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RESEARCH ARTICLE

Administration of HeberFERON in Patients with Persistent Oropharyngeal SARS-CoV-2 Wuhan/D614G Strain Viral Shedding

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ABSTRACT

Study background: HeberFERON accelerates SARS-CoV-2 clearance in COVID-19 cases. Considering this we evaluated the employment of HeberFERON in patients with more than 14 days of viral shedding.

Methods: This is a case series study of mild or moderate ill patients with laboratory-confirmed SARS-CoV-2 from one hospital in Havana, Cuba. We evaluated the effect and safety of HeberFERON in patients previously treated with Heberon Apha R that resulted with prolonged viral shedding. All patients received lopinavir-ritonavir 200/50 mg every 12 h and chloroquine 250 mg every 12 h. The primary endpoint was the time to negativization of viral RNA in patients with persistent viral shedding. The protocol was approved by the Ethics Committee of the Luis Diaz Soto Hospital.

Results: The characteristics of the individuals included the age ranged from 19-87 years with a mean of 40 years, (Study and Control I groups), while in the Control group II the mean age was 43.8 years. Leukocytes, platelets, neutrophils, and eosinophils, show a significantly lower counts in the groups with viral persistence.

Under IFN treatment the median viral shedding duration from diagnosis were 21 days and 19 days in Study group and Control group II, respectively. The Control group I showed a median viral shedding of 11 days (log-rank $p = 0.000$). Significant longer median viral negativization time (19 days) of symptomatic than asymptomatic patients (11 days, Long-rank $p = 0.004$), was observed. In patients under Heberon Alpha R treatment that resulted persistent for viral presence, the median time to viral negativization was 7 days for the period of administration of HeberFERON. Being symptomatic at diagnosis was significantly associated with viral persistence. The HeberFERON showed an adequate safety profile.

Conclusion: HeberFERON showed a safe and rapid negativization of patients with viral persistence, achieving negativization in more than 50% of patients in 7 days.

INTRODUCTION

The world is living a major devastating pandemic of Coronavirus Disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus- 2 (SARS-CoV-2) with disease manifestations from no symptoms through mild to severe

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disease including death [1]. The first cases of Wuhan SARS-CoV-2 in Cuba were detected on March, 2020 [2]. Recently emerging variants of SARS-CoV-2 (Delta variant) with increased transmissibility or immune escape [3,4] have been causing large outbreaks of COVID-19 infections across the world.

Symptoms of COVID-19 commonly include cough, loss of smell, nausea, vomiting, and diarrhea that are less common with the Delta variant, which is characterized by more frequency in sore throat, runny nose, headache, and fever [5]. Subjects with diabetes mellitus, hypertension, cardiovascular disease, and respiratory disease, are at increased risk for severe COVID-19, and case fatality rates increase steeply with age [6].

Viral nuclei acid release vary widely, from 6 to more than 100 days after symptoms with a median duration of 20 days that may include a period of symptoms cessation [7-11]. Additionally, patients could asymptomatically maintain a replicative SARS-CoV-2 for weeks [12,13].

Persistent viral RNA shedding in patients with COVID-19 potentially increases the risk of spread, resulting in the consumption of additional hospital resources and greater economic costs, as well as the increase of risk of worsening of disease symptoms [14]. Severe disease has a higher viral peak and longer duration of viral release in nasopharyngeal swabs [14-16]. However, other studies showed no correlation between the duration of viral shedding and the severity of COVID-19 [17-19]. Factors associated with prolonged and increased risk of transmission remain unclear [20].

Accordingly, we need to develop different treatment strategies for persistent viral infections. For these patients, it would be worth to further explore a combination of antiviral therapy (for example, Interferons (IFNs) α and γ, remdesivir [23] or convalescent plasma), immune activation therapy (anti-PD-L1 or CTLA-4 antibody), or more recently developed neutralizing antibodies to SARS-CoV-2 [21-26].

The key point in SARS-CoV-2 persistent viral nuclei acid release could be the depletion of adaptive and innate immunity antiviral defenses. IFNs type I (α/β/λ) and type II (γ), trigger intracellular and T and B cells antiviral responses in the elimination of viral infections [27-31].

Early studies with combination treatments of a type I IFN and IFN-γ revealed synergistic inhibition of SARS-CoV replication *In vitro* [21,22]. Recently a phase II randomized clinical trial demonstrated the potent anti-SARS-CoV-2 activity of combination of IFN-α2b and IFN-γ in mild to moderate patients positive to SARS-CoV-2 by RT-PCR [32].

We conducted a case series cohort study aimed to evaluate the impact of the combination of IFNs in reducing the time to cessation of viral shedding in laboratory-confirmed COVID-19 patients with mild to moderate disease from the Hospital Luis Soto, in Havana, Cuba, during the course of antiviral treatment and to investigate the associations with characteristics of COVID-19 patients with persistent SARS-CoV-2 infection.

METHODS

Study design and participants

Adult (19-87 yrs), PCR(+) confirmed SARS-CoV-2, of mild or moderate symptoms, were consecutively enrolled in this case series, single center study at Military Central Hospital Luis Diaz Soto Hospital, Havana, Cuba. The study included three cohorts of inpatients, who were hospitalized between March and July 2020.

Patients of the Study group were those treated with Heberon α R (Center of Genetic Engineering and Biotechnology (CIGB), Havana, Cuba) that resulted positive to viral RNA 14 days after the diagnosis, and per doctor decision were treated with HeberFERON (combination of IFN-α 2b and IFN-γ, CIGB, Havana, Cuba) for viral clearance. Patients treated with Heberon alpha R that became negative before or day 14 after diagnosis composed the Control group I; and patients treated with Heberon Alpha R, positive at day 14 for viral presence, that continued to receive Heberon Alpha R to obtain viral clearance were selected for Control group II. Additionally, all patients received lopinavir-ritonavir 200/50 mg every 12 h and chloroquine 250 mg every 12 h (Figure 1). The primary endpoint was the time to negativization of viral RNA in patients with persistent viral shedding.

The study execution followed the ethical principles of the Declaration of Helsinki and the International Council for Harmonization–Good Clinical Practice guidelines. The authors were responsible for designing the trial and for collecting and analyzing the data, and assured the completeness and accuracy of the data collection and the adherence to the protocol. All patient data used in this study were collected in the context of routine clinical patient care.

