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Aprotinin treatment against SARS-CoV-2: A randomized phase III study to evaluate the safety and efficacy of a pan-protease inhibitor for moderate COVID-19

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Abstract

Background: SARS-CoV-2 virus requires host proteases to cleave its spike protein to bind to its ACE2 target through a two-step furin-mediated entry mechanism. Aprotinin is a broad-spectrum protease inhibitor that has been employed as antiviral drug for other human respiratory viruses. Also, it has important anti-inflammatory properties for inhibiting the innate immunity contact system.

Methods: This was a multicentre, double-blind, randomized trial performed in four Spanish hospitals comparing standard treatment versus standard

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treatment + aprotinin for patients with COVID-19 between 20 May 2020 and 20 October 2021. The primary efficacy outcomes were length of hospital stay and ICU admission. The secondary endpoints were each of the primary efficacy outcomes and a composite of oxygen therapy, analytical parameters and death. Safety outcomes included adverse reactions to treatment during a 30-day follow-up period. Treatment was given for 11 days or till discharge.

Results: With almost identical analytical profiles, significant differences were observed in treatment time, which was 2 days lower in the aprotinin group ($p = .002$), and length of hospital admission, which was 5 days shorter in the aprotinin group ($p = .003$). The incidence of discharge was 2.19 times higher (HR: 2.188 [1.182–4.047]) in the aprotinin group than in the placebo group ($p = .013$). In addition, the aprotinin-treated group required less oxygen therapy and had no adverse reactions or side effects.

Conclusion: Inhaled aprotinin may improve standard treatment and clinical outcomes in hospitalized patients with COVID-19, resulting in a shorter treatment time and hospitalization compared with the placebo group. The administration of aprotinin was safe.

KEYWORDS

antivirus, aprotinin, clinical trial, COVID-19, inhalation therapy, pneumonia, protease inhibitor, SARS-CoV-2

1 | INTRODUCTION

The COVID-19 pandemic, which is caused by infection with a highly airborne betacoronavirus called SARS-CoV-2, has become one of the world's greatest public health challenges. Although the infection can be asymptomatic or mildly symptomatic for many individuals, approximately 20% of cases progress to severe acute respiratory distress syndrome (ARDS),[1] which is characterized by lower respiratory tract involvement and activation of a major inflammatory process referred to as a 'cytokine storm.'[2] Of the patients who develop ARDS, approximately 5% will continue to progress to a more critical illness that is characterized by extrapulmonary systemic and multi-organ involvement.[1]

In these cases, the infection fatality rate, which depends on the capacity of care services, is estimated from 0.002% at age 10 to 15% at age 85.[3] Hospital occupancy rates, which are often high during epidemic peaks, and the average length of hospital stay are important factors in hospital management and quality of care, and it is important to reduce both as much as possible.[4]

Although vaccines help to mitigate the severity of infection, the emergence of new genetic variants with a greater capacity for transmission and evasion of the immune system has diminished their effectiveness in terms of preventing infection.[5] Therefore, it is essential to complement vaccination with antiviral drugs. However, although several antivirals, such as molnupiravir (Lagevrio®), nirmatrelvir/ritonavir (Paxlovid®) and lopinavir/ritonavir (Kaletra®), have recently been used to treat COVID-19,[6] these therapies display several disadvantages: (i) they do not have ideal pharmacokinetic characteristics (low bioavailability for oral route, high metabolism and interactions, and high interindividual variability in therapeutic response) on an outpatient basis; or (ii) they are too expensive to be afforded in countries with low economic capacity; or (iii) they are not active during the inflammatory phase of SARS-CoV-2 infection, which are important factors to overcome to provide useful solutions to this pandemic.[7]

In the search for new drugs, it is important to understand the mechanisms of viral infection. Coronaviruses such as SARS-CoV-2 have evolved a two-step activation process that requires proteolytic cleavage of the spike (S) glycoprotein into the S1 and S2 subunits to allow them to function independently during cell entry.[8] The cleavage of the S1 and S2 subunits occurs at a furin-like domain that is recognized by proteases and is a key process facilitating both viral entry into the cells of the respiratory tract and viral replication.[9] The proteases that can catalyse this cleavage include trypsin, plasminogen, kallikrein, cathepsin, elastase, and members of the TMPRSS family of serine proteases, which are in abundance in both type I and II alveolar cells and vascular endothelial cells.[10] This cleavage is a critical step that

occurs prior to viral entry and leads to a conformational change in the S protein that exposes key amino acids required for the binding of viral S1 protein to the ACE2 receptor on host cells.[11]

The fact that proteases present in the human respiratory tract are involved in SARS-CoV-2 infection also helps to amplify the infection and inflammatory processes through a vicious cycle in which the virus stimulates the action of proteases on the surface of the host epithelial cells, triggering both more rapid activation of progeny with infective capacity, which enhances the viral cycle and proinflammatory reactions through activation of protease-dependent cascades, such as plasmin, kallikrein, trypsin, C-reactive protein, and neutrophil elastase, which can affect the respiratory, myocardial, and haematopoietic systems (coagulation). This involvement is most evident in patients with SARS-CoV-2 and is a consequence of activation of the contact system (kallikreins) of innate immunity, which involves the aforementioned proteases and the complement activation system.[12] It is also important to note that, under normal conditions, a regulatory system in the respiratory tract, consisting of natural protease inhibitors and antiproteases, maintains proteolytic balance. These control systems have been shown to be negatively regulated by various viruses. Both virus-induced antiprotease deficiency and protease dysregulation aggravate the pathology. These processes are particularly altered in SARS-CoV-2 patients, and a large portion of the pathology can be explained by dysregulation of the activation of the contact system of innate immunity.[12]

