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Title: Effect and safety of combination of interferon alpha-2b and gamma or interferon alpha-2b for negativization of SARS-CoV-2 viral RNA**.** Preliminary results of a randomized controlled clinical trial.

Authors: *Idelsis Esquivel-Moynelo I[1], *Pérez-Escribano J[2]. *Duncan-Roberts Y[3], *Vazque-Blonquist D[4], *Bequet-Romero M[4], Baez-Rodríguez L[5], Castro-Ríos J[6], Cobas Cervantes L[7], Pagé-Calvet E[5], Travieso-Pérez S[5], Martinez-Suarez C[5], Campa-Legra I[3], Fernandez-Masso JR[4], Camacho-Rodriguez H[4], Díaz-Gálvez M[1], Sin-Mayor A[1], García-Sánchez M[1], Martínez-Martín SM[1], Alonso-Valdés M, Hernandez-Bernal F[3], Nodarse-Cuni H[3], Bello-Garcia D[8], Beato-Canfuk A[9]. Vizcaino-Cesar MT[1], Guillen-Nieto GE[4], Muzio-Gonzalez VL[3], Bello-Rivero I[3].

* These authors contributed equally to this work

Corresponding Authors

-Iraldo Bello Rivero, MSc, Ph.D. Department of Clinical Investigations, Center for Genetic Engineering and Biotechnology, Ave 31 e/ 158 and 190. Cubanacan, Playa, 10600, Havana, Cuba. Email: iraldo.bello@cigb.edu.cu

-Idelsis Esquivel Moynelo, MSc. Department of Medical Investigations, Military Central Hospital Luis Díaz Soto, Havana, Cuba. Email: idelsisem@infomed.sld.cu.

-Abrahan Beato Canfuk MD, PhD, Direction of Technics-Science, Military Central Hospital Luis Díaz Soto, Havana, Cuba. Email: abeato@infomed.sld.cu.

Affiliations: Department of Medical Investigations[1], Department of Clinical Laboratory[2], Department of Internal Medicine[5], Department of Neurosurgery[6], Department of Endocrinology[7], Department of Medical Investigations, Direction of Technics and Science[9], Military Central Hospital Luis Díaz Soto; Department of Clinical Investigations[3], Direction of Biomedical Research[4], Center for Genetic Engineering and Biotechnology; Faculty Victoria de Giron, Scholl of Medicine[8]; Havana, Cuba.

Abstract

Objectives: IFN-α2b and IFN-γ combination has demonstrated favorable pharmacodynamics for genes underlying antiviral activity which might be involved in the defense of the organism from a SARS-CoV-2 infection. Considering this we conducted a randomized controlled clinical trial for efficacy and safety evaluation of subcutaneous IFN -α2b and IFN-γ administration in patients positive to SARS-CoV-2.

Methods: We enrolled 19-82 years-old inpatients at the Military Central Hospital "Luis Diaz Soto", Havana, Cuba. They were hospitalized after confirmed diagnosis for SARS-CoV-2 RNA by real-time reverse transcription polymerase chain reaction. Patients were randomly assigned in a 1:1 ratio to receive either, subcutaneous treatment with a co-lyophilized combination of 3.0 MIU IFN-α2b and 0.5 MIU IFN-γ (HeberFERON, CIGB, Havana, Cuba), twice a week for two weeks, or thrice a week intramuscular injection of 3.0 MIU IFN-α2b (Heberon® Alpha R, CIGB, Havana, Cuba). Additionally, all patients received lopinavir-ritonavir 200/50 mg every 12 h and chloroquine 250 mg every 12 h (standard of care). The primary endpoints were the time to negativization of viral RNA and the time to progression to severe COVID-19, from the start of treatment. The protocol was approved by the Ethics Committee on Clinical Investigation from the Hospital and the Center for the State Control of Medicines, Equipment and Medical Devices in Cuba. Informed consent was obtained from each participant.

Results: A total of 79 patients with laboratory-confirmed SARS-CoV-2 infection, including symptomatic or asymptomatic conditions, fulfilled the inclusion criteria and underwent randomization. Thirty-three subjects were assigned to the HeberFERON group, and 33 to the Heberon Alpha R group. Sixty-three patients were analyzed for viral negativization, of them 78.6% in the HeberFERON group negativized the virus after 4 days of treatment versus 40.6% of patients in the Heberon Alpha R groups (p=0.004). Time to reach the negativization of the SARS-CoV-2 measured by RT-PCR in real time was of 3.0 and 5.0 days for the HeberFERON and Heberon Alpha R groups, respectively. A significant improvement in the reduction of time for negativization was attributable to HeberFERON (p=0.0027, Log-rank test) with a Hazard Ratio of 3.2 and 95% CI of 1.529 to 6.948, as compared to Heberon Alpha R treated group.

Worsening of respiratory symptoms was detected in two (6.6%) and one (3.3%) patients in HeberFERON and IFN-α2b groups, respectively. None of the subjects transit to severe COVID-19 during the study or the epidemiological follow-up for 21 more days.

RT-PCR on day 14 after the start of the treatment was negative to SARS-CoV-2 in 100% and 91% of patients of the combination of IFNs and IFN-α2b, respectively. Negativization for HeberFERON treated patients was related to a significant increase in lymphocytes counts and an also significant reduction in CRP as early as 7 days after commencing the therapeutic schedule.

All the patients in both cohorts recover by day 14 and were in asymptomatic condition and laboratory parameters return to normal values by day 14 after treatment initiation. Adverse events were identified in 31.5% of patients, 28.5% in the control group, and 34.4% in the HeberFERON group, and the most frequent were headaches (17.4%).

Conclusions: In a cohort of 63 hospitalized patients between 19 to 82 years-old with positive SARS-CoV-2, HeberFERON significantly negativized the virus on day 4 of treatment when comparing with IFN-α2b. Heberon Alpha R also showed efficacy for the treatment of the viral infection. Both treatments were safe and positively impact on the resolution of the symptoms. None of the patients developed severe COVID-19.

Key words: COVID-19, treatment, drug, virus negativization, antiviral, interferon combination, SARS CoV-2.

Introduction

Coronavirus disease (COVID-19) is an infectious disease caused by SARS-CoV-2 that has spread to more than 185 countries of the World and generated more than 644 832 deaths, with great social and economic consequences. Since effective vaccines are not available, it is urgent to find and develop strategies to ameliorate this virus effect[1]. The first cases of the disease in Cuba were confirmed on March 11, 2020: three tourists from the Italian region of Lombardy, who were immediately hospitalized[2].

In Cuba, 60.1% of people diagnosed with COVID-19 are less than 20-year-old. In the country 53.9% of patients are asymptomatic, and even in the most vulnerable population of patients, comprising the 80-year-old group, 51.7% of those infected present with no symptoms at virus confirmation[3].

Due to its antiviral nature, interferons (IFNs) have been used for the treatment of viral infections. Their therapeutic use is justified by the antiviral and immunomodulatory

properties of these molecules[4]. In fact, severity of COVID-19 disease correlates with the failure to implement an IFN response to SARS-CoV-2 infection[5].