Eligibility criteria

Patients age ≥ 19 were included if they were SARS-CoV-2 positive by RT-qPCR at diagnosis, with persistent viral shedding or not, after 14 days of antiviral treatment. This study included three cohorts of inpatients, who were hospitalized between March and July 2020. We evaluated the effect and safety of HeberFERON in patients previously treated with Heberon Apha R that resulted with prolonged viral shedding.

The protocol was approved by the Ethics Committee on Clinical Investigation of the "Luis Diaz Soto" Hospital (01/09/2020).

Procedures

Patient diagnosis for SARS-CoV-2 was undertaken using a single swab with oropharyngeal sampling and defined as

positive if they had two consecutive positive results, by RT-PCR targeting amplifications of E and /or RdRP genes, the details are provided by Idelis, et al. [32]. Patients positive at diagnosis for SARS-CoV-2 were treated with 3.0 million international units (MIU) IFN-α2b (Heberon Alpha R), thrice a week, intramuscularly and lopinavir-ritonavir 200/50 mg every 12 h and Chloroquine (KCh) 250 mg every 12 h. Those that resulted negative for SARS-CoV-2 at day 14 or before, discharged in the absence of symptoms, were selected for Control group I.

The patients with more than 14 days with persistent SARS-CoV-2 presence after Heberon Alpha R schedule, received either, 3.5 MIU IFN-α 2b and 0.5 MIU IFN-gamma (HeberFERON), twice a week for two weeks, subcutaneously and lopinavir-ritonavir 200/50 mg every 12 h and Chloroquine 250 mg every 12 h (Study group), or continued treatment with Heberon Alpha R as described, and constituted the cohort of control group II (Figure 1).

The medical records of patients were analyzed by the research team of "Luis Diaz Soto" Hospital, Havana, Cuba. Patient data were collected with standardized case report forms from electronic medical records. These data were checked by a trained team of physicians. Information recorded included demographic data, medical history, exposure history, underlying comorbidities, symptoms, signs, laboratory findings, and treatment measures. Routine blood examinations included whole blood count, serum biochemical, C-reactive protein and ferritin. Inflammatory parameters were calculated.

Outcomes assessment

The primary outcome consisted in the time to SARS-CoV-2 RNA negativization (absence of the virus according to the qRT-PCR) in patients with viral persistent shedding after starting HeberFERON antiviral therapy. Secondary outcomes included safety, proportion of patients who developed adverse events and the frequency, type, and intensity. Risk factors for prorogued viral shedding were also assessed.

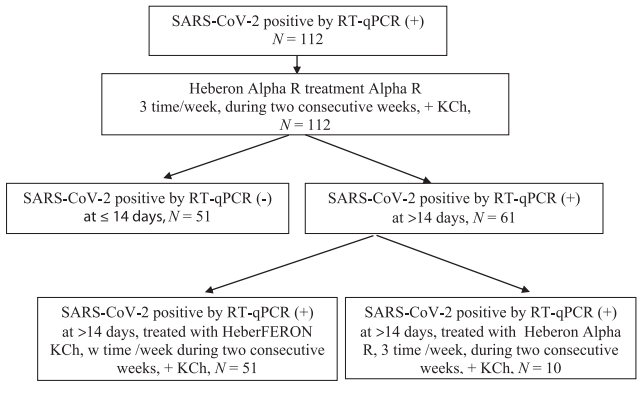

Figure 1 Trial flowchart.

Statistical analysis

Data analysis was performed using Statistical Package Stata version 11. Categorical variables were reported as absolute (relative frequencies) and compared by χ^2 tests or Fisher's exact tests. Continuous variables were expressed as mean (SD) if they are normally distributed or median (IQR) if they are not and compared by independent group T Student depending of normality data distribution or not, or Mann-Whitney U tests, respectively. $p < 0{\cdot}05$ was considered as statistically significant.

To investigate the risk factors associated with the prolonged viral shedding, univariate and multivariate logistic regression models were used. Spearman non parametric correlation between viral clearance and levels of lymphocytes, neutrophils, and Systemic Inflammation Index (SII), and thrombin time were calculated. Kaplan-Meier method was used for cumulative proportion of patients with detectable SARS-CoV-2 at in hospitalization days. A two sided α of less than 0.05 was considered statistically significant. Sensitivity and specificity calculations (for some laboratory values), as possible predictors of viral persistence was conducted using Receiver Operator Curve (ROC) analysis to identify a cut-point detection that minimized the Euclidean distance between the ROC curve and the (0,1) point in the ROC plane, to maximize sensitivity and specificity.

RESULTS

Patients and baseline features

The duration of virus shedding was defined as the interval from diagnosis/hospitalization until successive negative detection of SARS-CoV-2 RNA. Between March 11, 2020, and July 13, 2020, 61 PCR (+) COVID-19, symptomatic or not, patients exhibited long duration of viral shedding (> 14 days). 51 of these patients were treated with HeberFERON (Study group) or 10 inpatients continued the treatment with Heberon alpha R (Control group II). We also collected data from 51 COVID-19 inpatients treated with Heberon Alpha R, whose viral shedding durations were ≤ 14 days for comparison (Control group I). All the patients were identified as laboratory confirmed SARS-CoV-2 infected patients at Luis Diaz Soto, Havana, Cuba.

The basic demographic information and clinical parameters of these patients are detailed in tables 1 and 2, respectively. The mean age of the 112 included patients was 49.7 (± 15.6). The age ranged between 19-87 years with a mean of 40 years, for the study and the control groups I, while in control group II the age was from 21-76 with a mean age of 43.8 years. Medical staff members and health workers predominated in the group that used HeberFERON (23.5%, $p = 0.045$). In Control group I, patients who acquired the virus in their community showed the higher frequency ($p = 0.011$). Symptomatic patients after diagnosis were more represented in the Study group ($p = 0.000$, table 1).

Table 1: Demographic and clinical characteristics of the participants at baseline.