For the reasons described above, selective serine protease inhibitors that target the host protease TMPRSS have been proposed as antivirals for the treatment of SARS-CoV-2.[13,14] However, because they do not target the full spectrum of proteases that can be used by the coronavirus, their efficacy is limited, and they do not prevent thromboinflammation. Compared with selective inhibitors, broad-spectrum protease inhibitors, such as aprotinin, showed greater efficacy in inhibiting SARS-CoV-2 activation, entry and replication in epithelial cell lines.[11] In addition, SARS-CoV-2 increases the transcription of proteases such as TMPRSS2 in host cells to promote their infective capacity and increase the entry of new virions.[15] The activation of these proteases, together with other toxic insults (e.g. viral antigens, NETs or endotoxins), activates the plasma contact system (also called kallikrein–kinin system [KKS]). This system encompasses three plasma proteins, namely coagulation factor XII (FXII), prekallikrein and high molecular weight kininogen. This allows the activation of the intrinsic coagulation pathway, as well as the proinflammatory system of KKS, together with the generation of bradykinin.[16] The latter causes inflammation, mucosal irritation and a dry cough that is characteristic of COVID-19 patients. In addition, FXII stimulates the aggregation of neutrophils, the release of proteases from their granules (e.g. elastase), and the formation of NETs that help in a feedback process of the thromboembolic and inflammatory processes.[16] Aprotinin is a potent inhibitor of a wide range of proteases, including KKS, plasminogen or thrombin PAR-1 receptors, and therefore has important actions on the thromboinflammation caused by SARS-CoV-2. Other anti-inflammatory actions of aprotinin include inhibition of: (i) mediator release (e.g. interferon-alpha); (ii) granulocyte and monocyte adhesion molecule expression; (iii) nitric oxide synthase (NOS); (iv) tracheobronchial secretion; and (v) plasminogen preventing activation of complement proteins such as C3a and C5a.[17] These mechanisms make aprotinin a candidate drug for treating SARS-CoV-2 infections.

Prior to the COVID-19 pandemic, the efficacy of aprotinin as an antiviral for viruses with two-step entry mechanisms was demonstrated both in experimental animals and in human clinical trials by inhalation administration.[18] In the present study, we present the main results from a clinical trial examining the efficacy and safety of inhaled aprotinin for the treatment of SARS-CoV-2 infections in patients with moderate COVID-19.

2 | METHODS

2.1 | Study design

This double-blind, multicentre, parallel-arm randomized phase III trial was conducted at four hospitals in the Castilla-La Mancha region of Spain following the protocol of local standard care and using electronic medical records.

This randomized clinical trial was approved by the institutional ethics committees and the Spanish Drug Agency (AEMPS; reference number EudraCT 2020–002434–33). The protocol was amended on 17 July 2021, based on an emerging understanding of the clinical presentation and evolution of COVID-19. The last version of the study protocol, along with a summary of the changes, is included in the Appendix S1. All versions allowed the use of other treatments with presumptive activity against SARS-CoV-2 if their use was part of the approved standard of care, including in vaccinated patients.

Patients provided written informed consent, and the trial protocol was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice. Reporting of the study conforms to broad EQUATOR guidelines.[19]

2.2 | Participants

The study comprised patients admitted to medical wards with SARS-CoV-2 infection confirmed by polymerase chain reaction assay within 48 h prior to randomization and moderate COVID-19 pneumonia. Key inclusion criteria were as follows: >18 years of age, radiographic evidence of pulmonary infiltrates, oxygen saturation >90% and oxygen therapy using nasal spectacles at 2–3 L/min.

Patients were excluded if they were enrolled in any other interventional study, did not provide written consent, had serum creatinine >2.5 mg/dl, were on anticoagulant treatment for prior indications, were pregnant or a female of child-bearing age potential, were directly admitted to the intensive care unit (ICU), were patients with asthma or COPD, or had any other condition that could put them at risk as a participant in the trial.

2.3 | Randomization

Randomization was done using the EPIDAT software, and patients were assigned in a 1:1 ratio to either the placebo or the aprotinin group. The dummy randomization list was reviewed and validated by the statistics team. A separate list of sequential numbers within each treatment group was provided to the Pharmacy team. To assure double-blind conditions, aprotinin or placebo kits were prepared and sent in the same format to the internist physicians at the participating hospitals. Specifically, once the informed consent was signed, the Internal Medicine Service of each participating hospital sent the document of acceptance to the Pharmacy Service of the University General Hospital of Ciudad Real. This service was responsible for the coding and randomization of the patient's clinical record number, and they registered it in the electronic management and prescription computer system. The system performed the randomization of the patients. When the pharmaceutical form had to be made, it was done in a codified way, without any operator knowing the treatment. The characteristics of the pharmaceutical forms did not allow distinguishing placebo from aprotinin. The treatment was sent to the Internal Medicine Services of each hospital, perfectly codified and without the possibility of recognizing between groups.

2.4 | Procedures

Participants randomized to the aprotinin group were treated with aprotinin by inhalation on Day 1 (500 KIU every 6 h over 10 min until 2000 KIU/day). Patients in the placebo group received a physiological saline solution by inhalation. In both groups, therapy was administered immediately after randomization and for 11 days or until discharge. The main clinical criteria for hospital discharge were as follows: (i) overall improvement in the fever curve without antipyretics. No spikes for 48 h; (ii) improvement or stability of respiratory symptoms (no dyspnoea, no cough, no tachypnoea or work of breathing) and improvement or stable oxygen requirement for 48 h; and (iii) improvement or stability of laboratory data, including inflammatory markers, if followed during the admission (especially C-reactive protein and ferritin).

The device used for inhalation therapy was a Mesh Nebulizer MicroAIR U100 (NE-U100-E; Omron®), which is a vibrating mesh nebulizer capable of generating Mass Median Aerodynamic Diameter (MMAD) particles of 2–5 μm. This type of nebulizer is recommended by scientific societies for use by COVID-19 patients as it can reach distal alveolar areas.[20] In our case, a vibrating mesh nebulizer with a pipette was used. The vibrating mesh technology transforms the liquid drug into a fine vapour, with atomization into small particles that reach the bronchi and alveolae, while avoiding dispersion and possible environmental contamination.[21]

Demographic information, pre-existing comorbidities (e.g. high blood pressure, diabetes and obesity) smoking habit, concomitant medications (remdesivir, corticosteroid, anticoagulant or tocilizumab) during or before the study, vaccination status, oxygen therapy with different devices (nasal spectacles, Venturi-type oxygen mask [Ventimask], oxygen mask reservoir bag or high-flow nasal cannula oxygen), adverse events due to the protocol, and respiratory and cardiovascular status were recorded. On study days 1, 5 and 10, blood samples were obtained for measurements of complete blood cell counts; coagulation activity; renal, respiratory and cardiovascular function; glucose; and inflammation responses. If the clinical status of a patient changed on a particular day, the worst score was documented.

