplet-based digital PCR (ddPCR) of the SARS-CoV-2 RNAemia and the plasmatic release of a ubiquitous human intracellular marker, the ribonuclease P (RNase P) in order to evaluate tissue injury and cell lysis in the plasma of 139 COVID-19 hospitalized patients at admission.

Results: We confirmed that SARS-CoV-2 RNAemia was associated with clinical severity of COVID-19 patients. In addition, we showed that plasmatic RNase P RNAemia at admission was also highly correlated with disease severity (P<0.001) and invasive mechanical ventilation status (P<0.001) but not with pulmonary severity. Altogether, these results indicate a consequent cell lysis process in severe and critical patients but not systematically due to lung cell death. Finally, the plasmatic RNase P RNA value was also significantly associated with overall survival.

Conclusion: Viral and ubiquitous blood biomarkers monitored by ddPCR could be useful for the clinical monitoring and the management of hospitalized COVID-19 patients. Moreover, these results could pave the way for new and more personalized circulating biomarkers in COVID-19, and more generally in infectious diseases, specific from each patient organ injury profile.

Key words : SARS-CoV-2 and RNAseP RNAemia, tissue/cell lysis biomarker, predictive and prognostic biomarkers, hospitalized COVID-19 patient, ddPCR,

Introduction

Coronavirus disease 2019 (COVID-19) is a global public health problem that has already caused more than 3 million deaths worldwide. A wide spectrum of disease severity was rapidly described ranging from asymptomatic or mild diseases to respiratory failure and multiple organ dysfunction syndromes, or failure requiring intensive care management of patients and leading to a high mortality rate. In severe cases, clinical observations rapidly described a two-step disease progression, starting with a mild-to-moderate presentation followed by a secondary respiratory worsening 9 to 12 days after the onset of first symptoms [1–3]. Clinical deterioration is typically dominated by worsening of respiratory symptoms, which are potentially concomitant with severe systemic organ failure, including cardiovascular, renal and/or liver injuries [4–8]. Evidence tended to demonstrate that the second phase of COVID-19 was associated with a cytokine storm contributing to the development of acute respiratory distress syndrome (ARDS), systemic tissue injury, and multi-organ failure observed in severe cases of COVID-19 [9]. Approximately 5% of patients infected with SARS-CoV-2 require intensive care and admission for severe lung damage [7,10] and 15-30% of patients hospitalized with COVID-19 develop ARDS [11,12] leading to death in around 45% of cases [13]. Therefore, biomarkers that can quantify tissue injury, analyze disease pathogenesis, predict clinical outcomes, and guide the clinical management of hospitalized COVID-19 patients are clearly needed.

In recent years, the democratization of ultra-sensitive technologies, such as droplet-based digital PCR (ddPCR), has fostered the development of circulating markers, making them suitable for several clinical applications. Recently, we and others provided evidence that highly sensitive quantification of SARS-CoV-2 RNAaemia by ddPCR in peripheral blood could be a reliable marker of disease severity and that it could be used as a potential predictive biomarker of clinical worsening in COVID-19 patient follow-up in the second

phase of COVID-19 pathology [14,15]. Besides SARS-CoV-2 RNAaemia, plasmatic release of ubiquitous human intracellular markers could be an accurate biomarker to evaluate tissue injury and cell lysis induced by COVID-19. Thus, in addition, we monitored the plasmatic release of the intracytoplasmic ribonuclease P (RNase P), targeting its H1 RNA catalytic part [16] in order to evaluate tissue injury and cell lysis induced by COVID-19 in this global study bringing together two cohorts of comparable COVID-19 patients hospitalized for respiratory deterioration during the first wave in Paris, France.

We herein report the quantification of SARS-CoV-2 RNAemia and circulating RNase P in the plasma of 139 COVID-19 hospitalized patients at admission. We evaluated the interest of both markers in specifying the degree of clinical severity of COVID-19 at admission, and correlated them with clinical outcome of hospitalized COVID-19 patients during their medical follow-up.