Characteristic	N	Study group	N	Control I	N	Control II	*p*
Median age (min-max) yr¶	51	39 (19-85)	51	39 (19-87)	10	45 (21-76)	
Health worker	51	12 (23.5%)	51	4 (7.8%)	10	0 (0%)	0.046
COVID-19 acquired in the community	51	37 (72.5%)	51	47 (92.1%)	10	10 (100%)	0.011
Sex							
Male (%)	51	24 (47.06%)	51	25 (49.02%)	10	4 (40%)	0.886
Female (%)		27 (52.9%)		26 (50.9%)		6 (60%)	
Toxic habits							
Smoker	50	1 (2.0%)	50	3 (6.0%)	10	0 (0%)	0.430
Ex-smoker		3 (6.0%)		5 (10%)		2 (20%)	
Non-smoker		46 (92.0%)		42 (84.0%)		8 (80%)	
Drinker		6 (12.0%)	51	11 (21.5%)		1 (10%)	0.446
Non-drinker		44 (88.0%)		40 (78.4%)		9 (90%)	
Symptoms							0.000
Symptomatic	50	32 (62.7%)	51	15 (29.4%)	10	10 (100%)	
Asymptomatic		19 (37.2%)		36 (70.5%)		0 (0%)	
Comorbidity							
Cardiac disease	50	5 (10%)	51	1 (2.0%)	10	1 (10%)	0.152
Diabetes	50	6 (12.0%)	51	3 (5.8%)	10	3 (30%)	0.075
Hypertension	50	19(38.0%	51	10(19.6%)	10	4(40%)	0.100

Table 2: Baseline data of the clinical laboratory determinations.

Characteristic	N	Study group Mean (Std. Err)	N	Control I Mean (Std. Err)	N	Control II Mean (Std. Err)	*p*
Hemoglobin. g/l	47	141.78 (2.70)	44	141.36 (2.67)	10	129.9 (4.26)	0.9715
C-Reactive Protein. mg/dL	18	6.09 (2.49)	26	8.59 (4.45)	5	22.48 (18.89)	0.9619
Platelet count. × 10^9 per L	45	234.9 (11.06)	44	269.1(16.36)	10	273 (41.92)	0.0364
Leucocyte. × 10^9 per L	47	5.33 (2.36)	45	14.07(7.25)	10	5.99 (7.60)	0.0005
Neutrophils. × 10^9 per L	47	0.409 (0.22)	38	0.504 (0.03)	10	0.503 (0.08)	0.0118
Monocytes. × 10^9 per L	40	0.094 (.0071)	42	0.129 (0.025)	10	0.073 (0.012)	0.6866
Eosinophils. × 10^9 per L	39	0.014 (0.002)	42	0.064 (0.021)	9	0.061(0.010)	0.0031
Aspartate aminotransferase. U/L	48	33.3 (2.637)	36	23.4 (0.858)	8	31.3 (3.927)	0.0006
Alanine aminotransferase. U/L	48	49.09 (5.65)	36	30.78 (2.07)	8	46.94 (13.01)	0.0091
Alkaline phosphatase. U/L	40	180.63 (8.551)	31	196.54 (8.839)	7	159.69 (22.766)	0.1522
Ferritin. ng/mL	20	415.9 (64.49)	23	174.56 (25.90)	3	495.4 (337.41)	0.0030
Serum creatinine. µmol/L	45	96.59 (2.649)	35	97.82 (4.093)	8	90.10 (5.487)	0.8462
Urea. mmol/L	39	7.47 (2.090)	30	5.32 (0.219)	4	5.5 (0.283)	0.9807
Glucose. mmol/L	44	4.68 (0.167)	32	4.86 (0.268)	7	5.19 (0.672)	0.3854
Total protein. g/L	27	71.83 (1.093)	23	69.39 (1.410)	2	64.14 (8.58)	0.3552
Albumin. g/L	19	43.33 (1.128)	21	43.89 (0.809)	2	38.07 (8.294)	0.8390
Sodium mmol/L	14	135.85 (7.270)	9	142.36 (1.157)	4	135 (1.838)	0.8011
Potassium mmol/L	14	4.08 (0.174)	9	4.09 (0.125)	4	3.36 (0.166)	0.8749
Chloride mmol/L	14	102.5 (1.703)	9	94.47 (10.499)	4	97.15 (1.296)	0.7055
Prothrombin time. sec	14	13.2 (0.368)	10	13.8 (0.373)	2	13.8 (0.450)	0.3055
Thrombin time. sec	14	26.06 (4.244)	7	32.67 (1.943)	2	25.4 (3.4)	0.3706

Notably, there were no significant differences between groups, in sex, toxic habits, comorbidities, hemoglobin, monocytes counts, blood biochemistry (alkaline phosphatase, serum creatinine, urea, glucose, total protein, albumin, sodium, potassium, chloride, coagulation function (Prothrombin Time (PTT) and Thrombin Time (TT)), and inflammatory marker, C-reactive protein (Table 2). It can be noted that platelet (p = 0.0363), leukocytes (p = 0.0005), neutrophils (p = 0.0118), eosinophils counts (p = 0.0031); ASAT (p = 0.0006), ALAT (p = 0.0091), and ferritin (p = 0.0030); resulted significant different between the groups under study.

In the Study group, 46 patients (90.2%) were previously treated with KCh and Heberon Alpha R, 3 (5.88%) with Kaletra and Heberon Alpha R, 1 (1.96%) with Oseltamivir, KCh and Heberon Alpha R, and 1 patient (1.96%) treated only with KCh, according to the MINSAP protocol, version 1.6.[2] In the Control group II, 8 patients (80%) received KCh and Heberon Alpha R, and 2 (20%) received Oseltamivir, KCh and Heberon alpha R. In Control group I, 47 (92.2%) received KCh and Heberon Alpha R, 3 patients (5.8%) Kaletra and Heberon Alpha R and 1 patient received Oseltamivir, KCh and Heberon Alpha R.

Outcomes

Under IFN treatment the median viral shedding duration from diagnosis were 21 days (range: 12-40 days) and 19 days (range: 17-34 days) in Study group and Control group II, respectively (log-rank $p < 0.9962$, figure 2). The Control group I showed a median viral shedding of 11 days (log-rank $p = 0.000$, figure 2).

The median viral negativization of 11 days was observed for control group I. Control group II and treatment group (Treatment/Study group) showed a 19 days and 21 days of viral negativization, respectively.

Significant longer median viral negativization time (19 days, Long-rank $p = 0.004$) of symptomatic than asymptomatic patients (11 days), was observed (Figure 3).

Symptomatic patients showed a significant higher median viral negativization time (19 days, $p = 0.004$) than asymptomatic patients (11 days). Shown the cumulative proportion of patients with detectable SARS-CoV-2 RNA 21 day after admission, according to symptoms at diagnosis.