2.5 | Study outcomes

The primary efficacy endpoints were the reduction in hospitalization stay and ICU admission. The secondary efficacy endpoints included: the level of supplemental oxygen therapy, clinical and analytical parameters and death. Safety endpoints were analysed as the frequency of adverse events. A final check was conducted on Day 30 after randomization in person for hospitalized patients or by consulting electronic medical records for patients who had been discharged. Study outcomes were adjudicated by a clinical event committee that was blinded to treatment, and their definitions are listed in the Appendix S1.

2.6 | Sample size

The proportion of patients with pneumonia and ICU admission was expected to be 0.08, accepting an alpha risk of 0.05 and a beta risk of 0.2 in a bilateral contrast. Thus, 108 patients were estimated to be needed for the trial (54 in each arm) to show an estimated 25% reduction.

The recruitment period was projected to be 12 months, with a total duration of 15 months for follow-up, data collection, checking and analysis. However, after 12 months, only 75 patients had been enrolled. Considering the recruitment constraints and the very low likelihood of reaching the expected sample size at that time, the principal investigators decided to end the study on 20 October 2021. This decision was agreed by all involved investigators, ethics committees, and AEMPS.

2.7 | Statistical analysis

The analysis was conducted on an intention-to-treat principle. A test for differences in proportions was carried out to compare in-hospital composite events among patients randomized to the two groups. Clinical and laboratory examinations were compared by the t test or the Wilcoxon rank-sum test, as appropriate. Cox proportional hazard regression models were used to estimate hazard ratios (HRs), and the Kaplan–Meier survival curves were constructed for each group to estimate the cumulative outcome incidence, and 95% CIs for primary outcome evaluated at 30 days after randomization.

Safety analyses were performed using the safety data set, which included data from all participants who received study treatment. All tests were two-sided, and a p-value $<.05$ was considered statistically significant. Statistical analyses were performed using R software version 3.6.0 (R Project for Statistical Computing).

3 | RESULTS

The study cohort included 75 patients; 40 were randomly assigned to the placebo group, and 35 were assigned to the aprotinin group. During the study, eight patients from the placebo group and seven patients from the aprotinin group were excluded, leaving a total of 32 patients in the placebo group and 28 patients in the aprotinin group for inclusion in the analysis (Figure 1).

A repeated-measures ANOVA was performed to study the possible differences between the placebo and aprotinin groups considering two time points (Day 1 and Day 5). The results of this model and each of the multiple comparisons are shown in Table 1.

Comparing days 1 and 5 in the placebo group, there were significant decreases in dyspnoea, temperature, aTTP, fibrinogen, C-reactive protein, procalcitonin and IL-6 levels. On Day 5, there were significant increases in leucocytes, segmented, platelets, urea, ALT, potassium and lactic acid.

Comparison of days 1 and 5 in the aprotinin-treated group showed that on the fifth day, there were significant decreases in dyspnoea, cardiac frequency, fibrinogen, glucose, creatinine and C-reactive protein. Statistically significant increases were observed for platelets, ALT, potassium, pCO_2, bicarbonate and lactic acid (Table 1).

When comparing the analytical parameters of the placebo group with the aprotinin group on Day 1 and Day 5, there was only a statistically significant difference in platelets between both groups on Day 1.

Significant differences were observed in treatment time, which was 2 days shorter for the aprotinin group (7.7 ± 0.4 days for the placebo group vs. 5.8 ± 0.4 days for the aprotinin group, $p = .002$), and length of hospital admission, which was 5 days shorter for the aprotinin group (12.6 ± 1.4 days on placebo vs 7.5 ± 0.5 days on the aprotinin group, $p = .003$; Figure 2).

Admission time was significantly lower in the aprotinin-treated group when compared to the placebo group in a Kaplan–Meier model assuming hospital discharge as an event as shown in Figure 3.

Cox regression with a binary predictor was performed to assess the effect of treatment on admission to discharge. The incidence rate of discharge was 2.19 times higher (HR: 2.188 [1.182–4.047]) in the aprotinin group than in the placebo group ($p = .013$).

In addition, without being statistically significant, aprotinin-treated patients showed a more positive evolution in oxygen therapy (oxygen supplementation with different devices: nasal spectacles, Venturi-type oxygen mask [Ventimask], oxygen mask reservoir bag and high-flow nasal cannula oxygen) during treatment when compared to patients in the placebo group, as they did not need high-flow nasal cannula oxygen and required proportionally less Ventimask and oxygen mask reservoir bag use, as shown in Figure 4. These auxiliary therapy devices maintained mean O_2 saturations higher than 94% in both groups during hospitalization.

Two deaths occurred in the placebo group: one patient died at the hospital ward due to severe bilateral SARS-CoV-2 pneumonia with severe ARDS, and another died in ICU due to multi-organ failure associated with pulmonary fibrosis (haemodynamic failure, pneumonia due to *Serratia* spp. and pulmonary aspergillosis). No patients treated with aprotinin were transferred to ICU.