Taking these into consideration and the fact that therapeutics that target the coronavirus alone, might not be able to reverse highly pathogenic infections, the Cuban Protocol for Management of COVID-19[2] includes Heberon Alpha R and other antiviral treatment since the symptomatic phase. Cuban patients already showing symptoms or their near contacts are isolated in centers conditioned for that purpose and start receiving symptomatic treatments. As mentioned, this schedule incorporates Heberon Alpha R to lopinavir-ritonavir (Kaletra) and chloroquine (CQ). After confirmation of the positivity of SARS-CoV-2, they are hospitalized and continue to, or start to receive Heberon Alpha R, Kaletra, and CQ as established by Cuban Health Ministry guide-lines[2].

This has resulted in a favorable evolution of the patients in a cohort of 761 subjects confirmed for SARS-CoV-2 receiving Heberon Alpha R, where 95.4% fully recovered from COVID-19, with only 0.92% of case fatality rate[6].

Earlier studies of a combination of type I IFN and IFN-γ shown a synergistic inhibition of the SARSCoV virus replication in vitro[7,8,9,10]. IFN-γ is a key moderator in linking the innate immunity to adaptive immune responses[11], hence it is possible that a combinational therapy of IFNs and other antiviral drugs could significantly inhibit virus replication and modulates clinical variables related to the immune response with a positive outcome in terms of viral infection resolution[12,13,14].

In accordance to the previous comments we conducted this phase 2 randomized trial to establish whether a combination of IFN-α2b and gamma with the standard of care, can improve the viral load profile and clinical parameters in adults with COVID-19.

Methods

Study design

Hospitalized adult patients with RT-PCR confirmed SARS-CoV-2 were enrolled in this open-labeled, single center, prospective, randomized and controlled clinical trial at Military Central Hospital "Luis Diaz Soto" Hospital, Havana, Cuba.

Patients were randomly assigned to receive the combination of IFN-α2b and IFN-γ (HeberFERON, CIGB, Havana, Cuba) or IFN-α2b (Heberon Alpha R, CIGB, Havana, Cuba) based on a power of 80%, and a level of confidence set at 95%, while also considering a dropout rate of 5%. Patients were blocked randomized individually to one of two treatment arms by means of random computer-generated lists, with an allocation ratio of 1:1, with block sizes of six patients.

Heberon Alpha R (IFN-α2b) is a drug produced in Cuba by the Center for Genetic Engineering and Biotechnology (CIGB), which has remained a product with proven antiviral efficacy and an adequate safety profile for 34 years[15]. HeberFERON (IFN-α2b and IFN-γ, co-lyophilized in the same vial) is produced at CIGB, and registered in Cuba for the treatment of basal cell carcinoma.[16]

The study execution followed the ethical principles of the Declaration of Helsinki and the International Council for Harmonization–Good Clinical Practice guidelines. No compensation was provided for enrollment in the trial. Patient personal data were protected. The authors were responsible for designing the trial and for collecting and analyzing the data. The authors assured the completeness and accuracy of the data collection and the adherence to the protocol. The details about the trial are provided in the protocol that has been posted in TRIALS[17] and is in processing by the editors of the journal.

The primary endpoints were the time to viral RNA negativization from the start of treatment and the time to progression to severe COVID-19.

Eligibility criteria

The COVID-19 diagnosis was obtained by a positive real-time reverse transcription-polymerase chain reaction (RT-PCR) amplification of E gene and then confirmed by amplification of RdRP gene in throat swabs. Adult (≥19 years-old) patients with RT-PCR confirmed SARS-CoV-2, ECOG functional status ≥ 2 (Karnofsky ≥ 70%), and voluntariness by signing the informed consent were included. Patients with each of the following characteristics were excluded: decompensated chronic diseases at the time of inclusion

(severe arterial hypertension, ischemic heart disease, diabetes mellitus, etc.), with a history of autoimmune diseases, presence of hyper inflammation syndrome, serious coagulation disorders, known hypersensitivity to any of the components of the formulation under evaluation, pregnancy or lactation, and obvious mental incapacity to issue consent and act accordingly with the study.

The clinical trial protocol was approved by the Ethics Committee on Clinical Investigation of Military Central Hospital "Luis Diaz Soto", and the Center for the State Control of Medicines, Equipment and Medical Devices (CECMED) in Cuba. Patients were asked for written consent to participate after having been duly informed about the characteristics of the trial, objectives, benefits and possible risks. Likewise, they were informed of their rights to participate or not and to withdraw their consent at any time, without exposing themselves to limitations for their medical care or other retaliation. The study was registered on April 2020 at: registroclinico.sld.cu/en/trials/RPCEC00000307.

After a preliminary exploratory analysis of the outcomes of the first 79 patients, the monitoring board considered a preliminary report and early publishing of the RT-PCR results from the available throat swabs in 63 patients with available throat swabs, due to the significant effect of HeberFERON on the reduction of the time to viral clearance. The trial finally included 134 patients that are now in the process of data collection for definitive processes and analysis.

Treatment protocols

Patients received 3.0 million international units (MIU) IFN-α2b and 0.5 MIU IFN-γ (HeberFERON), twice a week for two weeks, subcutaneously and lopinavir-ritonavir 200/50 mg every 12 h and CQ 250 mg every 12 h (treatment group); or standard of care (3.0 MIU IFN-α2b (Heberon Alpha R), thrice a week, intramuscularly and lopinavir-ritonavir 200/50 mg every 12 h and CQ 250 mg every 12 h (control group).

Data collection: Demographic, clinical, laboratory, treatments and outcome characteristics of patients were extracted from medical records and registered in to CRF and then were entered in duplicate (independently by two operators) for the subsequent process of automatic comparison and correction of the databases, necessary for statistical analysis with accurate information from the trial. However the blinding was not feasible, it was maintained for laboratory SARS-CoV-2 RNA detection by RT-PCR that is one of the endpoint of the study.

Laboratory procedures: The hospital received patients from several zones in Havana city diagnosed in reference centers for SARS-CoV-2 infection following the Cuban Ministry of

Health guidelines for diagnostic testing. Patients were defined to have SARS-CoV-2 if they had two consecutive positive results, including the confirmatory test by RT-PCR targeting amplifications of E and /or RdRP genes. A cycle threshold up to value 40 was defined as positive.

Specimens were obtained from throat swabs of patients at the hospital following standard procedures and transported to a BSL2 certified laboratory at the CIGB for serial evaluation of SARS-CoV-2 viral nucleic acid detection by RT-PCR targeting after extraction by QIAamp® Viral RNA Mini kit (Qiagen, USA). A multiplexed detection by RT-PCR was carried out targeting E and/or RdPR genes plus EAV internal extraction control (TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany) as described before using Multiplex RNA Virus Master (Roche, USA).