Material and methods

Study design and patients. A first cohort of 60 COVID-19 patients admitted to the Cochin-Port Royal Hospital, Paris, France, was primarily included and to complete and extend our previous observations, we further retrospectively included another series of 79 patients admitted to the European George Pompidou Hospital (HEGP), Paris, France, between March 19, 2020 and June 26, 2020 for COVID-19 during the first wave of the epidemic in France, for the quantification of SARS-CoV-2 plasma RNAemia and RNase P RNAemia by ddPCR. Inclusion criteria for COVID-19 inpatients were: age between 18 and 80 years, diagnosis of COVID-19 according to World Health Organization (WHO) interim guidance (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/infection-prevention-and-control), and positive SARS-CoV-2 real-time PCR (RT-PCR) testing on a respiratory sample (nasopharyngeal swab or invasive respiratory sample).

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 19th, 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 and radiological findings of pneumonia on thoracic CT-scan, and requiring a maximum of 3 L/min of oxygen. Severe cases were defined as respiratory distressed patients requiring over 3 L/min of oxygen with no other organ failure. Critical cases were defined as patients requiring mechanical ventilation, into shock and/or with other organ failures that required management in an intensive care unit (ICU). Biological collection and informed consent were approved by the Direction de la Recherche Clinique et Innovation (DRCI) and the French Ministry of Research (N°2019-3677). The two cohorts conformed to the principles outlined in the Declaration of Helsinki, and received approval by the appropriate Institutional Review Board (Cochin-Port Royal Hospital, Paris, France; number AAA-2020-08018; European Georges Pompidou Hospital, Paris, France, SARCODO study: CPP 2020-04-048 / 2020-A01048-31 / 20.04.21.49318- ClinicalTrials.gov Identifier: NCT04624997).

RNA extraction. Total plasma 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, and 10.5 µL of the elution were added to the RT-PCR mix for amplification [15].

Quantification of plasmatic SARS-CoV2 RNA. Plasmatic SARS-CoV-2 RNAaemia in each COVID-19 patient from HEGP cohort was quantified at admission by droplet-based Crystal Digital PCR™ (Stilla Technologies, Villejuif, France) on a Naica™ System (Stilla

Technologies, Villejuif, France) using the following commercial RT-PCR amplification kit (Novel Coronavirus (2019-nCoV) Digital PCR Detection Kit, Apexbio™, Beijing, China), according to the manufacturer's instructions. The kit includes primers and FAM- and HEX-labeled probes specific to two distinct regions [ORF1ab and Nucleocapside (N) genes] of the SARS-CoV-2 positive strand RNA genome [15].

Quantification of plasmatic RNase P. Ubiquitous plasmatic RNase P RNAemia from all COVID-19 patients of both cohorts was quantified using 10.5 µL of eluted RNA at each time point by droplet-based Crystal Digital PCR™ (Stilla Technologies, Villejuif, France) on the Naica™ System (Stilla Technologies, Villejuif, France) using the following commercial RT-PCR amplification kit (Novel Coronavirus (2019-nCoV) Digital PCR Detection Kit, Apexbio™, Beijing, China) , according to the manufacturer's instructions. The kit includes primers and a Cy5-labeled probe for the detection of RNase P detected on the third channel of the Naica™ system. RNase P positivity was necessary to validate the RT-PCR assay prior to any further analysis. The results were automatically analyzed using the "Crystal reader" and "Crystal Miner" software (Stilla Technologies). RNase P concentrations were finally calculated considering the extracted volume of plasma and expressed in copies per milliliter of plasma (cp/mL).

Statistical analysis. Descriptive statistics were computed for the population at hospital admission. Quantitative variables were described as mean ± standard deviation (SD), if normally distributed, or median and inter-quartile range (IQR), otherwise. Categorical variables were described as group sizes and percentages.

Bivariate comparisons between clinical classes were computed using the Fisher test for categorical variables, one-way analysis of variance (ANOVA) for continuous variables when all groups were normally distributed, and the Kruskal–Wallis ANOVA otherwise. When comparing control and COVID-19 patients, the Student *t* test was used for quantitative

variables when both groups were normally distributed, and the Mann-Whitney rank-sum test was used otherwise.