Two skin reactions were reported in the placebo group, one maculopapular exanthema associated with the

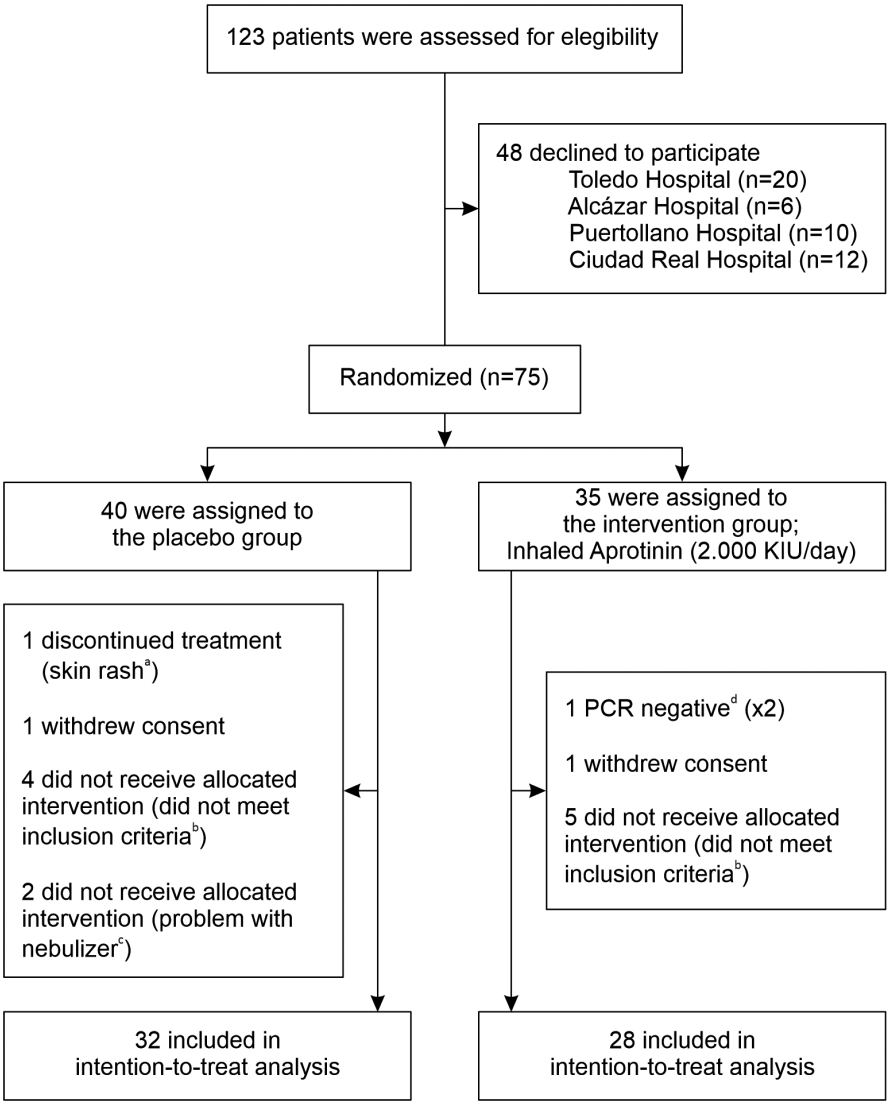

FIGURE 1 Study design flow chart. [a]The rash appeared right after the first administration, and the patient received practically no medication at all. [b]These patients were effectively excluded after randomization because in the interval between randomization and the start of treatment, they no longer met the inclusion criteria (more or less severity than stated in the inclusion criteria: oxygen saturation >90% with oxygen therapy using nasal spectacles at 2–3 L/min). [c]They were excluded because treatment administration was delayed due to the unavailability to supply the nebulizer to one of the participating hospitals. In no case, technical problems were reported with the nebulizer used. [d]The patient was initially included on suspicion of COVID-19 and then excluded after negative PCR

prescribed metamizole, and another undetermined. Both reverted within 24 h. Two patients had hiccups, which also reverted within 24–48 h. No adverse reactions or side effects were reported in the aprotinin-treated group.

4 | DISCUSSION

In this double-blind, randomized, multicentre, phase III trial of patients with moderate COVID-19 pneumonia due to SARS-CoV-2 infection, we found a statistically significant difference in the primary endpoint of length of hospitalization stay when comparing the placebo group to the inhaled aprotinin treatment group.

Given the evolution of the pandemic during the recruitment period, including a decrease in the number of hospital admissions and an increase in the percentage of vaccinated patients, the number of participants in our study was limited. Two patients died, both were in the placebo group, one in the hospital ward and one in the ICU. In addition, patients in the aprotinin group received proportionally less supplemental oxygen, and no patient required high-flow nasal cannula oxygen.

Regarding treatments for SARS-CoV-2 infection, so far, only the antivirals remdesivir, molnupiravir and nirmatrelvir/ritonavir[6,22] have demonstrated their efficacy on different parameters of disease progression in clinical trials. Administration of these drugs and corticosteroids such as dexamethasone reduces mortality in patients admitted to the ICU by acting in the proinflammatory phase,[23] and they are the only available pharmacological treatments with proven efficacy for treating COVID-19.

The dire pandemic situation calls for strategies to quickly identify drugs to effectively treat COVID-19. Therefore, we believe that drug repurposing is a useful approach to discover possible therapeutic options to fight SARS-CoV-2 infection, based on what is known about its pathophysiology. The effect of intravenous camostat mesylate, a TMPRSS2 protease inhibitor approved to treat pancreatitis, on SARS-CoV-2 was evaluated, and a

TABLE 1 Split-plot ANOVA comparing placebo (P) and aprotinin (A) groups over two time points (days 1 and 5)