Routine blood examinations were done at the Clinical Laboratory of Military Central Hospital "Luis Diaz Soto" and included whole blood count, coagulation profile, serum biochemical tests (including renal and liver function, electrolytes, and coagulation), C-reactive protein (induced by various inflammatory mediators such as IL-6[18]), and ferritin. Furthermore, all patients received chest X-ray. The frequency of examinations were defined in the trial protocol and consisted in weekly determinations at base line on days 2 and 4 of each week.

Outcomes assessment: The primary outcomes included virological and clinical evaluations. Time to SARS-CoV-2 RNA negativization (absence of the virus according to the RT-PCR) in positive patients after starting antiviral therapy was the virological endpoint. It was expressed as the percentage of patients negative to SARS-CoV-2 by RT-PCR in throat exudate tissue calculated at 48, 72, 96 and 120 hours. Days to reach the viral negativization were analyzed by Kaplan-Meier plots and compared with a Log-rank test. Hazard Ratio with 95% CI of ratio was also calculated.

The clinical evaluation considered the time to progression to severe COVID-19 and it was calculated by the percentage of patients who became severely ill after the end of the antiviral treatment under investigation.

Statistical analysis: Quantitative variables were described with the arithmetic mean and its standard deviation and the median with its range. We used the absolute and relative frequency (%) for qualitative variables. The hypothesis test used was Fisher's exact test. The viral negativization analysis was performed using the Kaplan-Meier plot representation and the comparison of factors was done with the Mantel-Cox Log Rank tests. The evolution of

laboratory parameters while under treatment was analyzed using a paired mixed model (which cannot handle missing values appearing due to patient release from hospital). Correlations between virus negativization and laboratory parameters were studied using a two-tailed non-parametric Spearman correlation with 95% confidence interval. $P<0.05$ was considered statistically significant. Statistical analysis was performed using the Windows software package SPSS (version 25) and GraphPad Prism v8.0.

Results

Patients and baseline features

We have screened 144 patients positive by RT-PCR to SARS-CoV-2. Fifty-seven patients did not fulfill the inclusion criteria, of them one with icterus, one with chronic decompensate renal insufficiency, two non-confirmed positive PCR for SARS-CoV-2, and fifty-three patients with 2 positive RT-PCR after more than 21 days of persistent virus shedding, were excluded. Patients with viral persistence were later treated with the HeberFERON out of the clinical trial (manuscript in preparation). Eight patients that did not consent were also excluded.

Finally, seventy-night subjects met the inclusion criteria and were randomly assigned (1:1) to either the HeberFERON group (41 patients) or the control group (38 patients). Twelve patients did not start the treatment, 7 patients refused to start the treatment, although they have been signed the consent, and 5 were excluded due to loss of inclusion criteria.

Seven patients withdrew by several causes: 3 due to worsening of respiratory symptoms (two of them in the HeberFERON group, with asthma as underlying diseases) that changed to other non-permitted in the study drug treatment; 3 patients from the control group with positive RT-PCR on day 14 were switched to receive HeberFERON out of the clinical trial by medical decision; and 1 with the appearance of an exclusion criterion (pregnancy) in the control group (see figure 1, flow chart of the study).

Four patients were not analyzed, three in the HeberFERON group, due to bad inclusions (were negative to viral RNA before the beginning of treatment as identified by the board of monitors), and one in the control group because refused the swabs sampling. Thirty and thirty-three patients were analyzed by intention to treat (ITT) in the HeberFERON and control group, respectively.

Finally, swabs samples to test for viral negativization were obtained from sixty-three patients. In this cohort 29 were symptomatic (46.0%), with a median from the beginning of symptoms

to the start of treatments with IFNs of 7.0 days (IQR: 2-13) in the control group and 7.5 days (IQR: 2-19) in the HeberFERON group.

Thirty-three patients were treated with standard of care which includes Heberon Alpha R (control group) and 30 with HeberFERON plus standard of care, excluding Heberon Alpha R. In the control group younger people prevailed with a median of 31.0 years-old (IQR: 19-57), while with 42.0 years-old (IQR: 19-82) for HeberFERON group (p=0.023). The sex distribution showed a prevalence of males in the control cohort (20/33, 60.6%) as compared with a similar distribution in the combination group for males (14/30, 46.7%) and females (see table 1). In the control group more symptomatic patients (51.5%) than in the HeberFERON group (40.0%) were present; however this difference was not statistical significant.

The median age of symptomatic subjects was higher [43 (IQR: 19-80)] than for asymptomatic [(34 (IQR: 19-82)], and for the HeberFERON group 50 (IQR: 19-80) versus 24 (IQR: 19-57). Symptomatic patients with more than 7 days from the symptoms onset were more common in the HeberFERON arm (50.0%), however, these numerically differences were not statistically significant (see table 1). In the HeberFERON group 66.6% of symptomatic were females and in the control symptomatic males were more frequent 70.6% (p=0.024).

The more common symptoms were fever and unproductive cough (16.4%), followed by headache (9.6%), decay (8.4%), odynophagia and nasal secretions (5.4%), diarrhea, dyspnea, chills and general malaise (4.1%), and others as sore throat and myalgia (2.7%).

Fifty percent of patients had any comorbidity; the most frequent were hypertension (22%), asthma (6.3%), diabetes and glaucoma (4.7%).
The vital signs at the time of hospital admission were not statistically different between groups.

Some imbalances existed at enrollment between the groups, including a higher median age in the HeberFERON than in the control group, as well as more patients with higher than 7 days from onset of the symptoms in the HeberFERON group.

No other major differences in symptoms, vital signs, laboratory results, disease severity, or treatments were observed between groups at baseline.

Outcomes

We analyzed 63 patients with available throat swabs samples. In the HeberFERON group 78.6% of patients were negative to the virus after 4 days of treatment versus 40.6% of patients in the control group (p=0.004). HeberFERON negativized SARS-CoV-2 in the 95.8% of patients at day 5, (p=0.0479), see tables 2 and 3.

Medians times to reach SARS-CoV-2 negativization by RT-PCR were 3.0 and 5.0 days for the HeberFERON group and control group, respectively. A Kaplan-Meier plot of percent of SARS-CoV-2 positive patients along the first five days after treatments start showed statistical differences between groups through a Mantel Cox log-rank (p=0.0022) test (table 2 and figure 2). A Hazard Ratio of 3.2 and 95% CI of ratio of 1. 529 to 6.948 were calculated for HeberFERON group as compared to control group.

When the viral RNA negativization was evaluated at 96 h after the treatment initiation, stratifying by the presence or not of symptoms (see table 3), it was observed a 91.7% and 37.5% of symptomatic patients negative for SARS-CoV-2 RNA in the group of HeberFERON and control groups, respectively, with statistical significance (p=0.006).
In asymptomatic patients a lower rate of negativization was observed for both IFNs. However, the HeberFERON showed a 70.6% of negativization in comparison to 46.7% for control group.
The worsening of respiratory symptoms was detected in two (6.6%) and one (3.3%) patients in HeberFERON and control groups, respectively. None of the patients transit to severe COVID-19. The RT-PCR after treatment with IFNs on day 14 for hospital discharges was negative to SARS-CoV-2 in 100% and 91% of patients of HeberFERON and control cohorts, respectively.

