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Evidence of significant difference in key COVID-19 biomarkers during the Italian lockdown strategy. A retrospective study on patients admitted to a hospital emergency department in Northern Italy

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Summary. *Background.* The Lombardy region, Italy, has been severely affected by COVID-19. During the epidemic peak, in March 2020, patients needing intensive care unit treatments were approximately 10% of those infected. This fraction decreased to approximately 2% in the second part of April, and to 0.4% at the beginning of July. COVID-19 is characterized by several biochemical abnormalities whose discrepancy from normal values was associated to the severity of the disease. The aim of this retrospective study was to compare the biochemical patterns of patients during and after the pandemic peak in order to verify whether later patients were experiencing a milder COVID-19 course, as anecdotally observed by several clinicians of the same Hospital. *Material and Methods.* The laboratory findings of two equivalent groups of 84 patients each, admitted at the emergency department of the San Raffaele Hospital (Milan, Italy), during March and April respectively, were analyzed and compared. *Results.* White blood cell, platelets, lymphocytes and lactate dehydrogenase showed a statistically significant improvement (i.e. closer or within the normal clinical range) in the April group compared to March. Creatinine, C-reactive protein, Calcium and liver enzymes, were also pointing in that direction, although the differences were not significant. *Discussion.* The laboratory findings analyzed in this study were consistent with a milder COVID-19 course in the April group. After excluding several hypotheses, we concluded that our observation was likely the consequence of the lockdown strategy enforcement, which, by imposing social distancing and the use of respiratory protective devices, reduced viral loads upon infection. (www.actabiomedica.it)

Keywords: COVID-19; lockdown; laboratory parameters; WBC; aminotransferase; lactate dehydrogenase; lymphocytes; neutrophils

Introduction

At the end of 2019, a novel highly transmittable acute respiratory disease has been characterized and defined as Coronavirus Disease 2019 (COVID-19) by the World Health Organization (WHO) (1). It is caused by a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), belonging to the Coronaviridae family (2), which has represented a global health concerns over the past decades, causing outbreaks of two different forms of lethal human respiratory diseases in 2003 and 2012 (3,4). Since its first identification, in China, SARS-CoV-2 has globally spread. On March 11th, 2020, the WHO declared COVID-19 pandemic. As of writing, 16 million people were infected and almost 700.000 died as a result (5) .

In Italy, one of the most affected country in term of infected people (over 240.000) and deceased (over 35.000) (5), the first autochthonous case appeared in Lombardy, where this study has been performed, on February 20th (6). In March, the number of COVID-19 patients exceeded 50.000, mainly located in the Northern Italy, and about 6000 of them died (5,6). Thus, a rigid lockdown strategy was enforced from March the 9th to May the 18th. During the first phase of the pandemic, patients needing intensive care unit (ICU) treatment were approximately 10% (March, 2020) and decreased over time to approximately 2%, in the second part of April, and further to 1% when the lockdown was released. Clinical manifestations consistent with a less severe form of COVID-19 disease, leading to a significant drop in both mortality and number of cases needing ICU, were also anecdotally observed by clinicians in the most recent months (7–9).

From a clinical point of view, COVID-19 is characterized by a broad range of clinical manifestations with different degrees of severity which range from asymptomatic patients to severe interstitial pneumonia evolving into lethal multiorgan disease (10,11). Several biochemical abnormalities have been associated with the SARS-CoV-2 infection and some of them can be detected as specific disease biomarkers (12,13). Recent studies showed that such laboratory parameters were also associated to disease severity (14,15). Significant increase of both white blood cells (WBC) and neutrophils count as well as lymphopenia and thrombocytopenia were described in the most severe forms of COVID-19 (13,16). High levels of transaminases and lactate dehydrogenase (LDH), as well as an increase of systemic C-reactive protein (CRP) were also associated with a worse clinical outcome (14,17). Recently, severe hypocalcemia has also been described in COVID-19 patients needing hospitalization (18,19).

Therefore, we performed a pilot retrospective study in order to assess whether patients' biochemical patterns were significantly different between early and later COVID-19 patients of the Italian pandemic. Biochemical parameters as well as demographical and clinical observations were analyzed and compared in two groups of 84 COVID-19 patients each, admitted to the emergency department (ED) of the San Raffaele Hospital (Milan, Lombardy, Italy), in the month of the outbreak peak (March) and the following month (April).