Variable	nP	nA	P1	A1	P5	A5	p GLM	P1 vs P5	A1 vs A5	P1 vs A1	P5 vs A5
Age (years)	32	28	56.0 ± 2.8	53.9 ± 2.1	–	–	–	–	–	0.564	–
Sex (woman)	32	28	8 (25)	13 (46.4)	–	–	–	–	–	0.083	–
BMI (Kg/m^2)	27	24	29.0 ± 0.8	28.5 ± 1.0	–	–	–	–	–	0.712	–
HBP	30	27	16 (53)	11 (41)	–	–	–	–	–	0.342	–
DM	30	27	8 (27)	9 (33)	–	–	–	–	–	0.583	–
Smoking habit	16	21	1 (6)	3 (14)	–	–	–	–	–	0.618	–
Pulmonary disease	23	23	2 (9)	3 (13)	–	–	–	–	–	0.636	–
Cardiac disease	24	23	5 (21)	3 (13)	–	–	–	–	–	0.701	–
Remdesivir	30	28	3 (10)	4 (14)	–	–	–	–	–	0.701	–
Corticosteroids	30	28	30 (100)	26 (93)	–	–	–	–	–	0.229	–
Anticoagulants	31	27	29 (94)	25 (93)	–	–	–	–	–	0.886	–
Tocilizumab	30	27	4 (13)	2 (7)	–	–	–	–	–	0.673	–
COVID vaccine prior to admission	30	27	12 (40)	8 (30)	–	–	–	–	–	0.413	–
Dyspnoea	32	28	24 (75)	19 (68)	13 (41)	10 (36)	–	**0.007**[a]	**0.022**[a]	0.540[b]	0.696[b]
SBP (mm Hg)	27	26	129.4 ± 2.9	129.1 ± 3.0	131.0 ± 3.5	128.4 ± 3.5	0.711	0.625	0.842	0.951	0.598
DBP (mm Hg)	27	26	80.2 ± 2.1	79.3 ± 2.1	81.0 ± 1.8	79.5 ± 1.9	0.603	0.727	0.946	0.766	0.567
MAP (mm Hg)	27	26	96.1 ± 2.1	95.5 ± 2.1	97.6 ± 2.1	95.3 ± 2.2	0.729	0.683	0.934	0.723	0.479
Cardiac frequency (beats/min)	27	25	79.6 ± 2.6	82.2 ± 2.7	76.0 ± 3.0	72.9 ± 3.1	0.227	0.259	**0.007**	0.503	0.479
Respiratory frequency (res/min)	4	5	21.0 ± 9.3	38.2 ± 8.3	19.00 ± 1.4	20.4 ± 1.3	0.179	0.840	0.076	0.211	0.482
O_2 saturation (%)	27	24	94.3 ± 0.5	95.5 ± 0.5	95.1 ± 0.4	95.4 ± 0.4	0.292	0.167	0.888	0.124	0.616
Temperature (ºC)	27	25	36.3 ± 0.2	36.2 ± 0.2	35.9 ± 0.1	36.1 ± 0.1	0.178	**0.008**	0.432	0.781	0.082
Leucocytes (mil/ml)	30	26	8.5 ± 0.7	8.9 ± 0.8	10.7 ± 0.6	9.8 ± 0.7	**0.035**	**<0.001**	0.169	0.708	0.341
Lymphocytes (mil/ml)	30	26	1.1 ± 0.6	2.3 ± 0.6	1.9 ± 0.4	2.3 ± 0.4	0.362	0.175	0.981	0.161	0.420
Segmented (mil/ml)	27	24	7.1 ± 0.6	6.8 ± 0.7	8.4 ± 0.6	7.1 ± 0.6	0.236	**0.035**	0.671	0.809	0.112
Eosinophils (mil/ml)	30	26	0.011 ± 0.011	0.030 ± 0.012	0.016 ± 0.013	0.044 ± 0.014	0.192	0.738	0.366	0.253	0.139
Haemoglobin (g/dl)	30	25	13.61 ± 0.21	13.70 ± 0.23	13.84 ± 0.23	13.88 ± 0.26	0.074	0.068	0.190	0.783	0.917
Haematocrit (%)	29	26	40.7 ± 0.6	41.1 ± 0.6	41.3 ± 0.7	41.5 ± 0.7	0.618	0.093	0.358	0.597	0.862
Platelets (mil/ml)	30	26	214.9 ± 16.7	267.4 ± 21.0	318.0 ± 20.2	338.2 ± 21.5	0.148	**<0.001**	**<0.001**	**0.041**	0.495

(Continues)

TABLE 1 (Continued)

Variable	nP	nA	P1	A1	P5	A5	p GLM	P1 vs P5	A1 vs A5	P1 vs A1	P5 vs A5
Prothrombin activity (%)	27	23	95.0 ± 3.1	91.0 ± 3.4	95.1 ± 2.2	95.5 ± 2.4	0.153	0.993	0.053	0.385	0.898
aTTP (s)	9	12	30.2 ± 1.8	29.7 ± 1.5	27.5 ± 1.4	27.5 ± 1.2	0.718	**0.038**	0.058	0.813	0.984
Fibrinogen (mg/dl)	27	22	627.9 ± 31.9	688.3 ± 35.4	543.8 ± 34.8	465.8 ± 38.6	**0.024**	**0.040**	**<0.001**	0.211	0.140
D-dimer (ng/ml)	25	24	464.0 ± 51.5	529.9 ± 52.6	733.0 ± 221.8	834.8 ± 226.4	**0.014**	0.221	0.175	0.376	0.749
Glucose (mg/dl)	29	25	151.6 ± 16.0	186.0 ± 17.2	131.0 ± 13.6	151.2 ± 14.7	0.389	0.071	**0.005**	0.148	0.318
Urea (mg/dl)	30	25	47.9 ± 4.1	48.8 ± 4.5	53.8 ± 3.7	51.9 ± 4.0	0.352	**0.005**	0.159	0.883	0.731
Creatinine (mg/dl)	30	26	0.85 ± 0.05	0.82 ± 0.06	0.81 ± 0.04	0.75 ± 0.04	0.574	0.120	**0.029**	0.697	0.310
Total bilirubin (mg/dl)	21	22	0.56 ± 0.06	0.60 ± 0.06	0.54 ± 0.05	0.62 ± 0.05	0.668	0.776	0.747	0.654	0.250
Glomerular filtration rate (ml/min/1.73)	25	24	78.2 ± 3.8	79.7 ± 3.9	80.9 ± 3.8	81.7 ± 3.9	0.090	0.075	0.180	0.786	0.874
AST (IU/l)	28	24	45.0 ± 6.5	34.2 ± 7.0	47.9 ± 11.6	33.8 ± 12.6	0.858	0.818	0.976	0.261	0.413
ALT (IU/l)	29	26	49.2 ± 7.4	42.9 ± 7.8	77.6 ± 11.9	69.0 ± 12.6	0.876	**0.007**	**0.019**	0.558	0.619
Potassium (nmol/l)	27	24	4.34 ± 0.10	4.57 ± 0.11	4.41 ± 0.09	4.60 ± 0.09	0.712	**0.008**	**0.042**	0.627	0.840
Chlorine (nmol/l)	27	25	101.6 ± 0.6	102.6 ± 0.7	98.9 ± 2.5	101.5 ± 2.6	0.671	0.313	0.700	0.274	0.470
C-reactive protein (mg/dl)	25	23	8.1 ± 1.3	6.7 ± 1.6	3.8 ± 1.0	1.3 ± 1.0	0.676	**0.024**	**0.007**	0.545	0.081
Ferritin (ng/ml)	21	18	695.7 ± 98.5	579.0 ± 106.4	715.3 ± 103.3	567.4 ± 111.5	0.775	0.791	0.885	0.426	0.337
pH	9	9	7.44 ± 0.01	7.46 ± 0.01	7.48 ± 0.01	7.45 ± 0.01	0.445	0.758	0.439	0.264	0.824
pCO_2 (mm Hg)	9	9	36.0 ± 2.2	31.9 ± 2.2	37.5 ± 1.4	38.3 ± 1.3	0.143	0.531	**0.012**	0.197	0.672
pO_2 (mm Hg)	9	9	76.1 ± 8.6	70.3 ± 9.0	81.0 ± 8.6	84.2 ± 9.0	0.266	0.646	0.797	0.693	0.291
Bicarbonate (nmol/l)	8	9	24.2 ± 1.3	22.4 ± 1.2	25.5 ± 0.9	26.4 ± 0.8	0.146	0.374	**0.006**	0.317	0.447
Lactic acid (mg/dl)	9	9	10.6 ± 1.4	12.3 ± 1.4	16.4 ± 1.6	16.9 ± 1.6	0.291	**0.002**	**0.008**	0.405	0.830
Troponin I ultras (ng/l)	21	15	7.4 ± 1.0	4.7 ± 1.2	6.7 ± 1.0	6.0 ± 1.1	0.166	0.432	0.245	0.087	0.642
Procalcitonin (ng/ml)	19	17	0.15 ± 0.04	0.11 ± 0.03	0.06 ± 0.01	0.07 ± 0.01	0.369	**0.014**	0.238	0.458	0.695
IL-6 (pg/ml)	18	18	28.8 ± 8.0	17.6 ± 8.0	9.8 ± 5.7	14.1 ± 5.7	0.164	**0.019**	0.655	0.325	0.597