Nevertheless, the kinetics for this recovery differ between treatments groups. Earlier increase in lymphocytes percentage was observed only for HeberFERON treated patients (p=0.0141) with a marked trend for increment in lymphocytes concentrations. Also a significant decrease in CRP (p= 0.0444) was notice for this group parallel to a trend in the reduction in CPK (Figure 3).

The correlation between laboratory data evolution and SARS-CoV-2 virus clearance data was analyzed using a two-tailed non-parametric Spearman correlation with 95% confidence interval. Table 2 summarized the parameters identified with significant direct or indirect relation with the reduction in the time needed to achieve a negative PCR result. A particular assessment of the same parameters is also included for the symptomatic patients included for

each experimental group (table 2). A statistically significant correlation between the viral negativization by HeberFERON on the first week of treatment for lymphocyte Concentration, C - reactive protein and the platelet to leukocyte ratio (PLR) was found. This kind of correlation was observed in the control group only for CPK. Further stratification of the data on patients showing or not symptoms of the disease indicates that such correlations were maintained for lymphocyte concentration, and PLR.

Adverse events were identified in 18 (31.5%) from 57 patients recorded, 8 (28.5%) in the control group and 10 (34.4%) in the HeberFERON group. The most common adverse events were headache (17.4%), nausea, hypertension, retroorbital pain and burning eyes (3.5%). Nighty-four percent of adverse events were mild and none severe. There were no differences between the incidence of any of the adverse events or duration between the treatment groups. No serious adverse events were reported. No patients died during the study (table 2).

Discussion

Asymptomatic incubation period with or without detectable viral RNA, followed by non-severe symptomatic step and viral presence, ending in a severe symptomatic stage with high viral load, characterizes the SARS-CoV-2 infection[19], that has been widely spread.

The global damage of COVID-19 could be partly explained by the median incubation time, from four to seven days to symptoms, a large window of time for transmission[20, 21]. Also, many infected patients remain completely asymptomatic and yet are fully capable of transmitting the virus[22,23,24].

Insufficient activation of the IFN system is referred to as the principal cause of innate immune failure to control viral persistence. Adaptive immune response is a fundamental factor for clearing and maintaining suppression of viral infections[25].

The present study is the only randomized open-label controlled trial reported so far assessing the efficacy and safety of the combination of IFNs alpha-2b and gamma versus IFN-α2b in patients with COVID-19 [26 , 27] .

Very early as 48h after the first administration of HeberFERON, an important negativization of SARS-CoV-2 was obtained (45%), a result that is consistent with a potent and rapid antiviral effect. This fast response and the nighty-six percent of viral negativization on day 5 of treatment with HeberFERON, has not been obtained for any other drug studied so far, even in a combinational design as described for the combined use of lopinavir 400 mg and ritonavir 100 mg every 12 h, ribavirin 400 mg every 12 h, and three doses of 8 MIU IFN

beta-1b[14]. Even 74% of negativization showed by Herberon Alpha-2b is superior to the reported by other authors[28].

Time to reach the negativization of the SARS-CoV-2 measured by RT-PCR in real-time was 3.0 and 5.0 days from the start of treatment with HeberFERON and Heberon alpha R, respectively, a difference statically significant. These results are in concordance with in vitro data about the greater sensitivity of SARS-CoV-2 to IFNs with respect to SARS-CoV[29, 30] and as compared to those treated with Heberon Alpha R.

Viral dissemination is determinant in the establishment of severe disease[31]. Therefore, the shortening of time to virus clearance as has been demonstrated for HeberFERON will impact very favorable in the disease outcome in COVID-19 infected patients.

The timing of initiation of antiviral therapy is another key factor in the treatment of viral infections. In the combat of SARS-CoV, no effect of several antiviral drugs was observed when the treatments were started 6–14 days after symptom onset[32]. It was suggested that administration of antiviral medications at the beginning of the infection might improve outcome of patients with COVID-19[33]. Early treatment with IFNs was recommended in the treatment of MERS[34]. Late therapy (10–22 days)[32] may contribute to poor outcomes[35].

The Cuban Protocol for Management of COVID-19[2] allowed us to include patients during a window of 7-8 days of symptoms onset which is a good moment to start to reinforce the host innate and adaptive immune response with the use of IFNs, mainly with the combination of IFN-α2b and gamma, where the last is a key player in linking the innate and adaptive immune response[36].

A recent open-label trial has demonstrated no benefit in hospitalized adult patients with severe COVID-19 treated with Kaletra. However, per-protocol analyses suggested possible reductions in time to clinical improvement particularly in those treated within 12 days of symptom onset[28].

The combination of Kaletra with other antiviral agents, as has been done in SARS[37], MERS-CoV,[38] and our trial, might enhance antiviral effects and improve clinical outcomes. The confirmation of this therapeutic approach remains to be determined. However, it has been recently shown the combination of IFNs with Kaletra is associated with more favorable clinical outcomes than the use of Kaletra alone in COVID-19 patients[35].

The presence of IFN-γ in the HeberFERON formulation additionally to its strong immune regulatory functions may restrict the angiotensin-converting enzyme 2 (ACE2) expression[39], a receptor for cell entry for SARS-CoV-2[40]. It has been reported that this cytokine can directly inhibit viral entry for several viral infections (HCV and HIV) [41] by controlling the

expression and/or distribution of their receptors. The effect of the combination of both IFNs on the ACE expression is under evaluation.

The results of viral negativization showed a more potent antiviral effect of HeberFRON over Heberon Alpha R, mainly in the symptomatic subjects. Our study includes a low amount of symptomatic patients (46.0%). In the control group more symptomatic patients (51.5%) than in the HeberFERON group (40.0%) were present. Asymptomatic cohorts constitutes the least manageable group of patients because they can spread the virus efficiently, as silent spreaders of SARS-CoV-2 which cause difficulties in epidemic control[22,42]. Symptomatic and asymptomatic patients had similar viral loads[43]. The asymptomatic patients had a significantly longer duration of viral shedding as well as abnormal radiological findings in more than 90% of patients[44].

Although countries did not pay adequate attention to asymptomatic people, this non-small population of infected patients negatively impacts the global outcomes of the disease; therefore the treatment and follow-up of these patients are important to control de pandemic. The use of IFNs may be a determinant factor in the control of the disease in symptomatic and asymptomatic patients with COVID-19, as reflect the results of this trial, where HeberFERON is the most effective in the elimination of viral replication in symptomatic and asymptomatic patients.

In MERS-CoV infected mice delayed IFN treatment was associated with increased infiltration and activation of monocytes, macrophages, and neutrophils in the lungs; and enhanced pro-inflammatory cytokine expression[34]. Additionally, soon, after infection in human, application of antiviral therapy with rapid viral clearance can delay pro-inflammatory cell development, activation and their infiltration that will contribute to spar human life[14].