Material and Methods

Cohort of analyzed COVID-19 patients

Between February 27th and April 25th, 2020, about 700 consecutive patients were admitted to the ED of the San Raffaele Hospital (Milan, Italy) and diagnosed with COVID-19. Among these, two groups of 84 positive individuals each, admitted to the ED respectively in March and April 2020, were included in this study. Patients were selected so that the distributions of age, gender, and severity of symptoms (defined in dichotomic terms as the clinical need of ICU within 24 from admittance) in the two groups did not present statistically significant differences. COVID-19 diagnosis was performed according to standard procedures: positive real-time reverse-transcriptase polymerase chain reaction (RT-PCR) test for SARS-CoV-2 from a nasal and/or throat swab (20), and clinical signs characteristic of COVID-19 pneumonia at conventional chest X-ray (12). Laboratory investigations and available clinical data, including clinical history, symptoms, and days from the onset of COVID-19 symptom to ED admittance, were retrospectively collected and analyzed according to the protocol BIGDATA-COVID19, approved by the Institutional Ethical Committee.

Laboratory testing

Routine blood tests were performed, on patients' admission to the ED, at the San Raffaele Hospital laboratory, according to IFCC recommendations (21). Hematological analyses (hemoglobin, hematocrit, red blood cell (RBC), WBC, platelets, basophils, eosinophils, neutrophils, lymphocytes and monocytes were performed on a Sysmex XE 2100 (Sysmex, Japan) (22,23). Creatinine (CREA), CRP, total bilirubin, urea, sodium (Na), potassium (K), glucose and enzyme activities (alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and LDH) were measured on a Roche COBAS 6000 (Roche Diagnostic, Basel, Switzerland) using Roche reagents, calibrators (C.f.a.s. / C.f.a.s. proteins) and control material at two different levels (Precicontrol ClinChem Multi 1 and 2). The following reagents were used: CRP Gen. 3, immunoturbidimetric assay (code 05172973 190); Creatinine plus vers. 2, enzymatic method (code 05168589 190); Glucose HK Gen. 3, Hexokinase enzymatic reaction (code 05168791 190); UREAL (Urease/GLDH) (code 05171873 190); Bilirubin Total Gen. 3 (code 05795419 190); ISE indirect Na-K-CL for Gen.2 (code Na 1082065 216; code K 1082065 216); ALTPM ALT (code 05531462 190), ASTPM AST (code 05531446 190), AP (code 05166888 190), g-GGT ver. 2 (code 05168775 190), LDH (code 05169330 190). All methods for enzyme activity measurement were standardized to IFCC reference measurement procedures. Point of care (POC) for K, Cloride (Cl), Ionized Calcium (Ca^{2+}), Na, anion gap and hemogasanalysis were performed on a Rapidpoint 500 (Siemens Healthcare).

Statistical analysis

Differences in the data distribution, for each laboratory parameter, were evaluated using the two-sample univariate Kolmogorov–Smirnov test (24) between the two groups of selected patients. Since the comparison between the two groups was assessed by multiple univariate tests, a statistical correction for family-wise errors using the Holm–Bonferroni correction (25) was performed. Statistical significance was assessed with a confidence level of 0.95 (alpha = 0.05) over the corrected *p-values*. In addition, a nonparametric multivariate bootstrap-based two-sample test for distribution equality (26) was performed by considering those biochemical features specifically altered in COVID-19, as lymphocytes, AST, LDH, Ca, platelets, CRP, CREA, in order to carry out a multivariate analysis. Statistical significance was assessed with a confidence level of 0.95 (alpha = 0.05) over the computed *p-value*. Statistical analysis was performed using the Python software. For the Kolmogorov-Smirnov test the implementation provided in the SciPy library (version 1.5.1) was used; whereas the implementations of the Holm-Bonferroni correction and the multivariate distribution equality test were from the authors (https://github.com/AndreaCampagner/uncertainpy/tree/master/utils).

Results

Clinical and demographic features

The two selected groups were adjusted and matched as described in Materials and Methods, in order to avoid statistical bias. Patients' demographic and clinical characteristics were summarized in Table 1. In the cohort of 168 analyzed patients, 61% were males with a median of age of 63 years old; 115 patients (68%) were affected by at least one comorbidity while 48% presented multiple pathologies. Total comorbidities were equally distributed, showing no statistically significant differences between the two groups (Table 1). When considering the type of comorbidities, all of them, with the exception of neurological, gastroenteric and renal diseases, were equally distributed between the two groups (Table 2). The time interval between symptoms onset and admittance to the Hospital ED was also statistically similar with a median of 5 days for both groups (Table 1).