Abbreviations: A1, aprotinin day 1; A5, aprotinin day 5; ALT, alanine aminotransferase; AST, aspartate aminotransferase; aTTP, acquired thrombotic thrombocytopenic purpura; DBO, diastolic blood pressure; DM, diabetes mellitus; HBP, high blood pressure; IL-6, interleukin-6; MAP, mean arterial pressure; nA, aprotinin sample size; nP, placebo sample size; P1, placebo day 1; P5, placebo day 5; pCO_2, partial pressure of carbon dioxide; pO_2, partial pressure of oxygen; SBP, systolic blood pressure.

[a]McNemar test.

[b]Pearson's chi-squared test. Data shown are mean ± SEM or *n* (%).

p-value is lower than 0.05, it was considered statistically significant and bolded.

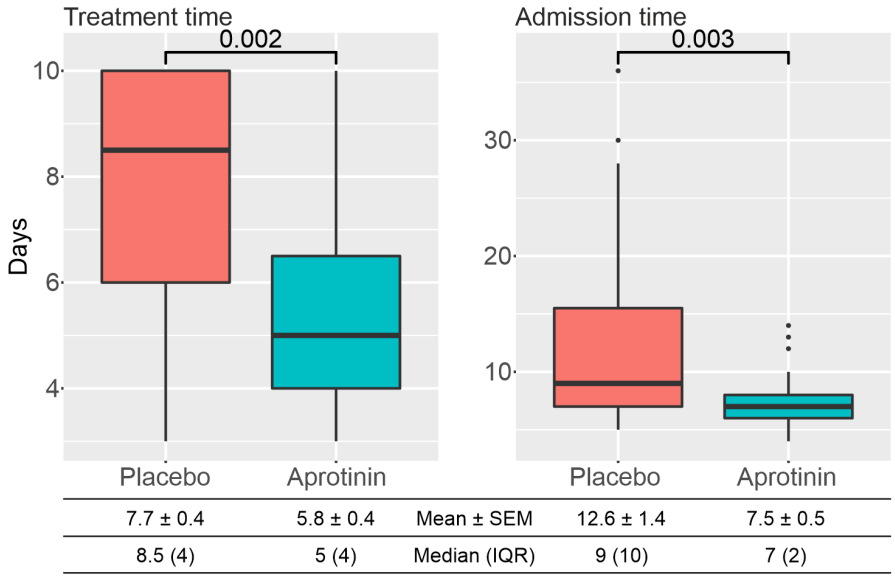

FIGURE 2 Treatment and admission time box-plot between placebo and aprotinin groups

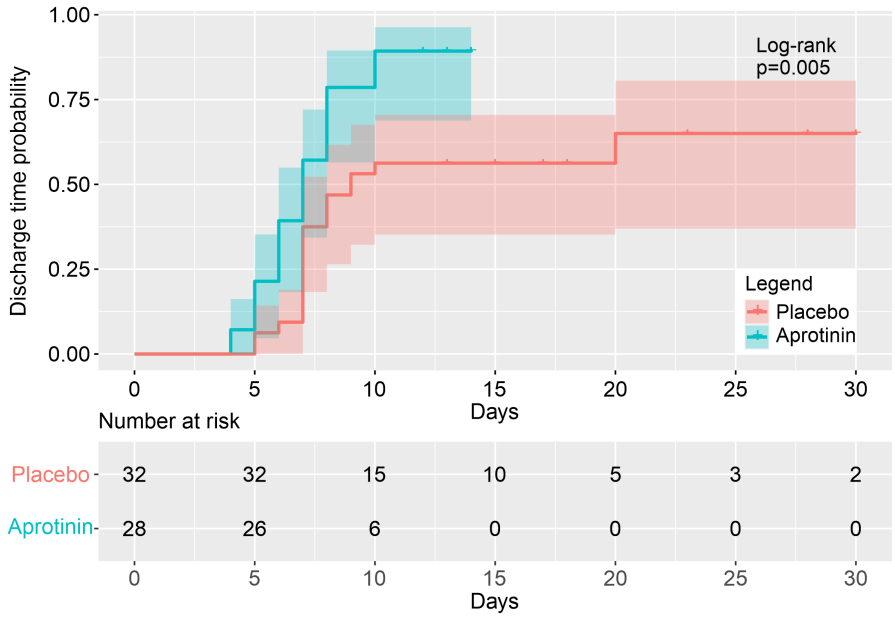

FIGURE 3 Kaplan–Meier model of length of hospital stay in the placebo and aprotinin-treated groups