Delayed IFN response can also cause inflammation and tissue damage. The host may benefit from IFN presence early in the disease course, particularly when IFN system is antagonized by viral proteins or is of low competence in older aged patients[24].

An important difference between treatments concerns their effects on lymphocytes percentages among leukocytes. Only for HeberFERON treated patients a significant increase of lymphocytes percentages was observed by week 1 and this fact, as well as lymphocytes concentrations, correlates significantly with the reduction in time to virus clearance (fig 3 and table 2).

It has been shown that lymphopenia predicts disease severity of COVID-19. In this context the restoration of lymphocyte population under the effect of a short and low dose of HeberFERON is very valuable. Further studies on the phenotype and functionality of peripheral blood mononuclear cell of patients are warrant since they will offer more clues on this regard.

Although, IFNs have a potential for the induction of an inflammatory response, its early use contributes to regulate the entering in the inflammatory scenario of COVID-19[4,Error! Bookmark not defined.]. Shreds of evidence have been given about the effect of IFN-α2b treatment in the reduction of inflammatory mediators IL-6 and CRP in COVID-19 cases[45].

Concentration level of CRP is not affected by factors such as age, sex, and physical condition, and correlate with the level of inflammation[46] and is an important index for the diagnosis and assessment of severe pulmonary infectious diseases[47,48]. In the early stage of COVID-19, CRP levels could reflect lung lesions and disease severity[49]. CRP levels are correlated with the level of inflammation.

Herein we detected a significant reduction in CRP in patients after two administrations of HeberFERON (see figure 3). CRP is correlated with the level of inflammation[46], and is an important index for the diagnosis and assessment of severe pulmonary infectious diseases[50,51]. In the early stage of COVID-19, CRP levels could reflect lung lesions and disease severity. The downregulation of CPR levels by IFNs in patients with COVID-19 early in the diseases could avoid acute inflammatory pathogenesis and disease severity[52].

Then the administration of the HeberFERON in patients primed with the antivirals may result in a further boosted antiviral effect that contribute to shortening the time for viral clearance and to lower the probabilities to develop a more severe conditions of the diseases, that implies at the end, a lower lethality rate.

Although with significant more aged patients in the HeberFERON and cohort with 40% of symptomatic patients with median age of 50 years-old, none of these patients became severe ill during the trial and all of them were discharged. However, two patients from HeberFERON group worsened the respiratory symptoms. These were asymptomatic men at admission, both 33 years-old, with asthma as comorbidity that received 3 and 4 doses of HeberFERON. They were negative to SARS-CoV-2 RT-PCR since 48h and 96 h since the first HeberFERON administration. During the days of symptoms worsening climate conditions were favorable to exacerbate asthma symptomatology. They recovered in 48 hours after anti-inflammatory therapy. In the control group a symptomatic man of 80 years-old,

with hypertension as comorbidity, that received only one dose of Heberon Alpha R also worsened the respiratory symptoms and was transfer to ICU. His RT-PCR for viral RNA was negative 20 days after symptoms worsening and discharged.

Altogether these results indicate that with high probability the rapid viral elimination detected for HeberFERON treated patients is translated into the reduction of systemic inflammation markers while inducing a significant increase in circulating lymphocytes concentrations that may explain the symptomatic improvement observed in the more risky patients in the HeberFERON group. These results adds to the anti-inflammatory effect described for IFNs in COVID-19 patients[45], and are in correspondence with the finding of gene signature involved in Type I and Type II response in mild-to-moderate COVID-19 patients[53].

About 15% of the confirmed COVID-19 cases progress to the severe phase, with a higher risk for patients over 65 years-old[54]. Using this estimate, in the 63 patients included in our study approximately 9 patients were expected to develop severe disease; however no patients became severely ill. No death was recorded in these mild or moderate patients. In similar cohort of patients, 0.9% of mortality was described with the early use of IFNs[35]. In our trial several clinical parameters known to be related to COVID-19 progression were significantly improved by the treatments or showed a trend to a favorable behavior.

These results confirm the validity of early intervention with the treatment of IFNs in patients with COVID-19, whereas demonstrated in the trial, the combination of type I and type II IFNs impacts strongly in the reduction of the risk for a severe disease likely through the efficient implementation of a timely controlled inflammatory antiviral response against the SARS-CoV-2 infection.

Before being approved recently by the FDA and EMA for severe COVID-19 patients, remdesivir, was not successful in two randomized clinical trials[55, 56]. Its approval was sustained on the reduction of the illness duration in a few days[57]. Still the role of this antiviral in the inflammatory processes that drives the transit to severe and critical condition in COVID-19 patients has not been described.

HeberFERON formulation that combines in one vial IFN-α2b and gamma results in an advantageous option for the treatment of COVID-19 patients. First, due to the demonstrated better pharmacodynamics[16] it is possible to administer less frequent and at lower doses than the other conventional IFNs, (IFN-α2b or IFN-β or IFN-λ) that need a thrice a week administration to have similar effect. IFN-β has been used at doses higher than 2 fold (of 12 MIU/mL[58] or 8 MIU/mL[14]) with respect to HeberFERON doses. Second, the simultaneous

administration of both types of IFNs will promote a faster and stronger innate and adaptive immune response. At least these two facts could be responsible for the quick clearance of SARS-CoV-2 detected in our trial.

It has been proposed that interferon is efficient only in patients who lacked comorbidities[59,60]; however we have obtained a high rate of negativitazation, resolution of symptoms, and hospital discharges for HeberFERON in a cohort of patients with 57% of coexisting comorbidities.

Moreover, it has been suggested that comorbidities like diabetes affect the response to IFN[60]. Two diabetic patients in our cohort negativized the virus on day 3 from the beginning of the treatment and the other at least before day 14.

Our study had several limitations. This trial was open label, without a placebo group with unbalanced demographics (age years) between treatment arms. In addition, sampling methods were most likely suboptimal using the throat sampling, because of inability to do sampling of lower respiratory tract secretions. Previous studies have shown that throat-swab specimens have lower viral loads[43].

Irrespective of these limitations the HeberFERON showed efficacy and was safe in shortening virus shedding, eliminating symptoms, and discharge of patients with COVID-19.

Based in our analyses of the clinical data and their correlation with the patient outcome we hypothesized that a potent antiviral response based in coordinated innate and adaptive immune responses mediated by the combination of type I and type II IFNs and an anti-inflammatory activity in the early steps of the diseases, are the main reason for the control of COVID-19 by HeberFERON treatment.

The use of HeberFERON in COVID-19 patients could be an effective therapeutic option to halt a second step of the disease characterized by a respiratory worsening approximately between 9-12 days after onset of symptoms[61], apparently related to an imbalance of inflammatory mediators[53].