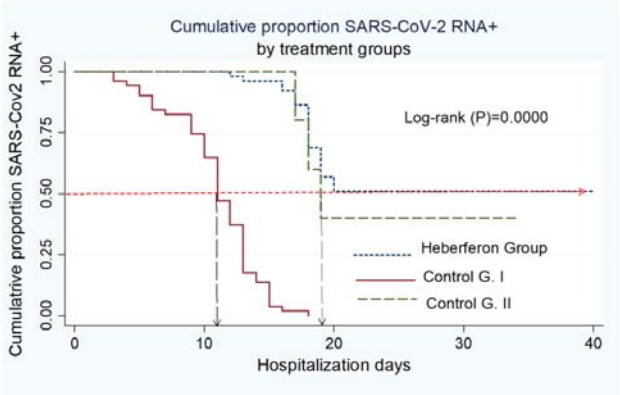

Figure 2 Cumulative proportion of patients with detectable SARS-CoV-2 in patients treated with Heberon Alpha R since diagnosis of SARS-CoV-2 by qRT-PCR.

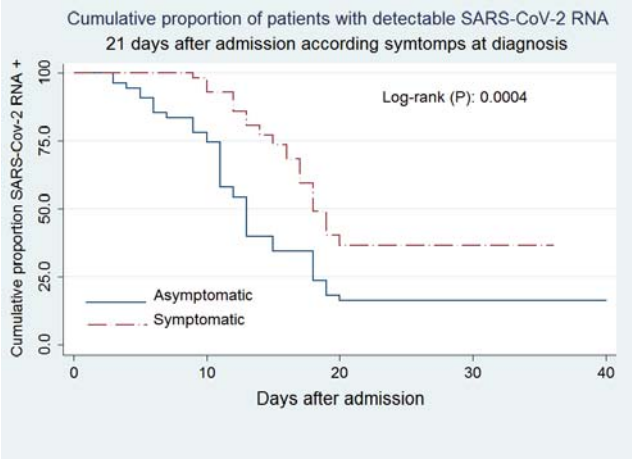

Figure 3 Symptomatic patients have more prolonged time of viral shedding.

In patients under Heberon Alpha R treatment that resulted persistent for viral presence, the median time to viral negativization was 7 days for the period of administration of HeberFERON (Table 3a,b).

According to the study design, the patients of study group (treated with HeberFERON) were exposed to a different magnitude depending on their negativization process, 68.3% of the patients used one dose of the drug to achieve negativity after viral persistence for more than 14 days, two doses received 11.8% of patients, three doses 7.8%, four doses 1.9%, five doses 5.9%, and seven or nine doses 1.9% of patients.

Of the 112 patients, 89.2% (100 patients) had at least one adverse event. In total, 259 adverse events were reported, of them only 4 of moderate intensity (1.55%). For the group of patients treated with HeberFERON the more common adverse events reported (≥ 10%) were: general malaise (73.3%) myalgia (40%), arthralgia (20%), headache (20%) and diarrhea (13.3%). Other events reported for this group, considered frequent (≥ 1% and <10%), are related to chills (8.9%), fever (6.7%), anemia, nasal congestion, loss of smell, pain at the injection site, depression , abdominal pain, tachycardia and decay; one patient for each event, which represents 2.2% in each case separately (Table 3a,b).

For the groups treated with Heberon alpha R, Control groups I and II, (Table 3a,b), the commonest adverse events reported (≥ 10%) were: general malaise (98.2%), pain at the injection site (54.5%), chills (38.2%), arthralgia (25.5%), myalgia (20%), vomiting (14.5%), headache (12.7%) and fever (10.9%). Other frequent (≥ 1% and <10%) were nausea (9.1%), diarrhea (9.1%), leukopenia (3.6%), anorexia, thrombocytopenia, retro-ocular pain, increased ASAT and ALAT and pruritus with 1.8% each. Two adverse events of moderate intensity were reported (1 diarrhea and 1 patient with pruritus).

Ninety-five percent of events were mild, 2 events were classified as moderate, which represents 4.5% of the total reported.

Risk factors for persistent viral shedding

Patients of the study were grouped as per viral persistence for < 21 days and the other with viral persistence ≥ 21 days. The data from the clinical laboratory determinations of these two cohorts are shown in table 4. It is observed that the blood levels of total leukocytes ($p = 0.0364$), neutrophils ($p = 0.0202$) and eosinophils ($p = 0.0225$) are significantly lower in patients with viral persistence for ≥ 21 days. Another factor significantly decreased in patients with viral persistence for ≥ 21 days is the TT with 17 seconds vs 32 in the group with viral persistence for < 21 days ($p = 0.0087$).

Table 3a: Viral shedding duration from the day 15 in patients defined with viral persistence.

Groups	N	Median (min-max)	IQR	Mean (±DS)	IC 95%
Study group	51	7 (0-26)	8	8.5 (6.3)	6.8; 10.3
Control group II	10	5 (3-20)	8	7.8 (5.6)	3.8; 11.8

Table 3b: Comparison of type and frequency of adverse events per patient for the formulations of IFN administered.

Type of events	HeberFERON (*N* = 51)	Heberon Alpha R (*N* = 61)
	Total (%)	
General malaise	33 (73.3)	54 (98.2)
Myalgia	18 (40)	11 (20)
Headache	9 (20)	7 (12.7)
Arthralgia	9 (20)	14 (25.5)
Diarrhea	6 (13.3)	5 (9.1)
Chills	4 (8.9)	21 (38.2)
Fever	3 (6.7)	6 (10.9)
Anemia	1 (2.2)	0
Injection site pain	1 (2.2)	30 (54.5)
Depression	1 (2.2)	0
Abdominal pain	1 (2.2)	0
Fatigue	1 (2.2)	0
Tachycardia	1 (2.2)	0
Vomiting	0	8 (14.5)
Nauseas	0	5 (9.1)
Leucopenia	0	2 (3.6)
Anorexia	0	1 (1.8)
Thrombocytopenia	0	1 (1.8)
Retro-ocular pain	0	1 (1.8)
Increase of ASAT	0	1 (1.8)
Increase of ALAT	0	1 (1.8)
Pruritus	0	1 (1.8)

Table 4: Baseline clinical laboratory determinations in patients with prolonged viral shedding.