The Cox proportional hazards model was used to evaluate the risk of death at inclusion between patients with low and high plasmatic RNase P concentration.

Patients' clinical outcomes are presented using Kaplan–Meier curves.

Computations were performed using the R software, and the survival package for the Cox proportional hazards model. P values < 0.05 were considered statistically significant

Results

Patient characteristics

Global and per-cohort demographic and clinical characteristics of the patients are shown in **Table 1**. Mean age was 58 years (SD = 14), and 78% were male. Patients in both cohorts were comparable, except for patients from Cochin-Port Royal Hospital cohort who were slightly younger (mean age 54±13 vs. 62±14 years) and presented less comorbidities (hypertension 30% vs 53%, chronic renal failure 3% vs 15%) at inclusion. The degree of severity of COVID-19 was categorized as mild-to-moderate in 37 (27%) patients, severe in 35 (25%) and critical in 67 (48%).

Correlation between SARS-CoV-2 RNAaemia, clinical severity, invasive mechanical ventilation (IMV) and pulmonary severity at admission

SARS-CoV-2 RNAemia by ddPCR was significantly correlated with clinical severity (respectively at a median of 25 (101) copy/ml ; 36 (330) copy/ml in severe patients and 113 (528) copy/ml in critical patients) in hospitalized patients at admission (**Figure 1A**; *P*=0.021). Plasma SARS-CoV-2 RNAemia was also correlated with mechanical ventilation status, with a higher concentration in IMV (median of 113 (528) copy/ml) than in non-ventilated COVID-19 patients (median of 36 (199) copy/ml) (**Figure 1B**; *P*=0.012). No

correlation was found between SARS-CoV-2 RNAemia and pulmonary severity objected by CT-scan at admission (**Figure 1C**; Kruskal–Wallis test, *P* =0.47).

Correlation between plasmatic RNase P concentration and clinical severity, invasive mechanical ventilation and pulmonary severity at admission

Plasmatic RNase P concentration was highly correlated with clinical severity classes (**Figure 2A**; *P* < 0.001 on log values) and the invasive mechanical ventilation status (**Figure 2B**; *P* < 0.001), with median plasma RNase P concentration of 14345 copy/mL (IQR=27500 copy/mL) in non-IMV patients and 103482 (192500) copy/mL in IMV patients. Median plasma RNase P concentration in the control group of no disease patients (n = 18) was 3053 (1051) copy/mL. No correlation was found between RNase P RNAemia and pulmonary severity objected by CT-scan at admission (**Figure 2C**; *P*=0.53).

Clinical outcome and correlation with baseline SARS-CoV-2 RNAaemia and plasmatic RNase P RNA concentration

During hospitalization and clinical monitoring of COVID-19 patients, 27 out of the 139 patients died. Plasma RNase P RNA concentration at hospital admission predicted overall survival of the hospitalized COVID-19 patients. Plasma RNase P RNA concentration greater than 4.63 log copy/mL at admission (median value of the log plasma RNase P RNA concentration in the dataset) was significantly associated with death during follow-up (Hazard Ratio (4,.6 95% CI [2.18; 9.80]), *P*=0.0039) (**Figure 3**), whereas SARS-CoV-2 RNAemia value did not predict mortality in our study (data not shown). Interestingly, the median of delay between elevated plasma RNase P RNA concentration (>4.63log copy/mL) and death was of 4 days. Intubated patients without deadly outcome despite a concentration of RNAse P superior to 4.63log cp/mL (n=31) were finally extubated. Among the 16

moderate and severe patients with a concentration of RNAse P superior to 4.63log cp/mL, 50% of them presented a clinical deterioration, such as intensive care unit transfer during their hospitalization but with a final favorable clinical outcome.

Discussion

We measured SARS-CoV-2 RNAemia and plasma RNase P RNA concentrations at admission in a cohort of 139 COVID-19 *patients referred at the time of disease worsening*. SARS-CoV-2 RNAemia was detectable in most hospitalized patients. These results confirmed previous data on the correlation between viral RNAemia and clinical severity, showing higher viral loads in severe and even more in critical patients compared to the mild-to-moderate patients [14,15,17,18].