decrease in viral entry into lung cells was observed in vitro. However, in a clinical trial, there was no improvement in the clinical course, duration of disease, progression to ICU admission or mortality.[24] Aprotinin has three mechanisms of action to potentially target SARS-CoV-2. Firstly, it is a potent antiviral that inhibits several human proteases (e.g. trypsin, subtilisin, granzyme, chymotrypsin and TMPRSS) expressed naturally in human bronchial epithelial cells. Proteases cleave the S protein of SARS-CoV-2 into two distinct domains, namely S1 and S2. This process is essential for viral entry into the host cell since the S1 region is responsible for increasing its ability to bind with host cell ACE2 receptor, where the S2 region is responsible for fusion of the viral mRNA and cellular membranes. Aprotinin inhibits this rate-limiting step of viral entry.[9-11]

The transcriptomic profile of ACE2 and TMPRSS2 expression in human cells was recently reported,[25] which showed higher expression of TMPRSS2 in the lower respiratory tract and at the alveolar level. Therefore, we believed that the inhalation route would be more effective than intravenous or oral administration. The nebulization method used in our study, which generates small particles (2–5 μm), can reach the bronchi and alveolae while avoiding dispersion and possible environmental contamination.[20] In a previous study,[26] a combination of intravenous and inhaled aprotinin with Avifavir® (favipiravir) for patients with moderate COVID-19 reduced viral load, ICU admission, and average hospitalization stay with improvement in lung lesions on the 14th day of treatment. However, it was not clear whether these results were due to aprotinin or Avifavir® or both.

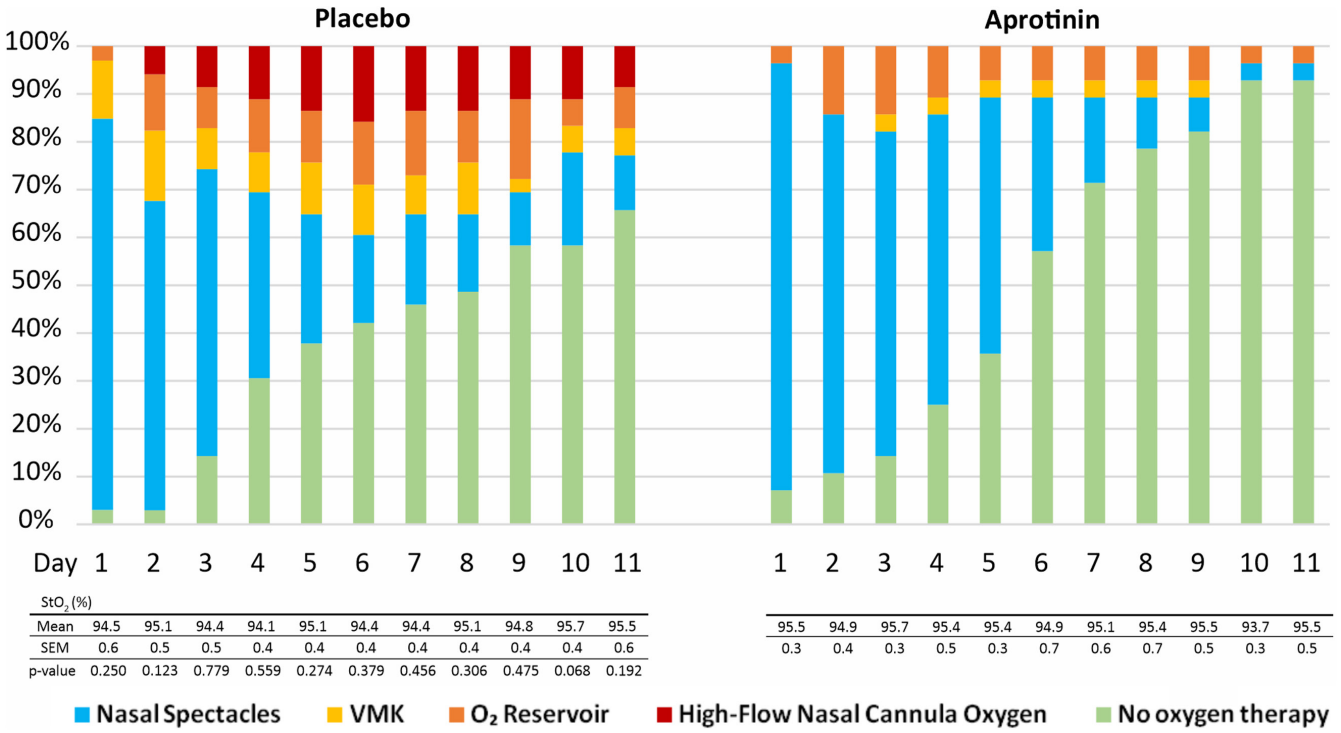

FIGURE 4 Daily evolution of oxygen therapy during treatment in the placebo and aprotinin-treated groups. This figure shows the frequency of the different types of devices used each day during the study period and the percentage of StO_2 achieved with the application of these devices. The chi-squared test was applied to compare between groups for each day. VMK: Ventimask, O_2 Reservoir: oxygen mask reservoir bag, StO_2: skeletal muscle oxygen saturation

Secondly, aprotinin could also exert anti-inflammatory effects by inhibiting the response to proinflammatory cytokines (IL-6, IL-1b and TNF-alpha) and metalloproteases. Therefore, the administration of aprotinin via the inhalation route may also reduce the tracheobronchial secretions that most COVID-19 patients present with.[11]

Thirdly, aprotinin could restore the imbalance in hypercoagulability (activation of kallikreins, plasmin, and complement and platelet aggregation) and hyperfibrinolysis in SARS-CoV-2-infected patients[27] and reduce endotheliopathy due to the expression of ACE2 and serine proteases in endothelial cells.[28] A clinical trial (DAWn-Antico) investigated the role that aprotinin may play in the coagulation contact pathway and the kallikrein–bradykinin pathway in severe COVID-19.[29] The results of this study may shed light on the role of aprotinin in the multi-level thromboinflammatory response of patients. In our study, the patients showed a statistically significant decrease in fibrinogen and C-reactive protein on Day 5 of aprotinin treatment and a statistically significant increase in platelet counts.