Parameters	Viral Clearance < 21 Days	Viral Clearance ≥ 21 Days	*p*
Hemoglobin (g/L)	141.26 ± 19.2	138.3 ± 14.5	0.4439
C-Reactive Protein. mg/dL	10.15 ± 23.8	3.7 ± 2.7	0.6263
Platelet count. × 10^9 per L	264.6 ± 108.04	228.4 ± 61.08	0.1289
Leucocytes count. × 10^9 per L	10.784 ± 3.8	5.220 ± 1.33	0.0364
Neutrophils. × 10^9 per L	0.489 ± 0.19	0.384 ± 0.18	0.0202
Lymphocyte. × 10^9 per L	0.520 ± 0.94	0.368 ± 0.18	0.6551
Monocytes. × 10^9 per L	0.116 ± 0.135	0.088 ± 0.044	0.9137
Basophils. × 10^9 per L	0.050 ± 0.148	0.008 ± 0.020	0.4719
Eosinophils. × 10^9 per L	0.051 ± 0.111	0.018 ± 0.020	0.0225
Aspartate aminotransferase. U/L	26.9 ± 8.94	34.4 ± 21.89	0.0812
Alanine aminotransferase. U/L	37.1 ± 21.63	51.7 ± 46.68	0.2673
Alkaline phosphatase. U/L	188.66 ± 56.29	175.93 ± 44.19	0.4272
Ferritin. ng/mL	281.74 ± 288.57	377.19 ± 188.5	0.0616
Serum creatinine. µmol/L	97.51 ± 21.75	94.42 ± 17.19	0.6005
Urea. mmol/L	5.47 ± 1.35	8.54 ± 16.63	0.3327
Glucose. mmol/L	4.85 ± 1.47	4.67 ± 0.92	0.8738
Total protein. g/L	70.25 ± 6.84	71.15 ± 5.33	0.7120
Albumin. g/L	43.85 ± 4.73	40.95 ± 4.06	0.0710
Sodium mmol/L	136.30 ± 23.16	141.68 ± 5.45	0.6139
Potassium mmol/L	4.04 ± 0.54	3.81 ± 0.67	0.3956
Chloride mmol/L	98.38 ± 21.88	100.57 ± 5.44	0.3956
Prothrombin time. sec	13.52 ± 1.22	13.53 ± 1.53	0.7378
Thrombin time (TT)	31.91 ± 10.97	16.96 ± 12.32	0.0087
Platelet to Lymphocyte Ratio (PLR)	4907.10 ± 24555	9199.45 ± 21422	0.3210
Neutrophils to Lymphocyte Ratio (NLR)	2.17 ± 2.89	5.17 ± 22.09	0.0529
Platelet × neutrophil /lymphocyte: Systemic Immune-inflammation Index (SII)	588.59 ± 709.32	1147.7 ± 4881.3	0.0211

The inflammatory response parameters, Platelet to Lymphocyte Ratio (PLR) and Neutrophils To Lymphocyte Ratio (NLR) were lower in the population with viral persistence of < 21 days, although without statistical significance with respect to patients with viral shedding ≥ 21 days. It could be noted that in the case of NLR, there is a strong tendency to significance (p = 0.0529), where the group of patients with viral persistence for ≥ 21 days, shows a value of NLR (5.17) higher by 2.4 times compared to that of viral persistence of < 21 days (2.17). The group with viral persistence has a Systemic Inflammation Index (SII) higher than the value shown by the group with viral persistence of < 21 days (p = 0.0211) (Table 4).

In univariate analysis we found that be a drinker (odds ratio [OR], 7.32; 95% confidence interval [CI] 0.87- 60.99; p = 0.0307) and be symptomatic (OR: 2.98; 95%CI: 0.016-1.13; p = 0.0149) were associated with increased risk of viral resistance. Then we analyzed 109 patients for all variables in the multivariate logistic regression analysis. We found that be symptomatic (OR: 5.18; 95% CI: 1.64-16.33; p = 0.005) is associated with increase odds of the prolonged viral shedding in COVID-19 patients (Table 5).

Spearman's non-parametric correlation was used to analyze the correlation between the time to negativization of viral RNA and hematological and biochemistry parameters. It was observed that the variables leukocyte count (r = -0.3460, p=0.004), neutrophils (r = -0.2364, 0.0211), SII (-0.2617, p = 0.0117) and TT (r = -0.5158p = 0.0118), correlate significantly and negatively with time to negativization, that is, the higher the values of leukocytes, neutrophils, SII and TT, the shorter it is the time to negativization. A graphic representation of these correlations is depicted in figure 4: A, B, C, and D.

The variables ASAT (r = 0.2520, p = 0.0154) and Ferritin (r = 0.4317, p = 0.0027) correlate significantly and positively