We also observed that RNase P RNA concentration, an ubiquitous and aspecific human intracellular RNA marker, was also highly correlated with disease severity and invasive mechanical ventilation status in hospitalized COVID-19 patients, indicating a consequent cell lysis process in severe and critical patients.

Moreover, correlation between plasma RNase P RNA concentration >4.63 log cp/mL at admission and overall survival pointed out the use of this quantitative biomarker as an accurate prognosis tool in hospitalized COVID-19 patients, in addition to routine collected clinical parameters. These observations reflect the powerful clinical value of plasma RNase P RNA as a surrogate biomarker of COVID-19-induced global cell/tissue damage and likely to underline the severity of COVID-19 pathology. The question of the influence of comorbidities on RNase P concentrations is important to ask. In our study, the proportion of patients with co-morbidities is equivalent the different clinical classes from moderate to critical classes (sup. Table 1 a,; test Chi 2 p= NS). Moreover, the percentage of patients with comorbidities in the group with elevated RNaseP concentration (> median of 4.63 log copy/mL) is not statistically different from the percentage of patients with comorbidities in

the group with RNaseP concentration below 4.63 log copy/mL (respectively 61% vs 48%; *Fisher test P*=0.12; supp. Table 1 b). Therefore, RNase P RNA alone appears to be an interesting biomarker to determine COVID19 severity. Finally, even if additional experiments are needed to confirm our data, circulating RNase P appears as a highly predictive and prognostic marker in COVID19.

Our data strongly support the use of this cell free RNA (cfRNA) quantification by ddPCR as a prognostic tool for early detection and monitoring of cell and tissue injury associated with COVID-19. Ordinarily, circulating endogenous RNA is considered to be extremely fragile and not sufficiently stable to represent a marker for monitoring, as compared to circulating DNA. However, in our study, the massive release of this endogenous marker seems to counteract the intrinsic weakness of RNA properties making it a marker of choice to quantify and monitor in parallel of the viral RNAemia the degree of cell lysis and systemic viral invasion simultaneously. Another hypothesis would be that this particular RNA, as a subunit of a protein complex, may be more protected from degradation and therefore more easily detected and quantitated than classical RNA.

Solely based on CT-scan imaging, a lytic and an inflammatory process cannot be distinguished. However, a high plasma level of RNase P RNA may more likely indicate a lytic process rather than an inflammatory one. In the case of a more frequent lytic pulmonary process in COVID-19, our data can also explain and predict the longer mean stay in ICU observed in critical COVID-19 patients (15 days) compared to critical seasonal influenza infected patients (8 days) [19] that could correlate with the highest degree of pulmonary cell lysis in COVID-19 patients and thus a longer time to recover functional lung cells. Distinction between pulmonary lytic and inflammatory lesions could be of great interest for the clinical management of COVID-19 patients, especially in a therapeutic perspective, considering that anti-inflammatory treatments would be more efficient in the context of

inflammatory process and maybe not in the context of lytic process. Finally, the delay between elevated RNase P (>4.63 log copy/mL) and death of patients of 4 days (from 0 to 28 days) also asks the question of refining timing therapeutics early to patients with elevated RNase P at admission. Obviously, such considerations could be applied to other pulmonary infectious pathologies.