Findings presented herein are the first to suggest therapeutic efficacy in COVID-19 disease of the combination of IFN-α2b and gamma with individual and public health impact with a shorter duration of virus shedding and preventing the worsening of the disease. Moreover, this is the expression of an accurate understanding of the biology of IFNs and their combination[16] that we have translated into safe and effective antiviral therapy.

Conclusions

HeberFERON was a safe treatment, superior to Heberon Alpha R in shortening the time to SARS-CoV-2 viral RNA negativization in a cohort of symptomatic or asymptomatic patients between 19 and 82 years-old, with more than 95% of patients negative to the SARS-CoV-2 in 5 days of treatment.

The rapid viral negativization contributes to implement an anti-inflammatory response that can protect the patients to enter in a more severe step of the disease.

Early isolation combined with early administration of antiviral treatments as IFNs is an efficient approach that could contribute to save the life of patients in the COVID-19 pandemic. The use of HeberFERON might be a distinctive element in the preventive and therapeutic strategy for current or future SARS outbreaks.

Tables

Table 1. Demographic and Clinical Characteristics of the Participants at Baseline.

Characteristic	HeberFERON (n=30)	Control group (n=33)	Total (n=63)
Median age (IQR) —yr ¶	42.0 (19-82)	31 (19-57)	38 (19-82)
Sex			
Male — no. (%)	14 (46.7)	20 (60.6)	34 (54.0)
Female — no. (%)	16 (53.3)	13 (39.4)	29 (46.0)
Current smoker— no. (%)	5 (15.1)	3 (9.0)	8 (12.7)
Symptomatic— no. (%)	12 (40.0)	17 (51.5)	29 (46.0)
Median age (IQR) —yr	50 (19-80)	23 (19-57)	43 (19-80)
Median time (IQR) from symptom onset to randomization — days	7.5 (2-19)	7 (2-13)	7 (2-19)
Male — no. /total no. (%) **$**	4/12 (33.3)	11/17 (70.6)	15/29 (51.7)
Female — no. /total no. (%)	8/12 (66.6)	5/17 (29.4)	13/29 (44.8)
Time from symptoms onset to start of treatment, days— no./total no. (%)			
>7 days	6/12 (50.0)	6/16 (37.5)	12/28 (42.9)
≤7 days	6/12 (50.0)	10/16 (62.5)	16/28 (57.1)
Asymptomatic— no. (%)	18 (60.0)	16 (48.5)	34 (54.0)
Median age (IQR) —yr	36.5 (23-82)	34 (19-50)	34 (19-82)
Male — no. /total no. (%) **$**	10/18 (55.5)	9/16 (56.2)	19/34 (55.8)
Female — no. /total no. (%)	8/18 (44.4)	8/16 (50%)	16/34 (47.0)
Coexisting conditions— no. (%)			
Any comorbidities	19 (57.5)	13 (39.3)	32 (50.8)
Cardiac diseases	2 (6.0)	2 (6.0)	4 (6.3)
Diabetes	3 (9.0)	0 (0.0)	3 (4.7)
Hypertension	7 (21.2)	7 (21.2)	14 (22.2)
Asthma	2 (6.0)	2 (6.0)	4 (6.3)
Vital signs			
Median vital signs (IQR)			
Temperature (°C)	36.0 (36-37.1)	36.2 (36-37.9)	
Respiratory rate (breaths/minute)	19 (15-22)	19 (16-24)	
Systolic blood pressure (mmHg)	120 (100-170)	120 (90-140)	
Baseline Treatment— no. (%)			
IFNα-2b	30 (100%)	33 (100%)	63 (100%)
Lopinavir-ritonavir at baseline	30 (100%)	33 (100%)	63 (100%)
Chloroquine at baseline	30 (100%)	33 (100%)	63 (100%)
Antibiotic treatment at baseline	1 (3.0)	4 (12.1)	5 (7.9)
Laboratory baseline data	**n=27**	**n=30**	**n=57**
Median laboratory values (IQR)			
White blood cell count (**WBC**), $\times 10^9$ per L	6.4 (3.8-11.7)	6 (2.4-12)	6 (2.4-11.7)
Lymphocyte count, $\times 10^9$ per L	2.45 (0.5-3.93)	2.3 (0.91-3.7)	2.45 (0.5-3.93)
Lymphocyte Percentage (%)	38 (11-57)	40 (8-72)	39 (8-72)
Neutrophils count, 10^9 per L	3.2 (1.42-8.66)	2.8 (0.62-9.9)	2.92(0.62-9.86)
Neutrophils Percentage (%)	50 (29-81)	48 (18-85)	49 (18-85)
Platelet count, $\times 10^9$ per L	224 (117-351)	235 (126-376)	226 (117-376)
Neutrophils to Lymphocyte Ratio (**NLR**)	1.34 (0.51-6.82)	1.2 (0.25-11)	1.25 (0.25-10.6)
Platelet to Lymphocyte Ratio (**PLR**)	107.3 (34-327.7)	103 (62-320)	107.3(34-327.7)

Systemic Immune inflammation Index (**SII**)	306 (115-2075)	275 (73-2879)	289 (72.5-2869)
Serum creatinine, μmol/L	99.6 (31.6-155)	103 (77-143)	102.4 (31.6-155)
Aspartate aminotransferase, U/L	28.1 (18.9-83)	26 (14-97)	25.7 (13.6-96.70)
Alanine aminotransferase, U/L	34.05 (17.3-133.2)	35 (14-78)	34.7 (13.8-133.2)
Creatine kinase, U/L	113 (31.09-2377)	110 (81-143)	109 (31.09-2377)
C-Reactive Protein mg/dL	4.135 (0.38-129)	2 (0.29-37)	3.58 (0.29-129)
Ferritin ng/mL	206 (40.7-873)	190 (12-600)	197 (11.6-10.2)
TP seg	13.95 (11-16.6)	13 (12-16)	13.5 (11-16.6)
Ionogran			
Potassium mmol/L	103 (95.5-113)	100 (96-109)	103 (92-112)
Sodium mmol/L	143 (136-151)	142 (136-147)	142 (134-151)
Chloride mmol/L	4.1 (2.66-5.4)	4.1 (2.9-5.1)	4.04 (2.66-5.4)

¶ p=0.023 by U de Mann-Whitney; $ p= 0.024 for the comparison between sex S and A by the Chi-square.