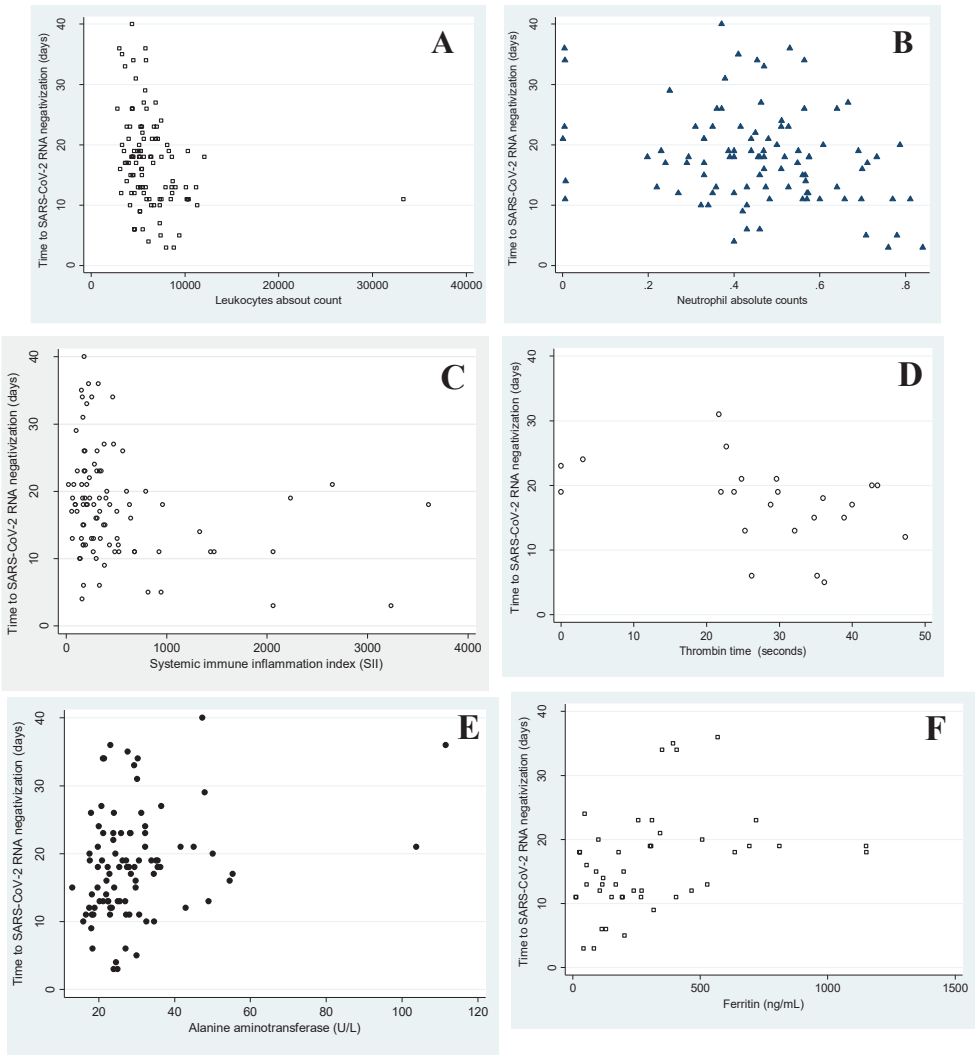

Figure 4 Correlation between viral clearance and levels of lymphocytes, neutrophils, Systemic Inflammation Index (SII), and thrombin time.

with the time to negativization of SARS-CoV-2. At higher values of ASAT and Ferritin, a longer time would be expected for the virus to become negative (See figure 4E and 4F).

A significant indirect (negative) relation was detected between (A) lymphocytes (r= -0.3460, p = 0.004), (B) neutrophils (r = -0.2364, 0.0211), (C) SII (r = -0.2617, p = 0.0117), (D) thrombin time (r = -0.5158p = 0.0118) and the days of negative PCR results. Relation to viral negativization in the case of (E) ASAT (r = 0.2520, p = 0.0154) and (F) Ferritin (r = 0.4317, p = 0.0027) was direct (positive) and significant.

Ferritin and TT have the highest values of Spearman's correlation coefficients: 0.4317 and -0.5158, respectively, so it was assessed whether any of these variables could be a prognostic marker of early negativization, using a ROC analysis, to identify cut-point for Ferritin concentration and TT, which maximize sensitivity and specificity of the test.

The ROC analysis for TT (Figure 5A) shows a large area Under the Curve (AUC). Values greater than 25 seconds of TT shows the cut-off point with the highest Sensitivity (83%) and Specificity (83%) values. Then, it could be affirmed that for a TT approximately value greater than 25 seconds could predict the negativization of the virus in 83% of the cases.

The ROC curve for Ferritin (Figure 5B) offers lower AUC values for serum Ferritin concentration, offering a "regular validity test". A Ferritin concentration approximately ≥ 257 ng/mL supposes a greater probability for viral resistance above 21 days, with a Sensitivity and Specificity of 88.89% and 62.16% respectively. It would be expected that with a Ferritin value ≥ 257 ng/ml, the probability of having viral resistance of 21 days or more is approximately 0.889 ~ 0.9.

ROC analysis of Thombin time (A) and Ferritin (B) for negativization of SARS-CoV-2 at 21 days of viral shedding. Thrombin time and Ferritin thresholds of 25 seconds and 257 ng/mL, respectively, maximized sensitivity and specificity for SARS-CoV-2 virus shedding duration.

DISCUSSION

The SARS-CoV-2 infection characterizes by a viral replication period from exposure period with or without detectable viral RNA followed by non-severe asymptomatic or symptomatic steps and viral presence, ending with an inflammatory period including the start of a coagulation disturbance and a transit to a severe stage [33,34].

Prolonged detection of SARS-CoV-2 has been demonstrated in previous studies, although this had been predominantly described in patients with severe disease [10,35]. Longer respiratory viral shedding was related independently with, fever, concomitant hypertension, steroid use, and lack of antiviral drugs [36-38] has been reported. Additionally, male sex, delayed admission to hospital after illness onset, and invasive mechanical ventilation were associated with prolonged SARS-CoV-2 RNA shedding [39].

Table 5: Risk factors associated with the prolonged viral shedding for at least 21 days.

Age. yr	Univariate OR (95%CI)	*p* value	Multivariate OR (95% CI)	*p* value
19-31	1 (ref)			
32-49	1.45(0.53-3.90)	0.4592	1.12 (0.35-3.56)	0.847
≥50	0.75 (0.24-2.27)	0.6129	0.50 (0.10-2.39)	0.388
Gender			0.63 (0.21-1.84)	0.402
Female	1.0 (ref)			
Male	0.45 (0.18-1.10)	0.0742		
Smoking			3.84 (0.54-26.96)	0.175
Non-smoker	1.0 (ref)			
Smoker	0.73 (0.18-2.86)	0.6552		
Drinking	1.0 (ref)		0.08 (0.006-1.16)	0.065
Non-drinker				
Drinker	7.32 (0.87- 60.99)	0.0307		
Asymptomatic	1.0 (ref)			
Symptomatic	2.98 (0.016-1.13)	0.0149	5.18 (1.64-16.33)	0.005
Comorbidity			0.34 (0.040-2.96)	0.334
Diabetes	0.53 (0.10-2.62)	0.4317	1.03 (0.16-6.45)	0.971
Hypertension	0.86 (0.33-2.23)	0.7699	0.86 (0.23-3.18)	0.829
Time from illness onset to Hospital admission. days			1.49 (0.39-5.68)	0.554
≤ 5	1.0 (ref)			
> 5	1.3 (0.42-3.94)	0.6425		

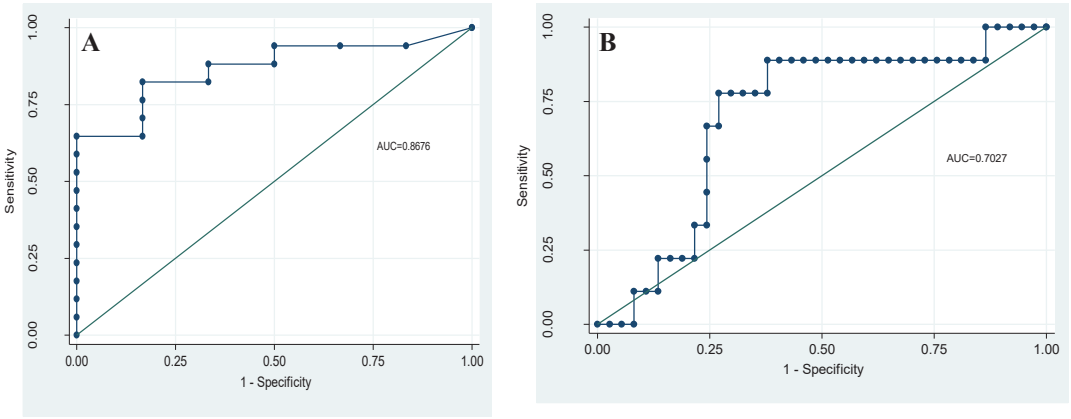

Figure 5 Thrombin time and blood ferritin predict the viral persistence shedding in patients with COVID-19.