The lack of correlation between RNase P RNA concentration and pulmonary severity estimated by the percentage of lung damage on CT-scan illustrates that radiologic lesions may not systematically reflect lytic process but both lytic and inflammatory lesions. This lack of correlation could also be explained by the fact that RNase P RNA level is not lung-specific and could therefore reflect extra-pulmonary tissue lysis. Therefore, we investigated the 9 patients (6 critical; 2 severe and 1 moderate, Figure 2C) with plasmatic RNase P concentration above 4.63 log copy/mL in the two less severe pulmonary groups (<10% and 10-25%). Very interestingly, in 6 of these 9 specific patients for whom other biological information were available, we found signal for other organ injury such as kidney, liver or heart with respectively, elevated blood creatinine, ASAT/ALAT or troponin levels. Finally, a possible hypothesis is that endogenous RNA release in plasma comes directly from infected cells lysis where SARS-CoV-2 replication occurs. However, we found 12 critical patients, with plasmatic RNase P levels above 4.63 log copy/mL but without any plasmatic SARS-CoV-2 RNA detection concurrently. In such patients, cfRNA plasmatic release could reflect the destruction of non-infected cells probably due to immunopathological mechanisms. As COVID-19 has been described as a systemic disease with multi-organ involvement, with regard to our preliminary results, we need further tissue-specific molecular markers to understand and specify the origin of observed cell lysis. Profiling organ-specific methylation markers within circulating cell-free DNA (cfDNA) to trace its origin and to quantify tissue-specific injury due to COVID-19 is possible. We aim to develop and validate such tissue-

specific biomarkers in further studies to determine the origin of hospitalized COVID-19 patients' complications. In this way, Cheng *et al.* recently reported a blood test to quantify cell-, tissue-, and organ-specific injury due to COVID-19 [20]. The authors assessed the utility of this test to identify subjects with severe disease and report an evidence of injury to the lung and liver and the involvement of red blood cell progenitors associated with severe COVID-19. In their study, the concentration of cfDNA correlated with the WHO ordinal scale for disease progression and was significantly increased in patients requiring intubation.

Such a reproducible molecular blood test allowing a more accurate assessment of clinical severity and prognosis in COVID-19 patients at hospital admission can also be used in clinical trials of candidate COVID-19 treatments to monitor their efficacy and select eligible patients.

Overall, monitoring of blood biomarkers could guide the management of hospitalized COVID-19 patients. Our results could pave the way for new and more personalized therapeutic options in infectious diseases, based on each patient specific organ injury profile.

NOTES

Author contributions.

Experimental strategy design, experiments: TB, GP, MW, NR, VT, DVand HP

Vital materials: AP, JH, SK, PLP, JLD, BT and DS

Manuscript writing: MW, LB, VT, DV and HP

Manuscript editing: TB, MW, GP, NR, AP, LB, WX, JLK, JLD, BT, VT, DV and HP

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Declaration of Interests: GP, PLP, VT, DV and HP have a pending patent application related to this work n°PCT/EP2021/065863 and PCT/ EP20305571.0. VT reports Evodrops ITN Network (JK) and Erganeo (Patent); reports serving on Scientific Board and as founder for Emulseo (no direct link to present work); reports serving on Scientific board and as founder for Methys Dx (no direct link to present work). SK reports research grants to their institution and consulting fees (2018-2019) from bioMérieux; reports payment/honoraria for education activites form bioMérieux (2019) and Accelerate Diagnostics (2018); reports travel support from bioMérieux (2019) and MSD (2019).

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Table 1. Demographic and clinical findings of 139 patients suffering from COVID-19 hospitalized in Paris during the first wave of the epidemic.

Figure 1

A. SARS-CoV-2 RNAemia concentrations in 139 patients suffering from COVID-19 according to clinical severity

B. SARS-CoV-2 RNAemia concentrations in 139 patients suffering from COVID-19 according to invasively mechanically ventilated (IMV) status

C. Correlation between SARS-CoV-2 RNAemia concentrations (log copy/mL) and pulmonary severity

Figure 2

A. Plasmatic RNase P concentrations in 139 patients suffering from COVID-19 according to clinical severity

B. Plasmatic RNase P concentrations in 139 patients suffering from COVID-19 according to the IMV status

C. Correlation between RNase P RNAemia concentrations (log copy/mL) and pulmonary severity

Figure 3. Overall survival regarding RNase P RNAaemia (log copy/mL)