Hematological parameters

Table 3 shows the averaged values of the hematologic biomarkers, known to be specifically altered in COVID-19 (16,17,27–30), recorded in the two groups. Statistically significant differences between the

Table 1. Demographic and clinical features of the two different groups of COVID-19 patients. *P-values* were calculated by means of a "N-1" Chi-squared test with MedCalc.

	All	March group	April group	P-value
Number of patients	168	84	84	
Age (median)	63	63	63	
Male (%)	61%	65%	57%	0.29
Days from symptoms (medians)	5	5	5	0.48
Patients with other comorbidities (%)	68%	66%	70%	0.58
Patients with 2 or more comorbidities (%)	48%	39%	55%	0.039

Table 2. Percentage of occurrence for the different class of comorbidities observed in the cohort of COVID-19 patients. *P-values* were calculated by means of a "N-1" Chi-squared test with MedCalc.

Comorbidities	All	March group	April group	P-value
Other	42%	39%	44%	0.51
Hypertension	34%	33%	35%	0.79
Cardiovascular disease	26%	26%	25%	0.88
Neurological disease	19%	13%	25%	**0.048**
Metabolic disease	17%	14%	19%	0.38
Gastroenteric disease	13%	6%	20%	**0.007**
Diabetes	10%	10%	11%	0.83
Oncologic disease	8%	11%	6%	0.25
Renal disease	6%	2%	10%	**0.029**
Respiratory disease	6%	7%	5%	0.59

groups were observed for WBC, platelets and lymphocytes count, which were increased in the April group when compared to the previous month counterpart. Figure 1 shows the violin plots for both platelets (Panel A) and lymphocytes (Panel B). In both cases the March values were located in the lower region of their normal clinical range (130-400 and 1.0-4.8 10^9/L for platelets and lymphocytes, respectively) whereas in April the averaged values were positioned more centrally.

Biochemical parameters

Among the biochemical parameters listed in Table 3, Na, Cl and LDH showed statistically significant differences between the two groups. Although Cl and Na were within the normal clinical range, the latter showed an averaged value, in the March group, near the lower limit whereas in April is positioned approximately in the center of the normal clinical distribution. This was further confirmed by the Na POC results (Table 3). In contrast, the averaged LDH values were above the normal clinical range in both groups. However, in April, the LDH value significantly shifted toward the normal clinical distribution (Table 3), as shown also in Figure 1 (Panel C). Our data showed that, in April, CRP, ALT, AST, GGT and CREA were, although not statistically different, more consistent, than their March counterpart, with a healthy individual clinical status (i.e. within the normal clinical range) (Table 3; Figure 1, Panel D-H). Ionized calcium, recently shown to be lowered in COVID-19 patients (19), was slightly increased in the April group and thus closer to the normal clinical distribution (Table 3; Figure 1, Panel I).

Table 3. Laboratory parameters obtained for the two groups of COVID-19 patients admitted at the ED on March and April, respectively.