In relation to the generalized inflammatory process in these patients, we found a twofold to fivefold increase in parameters such as ferritin, C-reactive protein, fibrinogen and leucocytes on admission (moderate clinical severity). By the fifth day of treatment, C-reactive protein (half-life 19 h) had normalized in both groups, but parameters such as ferritin (half-life 3 days), leucocytes and fibrinogen remained higher in the placebo group with respect to the aprotinin group, reflecting the persistence over time of the inflammatory process in the placebo group.[30,31] We also found an increase in lactic acid in both groups, which even augmented on the fifth day, probably due to the hypoxia that these patients underwent and the muscle lactic acid fermentation.[31]

The furin activation site primes the virus to enter the cell and facilitates transmission. Some mutations in the spike protein of the new SARS-CoV-2 variants (H655Y, N679K and P681H) that are close to the furin cleavage site appear to increase cleavage and make it more transmissible than previous variants.[32] These changes make the sequence less acidic, and it has been reported by others that the more basic the amino acid chain of the spike protein is, the more effective furin is at recognizing and cleaving it, leading to a broadening of the spectrum of hydrolysis and activation.[33] This would produce more spike protein that is ready to enter human cells and thus a greater capacity for virus transmission. As an inhibitor of these serine proteases, aprotinin could be even more effective against these new variants.

We did not observe any adverse events or reactions (defined as hypersensitivity or anaphylaxis after drug administration) in the treatment group. Inhaled aprotinin has

been used for the treatment of influenza and parainfluenza, with high overall tolerability. Even in clinical trials, no adverse reactions, either allergic or irritant in nature, have been observed in any patients treated with aerosolized aprotinin by inhalation.[18] Similarly, good tolerability and a marked therapeutic effect were documented in patients with chronic obstructive pulmonary disease treated with aprotinin inhalers.[34] One reason for the inexistence of adverse reactions in our aprotinin group is the low dose administered (2000 KIU/day) compared, for instance, with the intravenous dose that is needed in cardiac surgery ($2–6 \times 10^6$ KIU/day).[35]

Finally, the increase in hospitalizations for COVID-19 and the length of hospital stay has a great impact in the context of public health services, which, already in a non-pandemic situation, have difficulties in meeting the existing demand. This implies accommodating an additional burden associated with COVID-19, while maintaining essential health services unrelated to the pandemic by reallocating resources. Therefore, urgent, and time-dependent diagnostic and therapeutic interventions are delayed, with a significant impact on the population health and on the economic socio-sanitary budget. In addition, it has an impact on the patient's own context. A longer hospital stay implies a higher risk of nosocomial infection, other complications or psychological affectation due to social isolation. The fact of having a cost-effective antiviral drug that can also be self-administered on an outpatient basis would have a great impact in the context of this pandemic.

4.1 | Limitations

This clinical trial has a small sample size since it was stopped early due to vaccination of most of the population and the decreased number of admissions during the study period.

We designed this trial as an intention-to-treat analysis because of the scarcity of healthcare resources during the pandemic, and it seemed appropriate to allow patients to be discharged from the hospital as soon as medically indicated, regardless of whether they had completed the full course of aprotinin. Another important limitation is that we do not have SARS-CoV-2 viral load data during and after treatment due to variability in both local access to testing and the practices at different hospitals in the region.

5 | CONCLUSIONS

Based on our data, we can conclude that inhaled aprotinin seemed to improve clinical outcomes in hospitalized patients with COVID-19, as they required less oxygen therapy and shorter treatment time and hospitalization compared with the placebo group. Aprotinin administration was not accompanied by any adverse events or reactions. These results are a promising first step in the evaluation of inhaled aprotinin for COVID-19 and open the possibility of initiating an international multicentre randomized trial with a larger number of patients. Furthermore, given the type of nebulizer used, which is a small portable device that is easy to operate, the treatment could be extended to: (i) prophylaxis in people exposed to contagion; (ii) mild–moderate outpatients and/or associated risk of thromboembolism; and (iii) patients with moderate COVID-19 in hospital admission.

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CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

AUTHOR CONTRIBUTIONS

Redondo-Calvo and Padin had full access to all of the data in the study. They take responsibility for the integrity of the data and the accuracy of the data analysis. They contributed equally as co-first authors. Redondo-Calvo and Gomez-Romero conceived and designed the study. Muñoz-Rodriguez, Perez-Ortiz, Serrano-Oviedo, Lopez-Juarez and Manrique Romo acquired, analysed and interpreted the data. Porras Leal, Gonzalez Gasca, Muñoz Hornero, Barbera Farre, Dominguez-Quesada and Sepulveda Berrocal critically revised the manuscript for important intellectual content. Redondo-Calvo and Muñoz-Rodriguez performed statistical analysis. Parra Comino, Villegas Fernandez-Infantes and Manrique Romo provided administrative, technical or material support. Rodriguez Martinez, Perez Serrano, Sanchez Cadena and Bejarano-Ramirez supervised the study. Perez-Ortiz, Serrano-Oviedo, Lopez-Juarez and

Muñoz-Rodriguez contributed to monitoring of the study progress, supporting patient recruitment, data clarifications and data entry. All authors drafted the manuscript.

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APPENDIX 1

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