		N	%	Med	IQR
Age		139		58,61	17,30
Sex		139			
	Women	31	22		
	Men	108	78		
Classes		139			
	Moderate	37	27		
	Severe	35	25		
	Critical	67	48		
Delay from Symptoms Onset (DSO)		139		11	5
Tobacco status		138			
	Active smocking	4	2,9		
	Never smocker	109	79		
	Weaned smoker	25	18		
Cardiovascular history		135			
	No	114	84		
	Yes	21	16		
Hypertension		139			
	No	79	57		
	Yes	60	43		
Diabetes		139			
	No	110	79		
	Yes	29	21		
Cancer history		139			
	No	126	91		
	Yes	13	9,4		
Chronic renal failure		139			
	No	125	90		
	Yes	14	10		
Mechanical ventilation		67	48		
Pulmonary severity (% of lung involvement)		115			
	<10%	12	10		
	10-25%	22	19		
	25-50%	46	40		
	50-75%	29	25		
	75-100%	6	5,2		
Death		139			
	No	112	81		
	Yes	27	19		

Figure 1

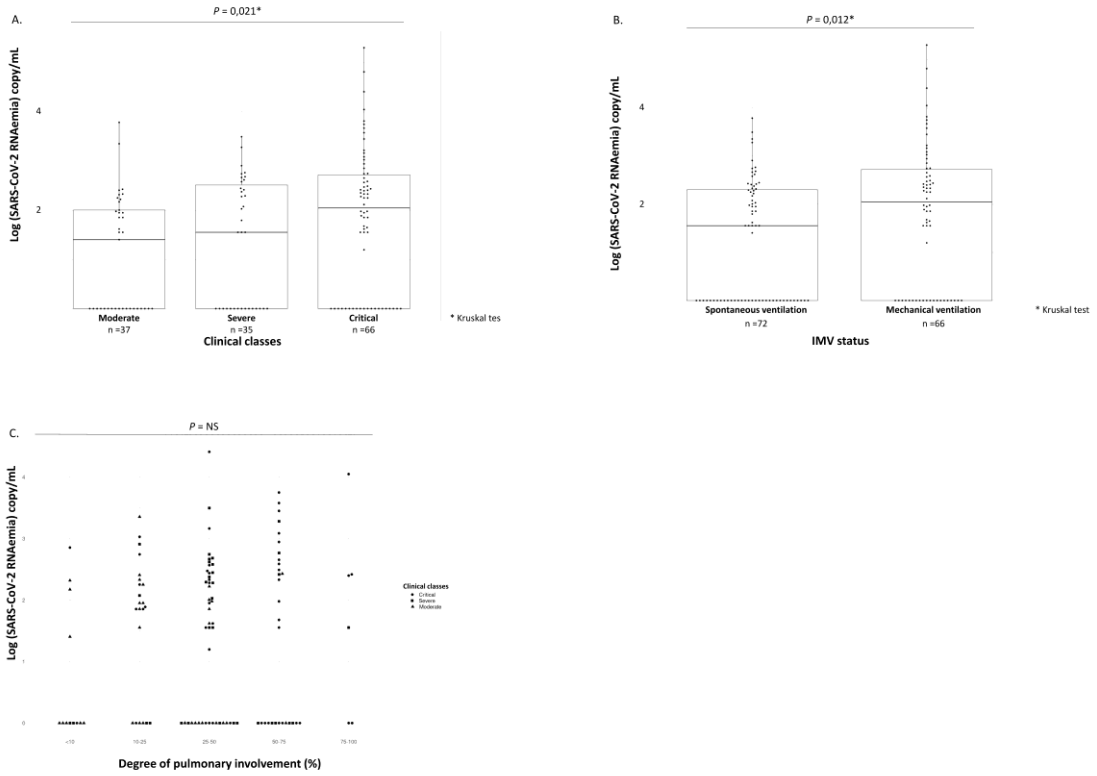
A.
P = 0,021*
Log (SARS-CoV-2 RNAemia) copy/mL
4
2
Moderate
n =37
Severe
n =35
Critical
n =66
Clinical classes
* Kruskal tes
B.
P = 0,012*
Log (SARS-CoV-2 RNAemia) copy/mL
4
2
Spontaneous ventilation
n =72
Mechanical ventilation
n =66
IMV status
* Kruskal test
C.
P = NS
Log (SARS-CoV-2 RNAemia) copy/mL
Clinical classes
Critical
Severe
Moderate
<10
10-25
25-50
50-75
75-100
Degree of pulmonary involvement (%)