Table 2. Outcomes in patients positive to SARS-CoV-2 treated with interferons

Outcomes			HeberFERON (n=30)	Control group (n=33)	p
Time to reach 50% the negativization of the SARS-CoV-2 (Log-rank test)	All patients		3 days	5 days	p= 0.002
	Symptomatic		2.5 days	Not reached	p= 0.003
	Asymptomatic		3 days	5 days	p= 0.123
Percent of patients negative to viral RNA after the start of treatment (Fisher test)		48 h	44.8	15.4	p= 0.022
		72 h	63.3	34.5	p= 0.037
		96 h	78.6	40.6	p= 0.004
		120 h	95.8	73.9	p= 0.047
Time to severe COVID-19			Non-reached	Non-reached	
Percent of patients with clinical symptom worsening			6.66%	3.33%	
Percent of hospital discharges at day 14			100%	91%	
Frequency of adverse events			34.4%	28.5%	
Correlation studies of laboratory parameters and time to negative viral RNA PCR results					
Parameters			**7 days on HeberFERON**		**7 days on control group**
			r	p	r	p
Lymphocyte Concentration All patients			-0.5220	**0.0336**	-0.0039	0.9849
Symptomatic			-0.7373	**0.0197**	0.2154	0.4768
C-Reactive Protein (CRP) All patients			0.5016	**0.0439**	-0.2393	0.5822
Symptomatic			0.5339	0.1160	-0.5372	0.0610
Creatinine Phosphokinase Kinase (CPK)_All patients			-0.020	0.9392	0.4097	**0.0304**
Symptomatic			-0.1653	0.6470	-0.3840	0.1749
Platelet to Lymphocyte Ratio (PLR)_ All patients			0.4978	**0.0439**	0.02942	0.8866
Symptomatic			0.7373	**0.0197**	-0.2882	0.3371

Table 3. Detection of SARS-CoV-2 in swabs by RT-PCR in the HeberFERON or control groups. Throat swabs were taken from 63 COVID-19 positive patients at 48h, 72h, 96h and 120h after their treatment with HeberFERON (30 patients) or standard of care (33 patients). Viral nucleic acid detection was carried out by RT-PCR resulting in positive or negative samples. For each analysis time, we represent the number of positive and negative patients, the percentage of negativization and the p value in a Fisher test analysis in an overall analysis (ALL) and splitting patients in Symptomatic (S) and Asymptomatic (A). *: p< 0.05 ; **: p> 0.01; ns: p> 0.05.

			HeberFERON	Control group
48h	ALL	Positives	16	22
		Negatives	13	4
		No Data	1	7
		% negativization	**44.8**	**15.4**
		p (Fisher test)	***0.0224 (*)***	
	S	Positives	5	11
		Negatives	6	2
		No Data	1	3
		% negativization	**54.5**	**15.4**
		p (Fisher test)	***0.0825 (ns)***	
	A	Positives	11	10
		Negatives	7	2
		No Data		4
		% negativization	**38.9**	**16.7**
		p (Fisher test)	***0.2487 (ns)***	
72h	ALL	Positives	11	19
		Negatives	19	10
		No Data		4
		% negativization	**63.3**	**34.5**
		p (Fisher test)	***0.0379 (*)***	
	S	Positives	3	9
		Negatives	9	5
		No Data		2
		% negativization	**75.0**	**35.7**
		p (Fisher test)	***0.0618 (ns)***	
	A	Positives	8	9
		Negatives	10	5
		No Data		2
		% negativization	**55.6**	**35.7**
		p (Fisher test)	***0.3075 (ns)***	
96h	ALL	Positives	6	19
		Negatives	23	13
		No Data	1	1
		% negativization	**78.6**	**40.6**
		p (Fisher test)	***0.004 (**)***	
	S	Positives	1	10
		Negatives	11	6
		No Data		
		% negativization	**91.7**	**37.5**

		p (Fisher test)	***0.0060 (**)***	
	A	Positives	5	8
		Negatives	12	7
		No Data	1	1
		% negativization	**70.6**	**46.7**
		p (Fisher test)	***0.2804 (ns)***	
120h	**ALL**	Positives	1	6
		Negatives	23	17
		No Data	6	10
		% negativization	**95.8**	**73.9**
		p (Fisher test)	***0.0479 (*)***	
	S	Positives	1	5
		Negatives	11	7
		No Data		4
		% negativization	**91.7**	**58.3**
		p (Fisher test)	***0.1550 (ns)***	
	A	Positives	0	0
		Negatives	12	10
		No Data	6	6
		% negativization	**100**	**100**
		p (Fisher test)	***1.0000 (ns)***	

Figures

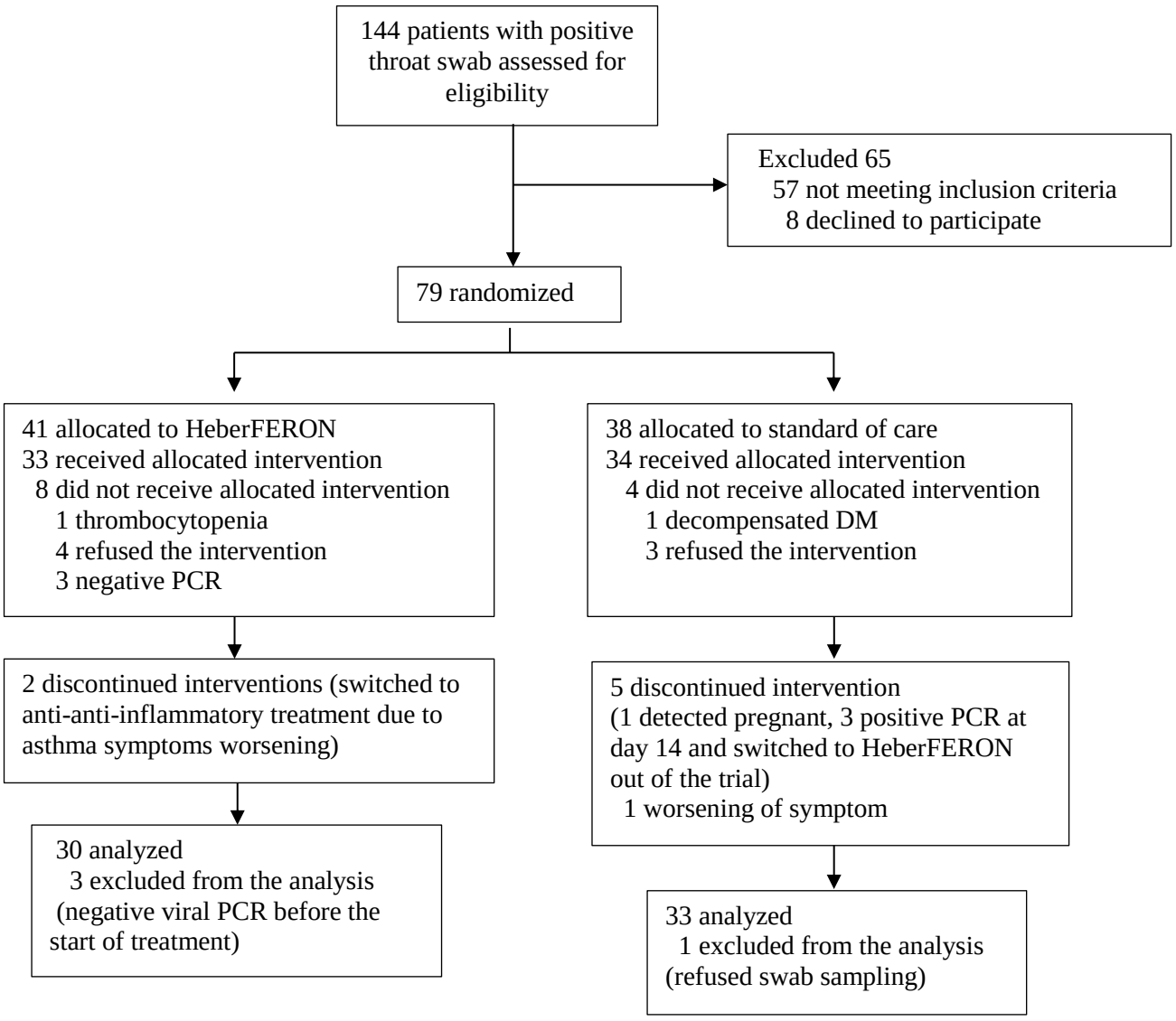
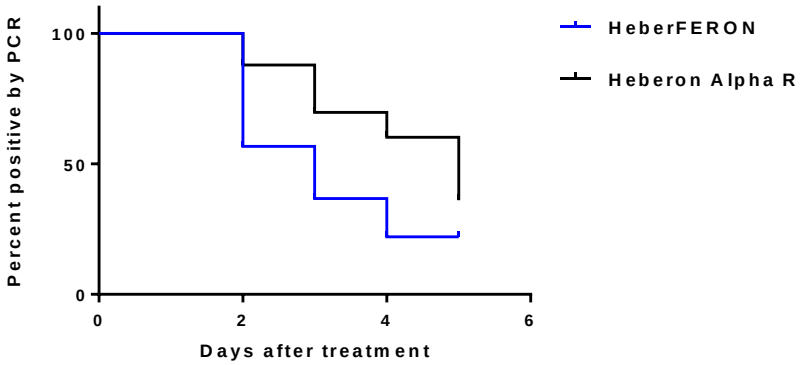