Type of Analyte	Measurand	Unit	Reference interval	Mean ($CI_{95\%}$), March Group~	Mean ($CI_{95\%}$), April Group~	p-value+
Henatological	Hemoglobin*	g/dL	14.0-18.0	13.5 (9.9-16.1)	13.4 (10.7-16.2)	1
	Hematocrit*	%	42.0-52.0	40.2 (30.7-47.7)	40.3 (33.3-47.8)	1
	Red Blood Cell (RCB)*	$10^{\wedge 12}$/L	4.7-6.1	4.6 (3.2-5.5)	4.6 (3.7-5.6)	1
	White Blood Cell (WBC)	**$10^{\wedge 9}$/L**	**4.8-10.8**	**5.6 (2.9-11.2)**	**7.0 (3.5-12.1)**	**0.034**
	Platelets	**$10^{\wedge 9}$/L**	**130-400**	**177 (91-275)**	**237 (109-388)**	**< 0.001**
	Basophils	$10^{\wedge 9}$/L	0.0-0.2	0.0 (0.0-0.0)	0.0 (0.0-0.1)	1
	Eosinophils	$10^{\wedge 9}$/L	0.0-0.5	0 (0-0.02)	0.04 (0-0.2)	0.09
	Lymphocytes	**$10^{\wedge 9}$/L**	**1.0-4.8**	**0.9 (0.4-1.7)**	**1.3 (0.4-2.6)**	**0.01**
	Monocytes	$10^{\wedge 9}$/L	0.2-0.8	0.5 (0.1-0.9)	0.5 (0.1-0.9)	1
	Neutrophils	$10^{\wedge 9}$/L	1.8-7.7	5.3 (1.9-11.8)	4.9 (1.7-10.8)	1
Biochemical	Glucose	mg/dL	60 - 100	117.5 (80.4-193.2)	120.7 (76.4-240.8)	1
	Creatinine*	mg/dL	0.5 - 1.25	1.34 (0.59-2.52)	1.19 (0.60-2.76)	1
	Total Bilirubin	mg/dL	0.1-1.0	0.5 (0.2-0.8)	0.6 (0.2-1.2)	1
	Urea	mg/dL	10.0-50.0	49.9 (11.8-103.7)	54.6 (16.4-196.8)	0.23
	Sodium	**mmol/L**	**135.0-148.0**	**137.0 (131.5-142.9)**	**141.5 (132.9-156.2)**	**0.002**
	Potassium	mmol/L	3.5-5.0	4.21 (3.26-5.21)	4.16 (3.36-5.20)	1
	Alanine aminotransferase*	U/L	6.0-59.0	45.6 (13.4-104.0)	41.2 (11.0-92.0)	1
	Aspartate aminotransferase*	U/L	5.0-35.0	53.1 (20.4-139.8)	50.5 (16.0-118.8)	1
	Alkaline phosphatase*	U/L	53-119	70.2 (39.9-113.9)	76.4 (42.0-115.6)	1
	Gamma-Glutamyl Transferase*	U/L	11.0-68.0	65.3 (11.4-143.4)	48.2 (12.0-116.2)	1
	Lactate dehydrogenase	**U/L**	**125-220**	**352 (225-543)**	**315 (165-578)**	**< 0.001**
	C- Reactive protein	mg/L	<6	74.9 (4.1-244.5)	63.4 (1.5-212.6)	1
Biochemical, POC	Potassium (POC)	mmol/L	3.0-5.0	3.87 (3.14-4.65)	3.84 (3.10-4.49)	1
	Chloride (POC)	**mmol/L**	**96 - 108**	**102 (96-108)**	**106 (98-120)**	**< 0.001**
	Ionized Calcium (POC)	mmol/L	1.18-1.30	1.08 (0.95-1.16)	1.11 (1.00-1.22)	1
	Sodium (POC)	**mmol/L**	**135.0-148.0**	**136.1 (130.6 – 141.4)**	**138.8 (130.6-152.5)**	**0.011**
	Anion Gap (POC)	mmol/L		14.2 (10.4-18.4)	13.4 (9.4-17.5)	0.93

CO-Oxymetry	Total oxyhemoglobin	%	14-18	13.8 (11.2-16.1)	13.7 (11.2-15.8)	1
	Methemoglobin	%	0-2	0.2 (0.0-0.4)	0.3 (0.1-0.5)	1
	Oxyhemoglobin	**%**		**90.1 (72.6-98.1)**	**92.5(74.6-98.2)**	**< 0.001**
	Carboxyhemoglobin	**%**	**< 5**	**0.37 (0.1-0.8)**	**0.59 (0.2-1.4)**	**< 0.001**
	O_2 Saturation (POC)	**%**	**95-98**	**91.4 (83.8-98.6)**	**95.0 (89.0- 99.0)**	**< 0.001**
	Deoxyhemoglobine	**%**		**8.6 (1.4-16.1)**	**4.9 (1.0-10.9)**	**< 0.001**
	hematocrit (POC)	%	37-47	40.7 (33.0-47.0)	40.3 (33.0-46.0)	1
Hemogasanalysis, venous blood gas	pH	U	7.35-7.45	7.44 (7.36-7.52)	7.44 (7.36-7.52)	1
	pCO_2	mmHg	34.45	36.5 (27.5-46.4)	35.1 (26.7-47.5)	1
	pO_2	**mmHg**	**80-110**	**74 (38-128)**	**81 (38-135)**	**0.001**
	HCO_3	mmol/L	22-26	24.0 (19.7-28.2)	23.4 (19.0-30.5)	0.373
	Excess Bases	mol/L	-2 - 3	0.3 (-3.6-4.3)	-0.2 (-3.9-5.3)	1

* The indicated reference intervals were chosen arbitrarily for male patients.
+ Bonferroni-corrected *p*-values.
~ Samples quota-controlled by gender, age, and disease severity.