Figure 2

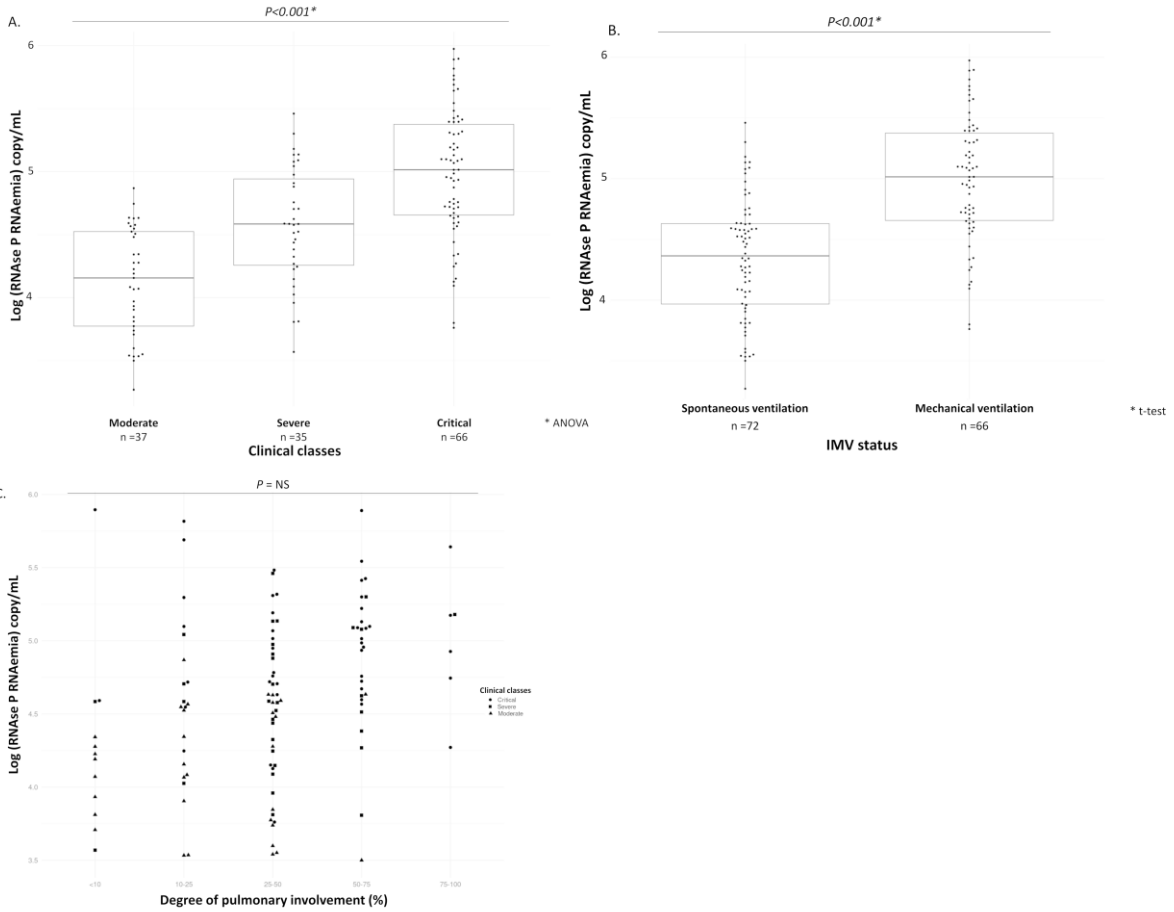
A.
P<0.001*
Log (RNAse P RNAemia) copy/mL
Moderate
n =37
Severe
n =35
Critical
n =66
Clinical classes
* ANOVA
B.
P<0.001*
Log (RNAse P RNAemia) copy/mL
Spontaneous ventilation
n =72
Mechanical ventilation
n =66
IMV status
* t-test
C.
P = NS
Log (RNAse P RNAemia) copy/mL
Clinical classes
Critical
Severe
Moderate
Degree of pulmonary involvement (%)

Figure 3