Figure 1. Randomization and treatment assignment.



Log-rank (Mantel-Cox) test	
Chi square	9.356
df	1
P value	0.0022

Hazard Ratio (Mantel-Haenszel)	
Ratio (and its reciprocal)	3.259
95% CI of ratio	1.529 to 6.948

Figure 2. Kaplan-Meier representation for viral negativization. Negativization curves were constructed using GraphPrism (version 8) from the available viral detection data at four analysis points for the 63 patients split in two treatment groups. The P value (* $p < 0.05$) represents statistic comparison of the two curves using a Log-rank Mantel-Cox test. Hazard Ratio (Mantel-Haenszel) and Median of negativization for the two treatments were also calculated.

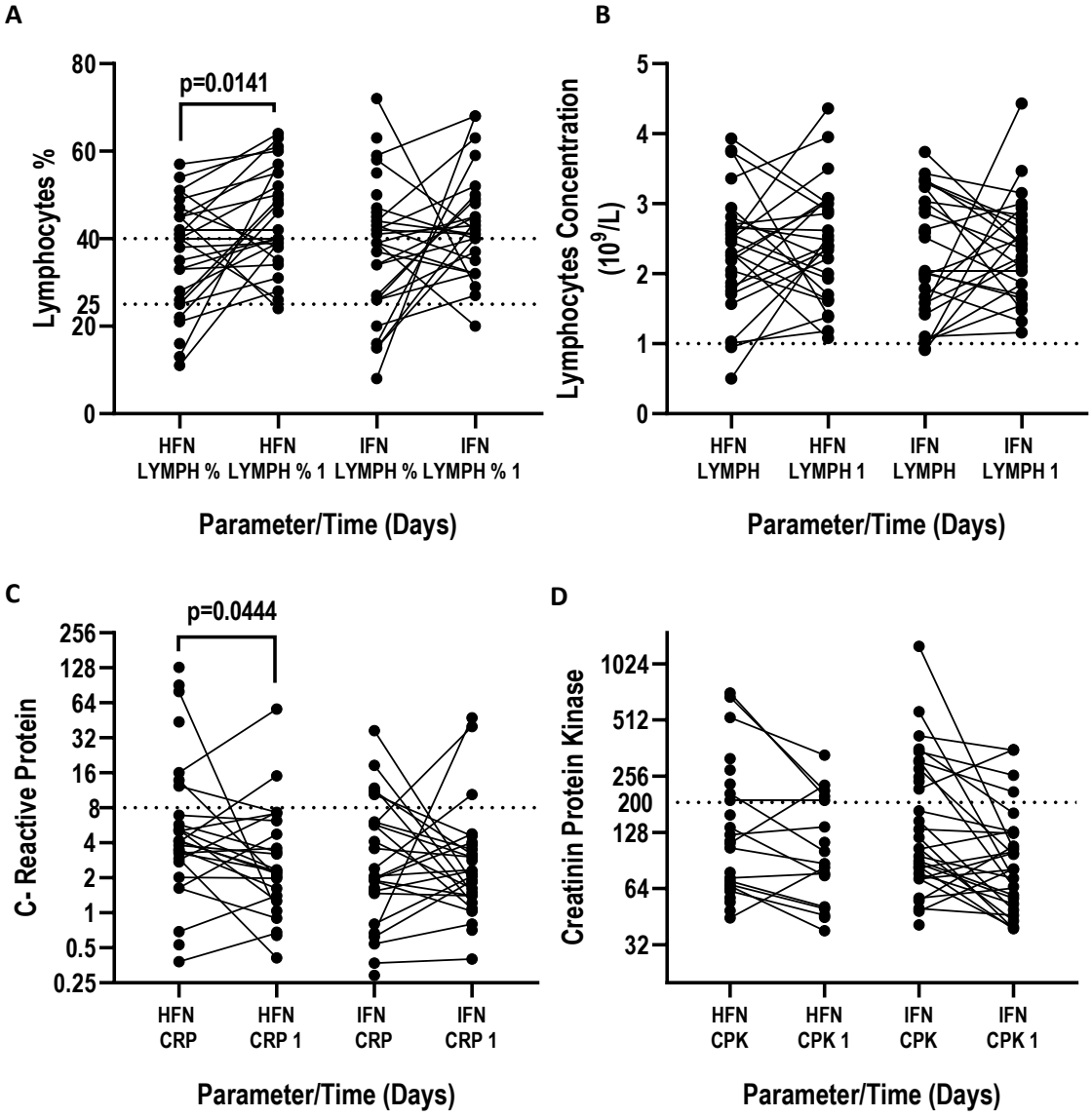


Figure 3. Comparative evaluation of clinical laboratory data a week after starting treatment in comparison to baseline. (A) Lymphocyte percentage among total leukocytes **(B)** Lymphocytes concentration **(C)** Levels of C - reactive protein and **(D)** Creatine phosphokinase levels. Differences were analyzed across three time points, and only week one is shown. "p" values are indicated in cases of significant changes for the results of a mixed model adjustment for pair data sets. HFN: HeberFERON. LYMPH: Lymphocytes. CRP: C-reactive protein.

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