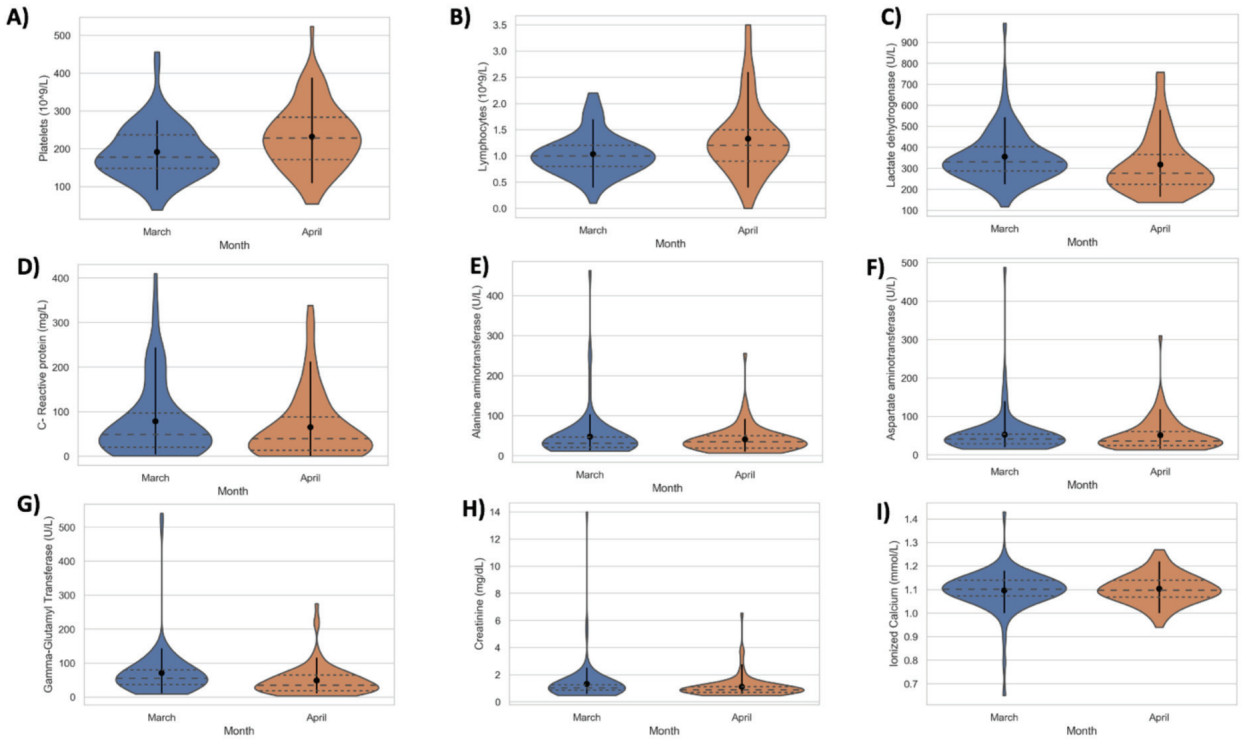

Figure 1. Violin plots of the distributions of the most significant laboratory findings analyzed in the study. Dashed lines inside the plots indicate (bottom-up) 1st, 2nd (i.e., median) and 3rd quartiles, respectively. Also means and their 95% confidence intervals were indicated as a vertical segment within each violin plot to facilitate March - April comparison.

CO-Oximetry and Hemogas analysis

The oxygen carrying state of hemoglobin as well as the partial pressure of oxygen (pO_2) were both consistent with an improved clinical state in April with respect to March. In particular, the POC O_2 saturation and pO_2 were outside their normal clinical ranges in March while returning to a normal distribution in April (Table 3).

Multivariate analysis

To further verify the presence of possible differences between the clinical state of the patients admitted at the ED during March and those admitted in April, Lymphocytes, AST, LDH, Ca^{2+}, platelets, CRP, and CREA, known to be COVID-19 biomarkers (16,17,27–31), were used to carry out a multivariate analysis as described in Materials and Methods. The results showed a statistically significant difference (*p=0.001*) between the March group and the April group.