Studies of patients with mild and moderate disease found viral persistence for a median of 21 days [11,20]. SARS-CoV-2 nasopharyngeal swaps cultures studies with the use of scanning and transmission electron microscopy, support the presence of viral particle even 70 days after diagnosis [24].

The pattern of SARS-CoV-2 RNA shedding during the course of antiviral treatment has not been well characterized. It was found that after 5 days of therapy 25% of patients had clear the virus from oral swabs [40]. Recently, Idelsi EM, et al. [32] found that with the use of HeberFERON the time to reach the negativization of the SARS-CoV-2 measured by RT-PCR was 3 days in a 50% of a cohort SARS-CoV-2 asymptomatic or mild to moderate symptomatic patients.

In patients treated with Umifenovir, corticosteroids, ribavirin, or mechanical ventilation, recovery is achieved in 92% of patients with less than 14 days of viral persistence and only 46% in those with viral persistence of more than 14 days. There were no deaths among patients with less than 14 days of viral persistence, and in-hospital mortality in the group with persistence of more than 15 was 2.6% [38].

In our study was assessed the effect of HeberFERON treatment in the duration of SARS-CoV-2 shedding in patients with mild and moderate COVID-19, which had persistent viral presence, median 21 days after qRT-PCR diagnosis, upon antiviral therapy with IFN-α 2b. The results did not stablish differences in age between the groups, observing median age of 39 years for Study and Control group I, and 45 years for Control group II. Similarly no differences in sex were observed. However, Kaijin Xu, et al. [38] showed that male sex (67%), advanced age (54.5 years), hypertension, delay in hospitalization, severe illness at the time of hospitalization, and mechanical ventilation; were significantly associated with viral persistence of more than 15 days. This population was characterized by illness duration of 21 days or more with virus shedding or death occurred within 21 days. Neither patient became severe or critical ill or died during the evolution of 21 or more days of viral persistence after treatment with HeberFERON.

The literature describes the median duration of viral presence for pre-symptomatic patients in 11.5 days, for asymptomatic patients in 28 days and for symptomatic patients in 31 days [41]. Fontana, et al. [42] reported the presence of the virus persistence of 18.4 days, with the highest viral load between weeks 1 and 2 from the onset of the disease, regardless of the presence or absence of symptoms, with a decrease after the second week. Patients with severe disease showed a longer viral persistence time (19.8 days, 16.2-23.5 days), compared to those with mild disease (17.2 days, 14.0-20.5 days).

In the article published by Vibholm and his colleagues [43], viral persistence is linked to patients with mild and asymptomatic disease. On the other hand, Seungjae Lee, et al. [11] found no significant difference in viral persistence between asymptomatic and symptomatic patients. In contrast to these results the multivariate analysis of our results identified symptomatic patients at diagnosis associated with viral persistence.

Our study had several limitations. The trial was a case series, of consecutive selected patients with persistent viral shedding. Our sample size is small, and the timing of the clinical presentation of the patients varied, which may influence their transcriptional landscapes. Irrespective of these limitations, the findings from the study showed that HeberFERON plus Kaletra and Chloroquine treatment for COVID-19 patients with prolonged viral shedding after IFN-α 2b is safe and results in viral clearance in more than 50% of patient with only one doses of HeberFERON.

Viral dissemination is determinant in the establishment of severe disease [44]. Therefore, the shortening of time to virus clearance as has been demonstrated by Idelis, et al. for HeberFERON [32] and the results from this study, will impact favorable in the disease outcome in COVID-19 infected patients, even in the era of vaccines.

Depletion of antiviral defenses related to immune response [45] and insufficient activation of the IFN system [46] are key points of innate immune failure to control viral persistence. The results reported herein are in concordance with a restauration or enforcement of adaptive immune response by the combination of IFNs, as a key factor for clearing and maintaining suppression of viral infections [47].

4.1. Observed adverse events are similar to described for the combination of IFNs alpha and gamma [48-50]. High frequency of general malaise was detected in the Heberon Alpha R group. Similarly, the intramuscular injection of Heberon Alpha resulted with frequent pain at the injection site with respect to subcutaneous administration of HeberFERON. Apparently differences for gastro-intestinal adverse events were noted between both IFN formulations, with more nauseas and vomiting in patients that received Heberon Alpha R. These data suggested a more tolerable use for HeberFERON in COVID-19 patients, a fact that can influence in the compliance of the treatment schedule and finally in the efficacy in the viral clearance. The incomplete antiviral therapy schedule may be a factor contributing to prolonged viral shedding in some patients**Risk factors for prolonged viral shedding**

Additionally, it was explored the existence of risk factors associated to prolonged viral presence after antiviral therapy with the combination of IFNs and Kaletra and Chloroquine, and found that lower blood concentration of leukocytes, neutrophils and eosinophils, as well as SII, are related with resistance to antiviral therapy when using Spearman's non-parametric correlation analysis.

Leukocytes, neutrophils, and eosinophils play an important role in controlling viral infections. Lymphopenia and eosinopenia are described for COVID-19 patients, whereas neutrophils are elevated [51], and have been associated with a restricted immune response and disease worsening [38]. In immunocompromised patients, viral persistence with demonstrated infectivity has been reported and may be associated with disease relapses [52,53]. Significantly higher levels of lymphocytes have been detected in survivors vs. non-survivors [35], as well as critical vs. severe [54,55] and severe vs. non-severe [56,57]. Moreover, a persistent stimulation of these cells by the virus leads to their exhaustion, with the corresponding dysfunctionality, with loss of cytokine production and reduced functionality [58].

Neutrophils have direct antiviral activity and are activators of the innate and adaptive response, and therefore contribute to an effective antiviral response [59,60]. Decreased levels of neutrophils at the beginning of the infection may contribute to the persistence of the virus, as observed in our study.