Discussion

A recently published study from the San Raffaele Hospital showed a ten-fold decrease in COVID-19 patients in-hospital mortality, from approximately 20% during February/March to a 2% observed at the end of April to mid-May (7). Furthermore, patients admitted in May, in the same Hospital, had viral loads, measured by RT-PCR on their first positive swab test, lower than those admitted in April (32). Taken together, these data are consistent with a time-dependent decreased severity in COVID-19 clinical presentation and progression. Different conjectures have been proposed in order to explain these findings, such as a better understanding of the pathophysiology of the disease, the establishment of a national lockdown from March 9th, the large use of respiratory protective devices, which can limit SARS-CoV-2 spread, and/or possible decrease of co-infection of respiratory pathogens (i.e. seasonal influenza viruses) due to the higher temperature (7). To better inquire into this topic we analyzed the laboratory parameters recorded in two groups that had been controlled by age, gender, and disease severity, admitted to the San Raffaele Hospital during two separated temporal frames: March and April, 2020.

Laboratory parameters like WBC, platelets, lymphocytes and LDH, known to be altered in COVID-19 and associated to disease severity (15), showed a statistically significant improvement in the April group when compared to March. Decreased lymphocyte count in severe COVID-19 patients have been recently explained through the expression of angiotensin-converting enzyme 2 (ACE2), the entrance target of SARS-CoV-2 at the surface of lymphocytes (33). Viral infection of these cells induces their gradual decline and, if not recovered, the lymphocytic dysfunction and immunosuppression lead patients to a worse prognosis often caused by bacterial/viral co-infections (34). Similarly LDH, known to be a marker of lung damage (35), was highly increased in severe COVID-19 patients because of the typical interstitial pneumonia caused by the disease (36). Sodium has been shown to be significantly lower in patients with severe COVID-19 (37). Its higher values in the April group suggest a possible clinical trend to a less severe COVID-19 form in this group when compared to March. CRP, ALT, AST, GGT and CREA, although not statistically different between the two groups, were consistent with a milder COVID-19 phenotype in the April group. It must be noted that CREA was out of the normal clinical range in March, whereas in April it returned to a normal distribution. Recently, hypocalcemia was described as a further COVID-19 biomarker and was associated with a worse clinical outcome (18,19). It is known that during viral infections, Ca^{2+} is essential for virus structure formation, entry, gene expression, virion maturation and release (38). The SARS-CoV *E* gene, encoding a small transmembrane protein with ion channel activity and permeable to Ca^{2+}, is upregulated during infection and might be responsible for hypocalcemia (19). It must be noted that the two groups of patients were matched for the presence of other comorbidities, such as hypertension, cardiovascular disease and diabetes, that have been associated with higher risk for severe COVID-19 infection (39). Because comorbidities and days between symptoms onset to hospital admission were similar in

the two groups, the above discussed data, as well as the CO-Oximetry and hemogasanalysis results, were all consistent with a slight amelioration of the COVID-19 manifestation in the April group.

Conclusions

Our data showed that the laboratory parameters from COVID-19 patients admitted to the San Raffaele Hospital, in Milan, during April 2020 were consistent with a milder disease course if compared with their March counterpart. This was in agreement with previous studies showing a higher mortality rate and increased viral loads in patients admitted at San Raffaele Hospital (Milan) at the early clinical outbreak (March) with respect to the following month (April) (7,32). Since our data were collected at ED admittance of the patients, we can exclude that the described differences in laboratory parameters were due to a better understanding of the pathogenic mechanism of the disease or to a more effective treatment. An alternative explanation might rise from a virus population diversity in time which might have evolved in less aggressive, yet more fitted, viral strains. However, recent studies showed that the Italian outbreak was mainly consistent with a single virus strain which accounted for the 90% of genome sequenced whereas the remaining 10% was attributed to a second strain (40,41). Thus, the hematochemical findings reported in this study, which are concomitant with the reduction of poor outcomes of SARS-CoV-2 infection that was observed in the second phase of the Italian pandemic and that was found related to reduced viral loads upon infection (32), are compatible with the enforcement of the lockdown strategy, to the large adoption of social distancing measures and to the widespread use of respiratory protective devices (42).

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