Eosinophils have a powerful inflammatory effect in a group of diseases. In addition, it has been described that they also perform other functions such as immunoregulation and antiviral activity [61]. Eosinophils expressing several TLRs in the endosome [62-64], allows to detect single-stranded RNA like those of coronaviruses, and to initiate an effective antiviral defense characterized by their granulation, the prolongation of their life in blood, concurrent with production of cytokines, the generation of nitric oxide and superoxide [62,63,65].

Eosinophils are also capable of mobilizing preformed Th1 cytokine granules (IL-2, IFN-γ), for mounting an antiviral response [66]. Low levels of eosinophils have been detected in COVID-19 patients, and levels below the normal range have been described as associated with the death of COVID-19 patients [56]. However, Du, et al. [67] related viral persistence to a strong adaptive response with a longer time for the elimination of the SARS-CoV-2 virus. It could be speculated that a strong IFN response can be associated with viral persistence in those patients where the antiviral effect is mediated by eosinophils and these are lowered by IFN-induced apoptosis [68].

SII, the inflammatory index that involve the relation between platelet, neutrophil and lymphocyte that defines the instability of the inflammatory response, has been noted significantly low in positive COVID-19 patients [69].

The ROC analysis suggests that with a ferritin value greater than or equal to 257 ng/mL the probability of having viral resistance of 21 days or more could be expected to be approximately 0.889 - 0.9. High levels of ferritin may identify a more aggressive subset of COVID-19 [70-72].

Serum ferritin is a reservoir for iron conservation, and its measurement serves as an indicator of iron status. Additionally, ferritin is also a marker of inflammation [73]. The production of pro-inflammatory cytokines (TNF-α, IL-6) in patients with COVID-19 can increase an early ferritin production [71,74]. One of immunological phenotypes characterizing COVID-19 severe respiratory failure, include elevated levels of ferritin [75]. It has been reported elevated levels of ferritin in non-surviving COVID-19 patients [35] and when patients begin to recover their ferritin levels decrease [76].

Elevated ferritin levels at the time of hospitalization as predictors of viral persistence can be interpreted as an early potent inflammatory response, inducing ferritin, in patients with apparently ineffective macrophage activation, with delayed virus elimination, and / or mediated by IL-6, with inactivation of the cytotoxic T cell response [43].

Moreover, high concentration of ferritin facilitated increased accumulation of iron molecules that has been described as negative regulator of an effective signaling mediated by IFN-γ / IFNGR2 / STAT1 [77], in concordance

with described insufficient activation of IFN-γ / STAT1-mediated signaling as a consequence of the interference of iron levels in IFNGR2 internalization [77].

It has been described that IFN-γ downregulates the expression of Ferritin receptor in monocytes as well as intracellular concentration of Ferritin [78]. These fats could explain the potential contribution of HeberFERON in the elimination of prolonged viral shedding observed in patients of Study group, negatively regulating the elevated ferritin levels.

Conversely, a TT of more than 25 seconds is related to the negativization of the virus in 83% of cases. Normal thrombin time values are between 14-21 seconds. In our cases, a decreased TT (16.96 ± 12.32 sec) is observed in the group of patients with viral persistence ≥ 21 days, and a better negativization of the virus is predicted for values greater than 25 seconds of TT (apparent TT prolongation) at the start of hospitalization / treatment with IFNs.

Clotting disorders have been reported in patients with COVID-19 [33,54,79]. Among these, shortening or prolongation of PT has been described, as well as elevation of D-Dimer, fibrin degradation products, abnormal platelet counts [80,81]. Coagulation parameters not only reflect homeostasis, but also the malfunction of the inflammation process and organs [82]. A normal PT (9-13 seconds) is associated with better survival than a prolonged PT. However, no difference was detected between survivors and deceased patients for TT [33].

In the last months the SARS-CoV-2 has been diversified. There are four variants of concerns (VOCs), Alpha, Beta, Gamma and Delta and four variants of interests (VOIs), Eta, Iota, Kappa, and Lambda [83]. Vaccinated individuals who become infected with the Delta variant may have the potential to transmit SARS-CoV-2 to others [84]. Recently it has been detected high virus loads in some individuals infected despite vaccination in England [85], Singapore [86], and Finland [87].

How will be the behavior of the viral shedding in these new conditions is a matter of investigational interest that will contribute to a better control of the disease in those patients at risk of prolonged shedding. The role of IFN therapy is necessary to be studied for SARS-CoV-2 variants of concerns to stablish the time of viral negativization and the impact in hospitalization time and the worsening of the disease.

CONCLUSION

Findings presented herein are the first to suggest that after IFN therapy, symptomatic patients have a highest risk for prolonged viral shedding and the thrombin time and blood ferritin concentrations are predictive for the control of viral replication. Therapeutic usefulness of HeberFERON for SARS-CoV-2 negativization in patients with prolonged viral shedding, in asymptomatic and symptomatic patients, was evidenced. The use of HeberFERON might contribute to counteract the risk of worsening of COVID-19 due to prolonged viral shedding and the cost of longer period of hospitalization.

DECLARATIONS

Study execution conformed to the ethical principles of the Declaration of Helsinki and the International Council for Harmonization of Good Clinical Practice guidelines. The authors were responsible for designing the trial and for collecting and analyzing the data. The protocol was approved by the Ethics Committee on Clinical Investigation of the "Luis Diaz Soto" Hospital.

AVAILABILITY OF DATA AND MATERIALS

Qualified individuals may request access to the de-identified participant data, anonymized clinical study reports, informed consent forms, through submission of a proposal with a defined research question to the corresponding author, Bello-Rivero Iraldo, provided that the necessary data protection and ethical committee approvals are in compliance with the trial. An agreement for transfer of these data will be required.

AUTHORS' CONTRIBUTIONS

ASM, MGS, SMM, JPE, were the clinical investigators from the Luis Diaz Soto Hospital that collected and guaranteed the fidelity of the data from medical records of each patient. JPE supervised the clinical laboratory evaluation and certified the data quality. IEM and ABC coordinated, supervised the execution of the study at hospital. MAV and CMS were responsible for data base and its quality. JL was the responsible of the statistical analysis, and contribute to the interpretation of the results. YDR was responsible for the quality of data and supervised the study. ICL designed, and wrote the protocol of the study and wrote the final clinical report of the study. IBR designed and contribute to the design of the protocol, interpreted and discussed all the data and results, and wrote the manuscript

All authors read and approved the final manuscript.

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