Inhaled aprotinin reduces viral load in mild-to-moderate inpatients with SARS-CoV-2 infection

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1 INTRODUCTION

In the fight against COVID-19, great steps have been taken to recover pre-pandemic socioeconomic activity. Despite this, the inevitable unsustainability over time of epidemiological norms and in a scenario of 'coexistence with the virus' produces the appearance of new infections that, although less serious, affect a significant percentage of persons. Within this population, it is important to highlight the one that is vulnerable to suffering a more severe COVID-19. There are many causes that can make an individual vulnerable to a more life-threatening form of COVID-19 and, therefore, require hospitalization (i.e. comorbidities, ageing or being at risk of social exclusion).

Redondo-Calvo FJ and Padín JF have contributed equally to the manuscript.

ATAC team members are listed in Appendix A.

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Therefore, vaccination as the single therapeutic measure is insufficient, and it is essential to develop antiviral drugs that help alleviate these problems.

In this sense, the recent introduction in antiviral therapy of the drugs molnupiravir (Lagevrio®), nirmatrelvir/ritonavir (Paxlovid®) and lopinavir/ritonavir (Kaletra®) has been viewed with great hope. However, these drugs have the handicap of interactions with other drugs, their economic cost, side effects, not acting on the inflammatory phase or not being effective in COVID-19 prophylaxis.1

A solution to these problems could be aprotinin, a broad-spectrum inhibitor of host proteases, such as the kinin–kallikrein system and fibrinogen.2 These proteases are used by the virus through a two-step mechanism to cleave the viral ‘spike’ protein and subsequently be able to recognize surface receptors on epithelial cells to infect.3

Recently, in a phase III clinical trial called ATAC (Aprotinin Treatment Against COVID-19), we showed that aprotinin is effective and safe to treat moderate COVID-19 by neutralization.4 This route of administration centres aprotinin’s action mainly to the lung, avoiding the appearance of systemic effects, which are common for other antiviral drugs used in COVID-19. Moreover, aprotinin has easy clinical handling, can be applied for infection prophylaxis and on an outpatient basis for mild cases and can be afforded by countries with low economic resources.

In addition, there is a great probability that contagion peaks will occur in a future scenario of the COVID-19 pandemic, increasing the risk of secondary infections and co-infections by other pathogens, which can trigger more severe infections.5 An example may be the coexistence with seasonal flu. The fact that aprotinin has a broad spectrum against respiratory viruses increases the interest of its clinical development.2 Thus, one of the most important proofs of concept demonstrating the efficacy of an antiviral is to measure its ability to lower viral load in patients. If reduced, it proves its potential in the prevention, progression and treatment of the most severe cases. Therefore, the objective of this work is to expand the information provided in the ATAC study, showing the viral load measurements by means of RT-qPCR in tracheobronchial samples from a group of patients with moderate COVID-19, before and after 5 days of standard treatment with aprotinin compared with standard treatment with placebo.

2 | PATIENTS AND METHODS

2.1 | Study design and participants

The samples of the current study were rescued from participants of a multicentre, double-blind, parallel-arm, randomized phase III trial, performed in four Spanish hospitals with the same inclusion criteria at baseline.4 The study included hospitalized patients with positive PCR for SARS-CoV-2 within 48 h prior to randomization and moderate COVID-19 pneumonia. Inclusion criteria were >18 years of age, radiographic signs of pulmonary infiltration, oxygen saturation >90% and oxygen therapy using nasal spectacles at 2–3 L/min. We compared 32 patients with standard treatment + placebo (physiological saline solution by inhalation) and 28 patients with standard treatment + aprotinin by inhalation (500 KIU every 6 h over 10 min until 2000 KIU/day) between 20 May 2020 and 20 October 2021. All patients gave their written informed consent for participation. The study was reviewed and approved by institutional ethics committees and the Spanish Drug Agency (AEMPS; reference number EudraCT 2020–002434–33).

2.2 | Follow-up

For the purpose of the present analysis, pre-existing co-morbidities, clinical and demographic information, concomitant medications during or before the study and respiratory and cardiovascular status were recorded at baseline and at 30 days of follow-up. All data were collected by the researchers through clinical interviews or from medical records, according to the usual clinical practice and without any additional procedure in relation to the study.

2.3 | Viral load procedures and treatment

Viral RNA was isolated from nasopharyngeal specimens using MagMAX Viral Pathogen II kit in a King Fisher Flex platform (both from Thermo Fisher Scientific, Lenexa, USA) with a 200 μl and 2-wash step protocol following manufacturer’s instructions. RT-qPCR was performed using TaqPath COVID-19 CE-IVD RT-PCR kit in a QuantStudio 5 Real-Time PCR system (Thermo Fisher Scientific). For quantification purposes, 10-fold dilutions from 10 to 10⁷ copies/ml of ddPCR quantified synthetic DNA were used as standards.

For inhalation therapy, the device used was a vibrating Mesh Nebulizer MicroAIR U100 (NE-U100-E; Omron®) with pipette, capable of generating mass median aerodynamic diameter particles of 2–5 μm, reaching distal alveolar areas. The vibrating mesh technology transforms the liquid drug into a fine vapour, with atomization into small particles avoiding dispersion and possible environmental contamination.
2.4 | Statistical analysis

Continuous variables were presented as mean with standard error of the mean (SEM) and compared by group using the Mann–Whitney U test after assessing normality with the Shapiro–Wilk test. Categorical variables were described using absolute frequencies and percentages and were compared by Fisher’s chi-square test. A p-value <0.05 was considered as statistically significant. Data were analysed using SPSS software version 28.0.

3 | RESULTS

Viral load was compared between placebo group and aprotinin-treated group at two time points. At the pre-time (i.e. day 0 before treatment), no significant differences were observed between groups. However, at post-time (i.e. treatment day 5), viral load levels were significantly lower in the aprotinin-treated group \( (p = 0.013, \text{Figure 1}) \).

Additionally, a comparative analysis was performed between the placebo group and the aprotinin group. A significantly shorter treatment time was observed in the aprotinin-treated group \( (p = 0.032) \), as well as a greater decrease in viral load \( (p = 0.016) \). The remaining variables showed no differences between both groups \( (\text{Table 1}) \).

4 | DISCUSSION

Recently, we presented 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. Based on that study, we concluded that inhaled aprotinin improved 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. Despite these promising results, a limitation of the ATAC study was that, due to the health situation at the time, we were unable to quantify SARS-CoV-2 viral load from all participants. However, we collected and preserved tracheobronchial samples from a subgroup of patients for further analysis. Now, we can show that aprotinin reduces viral load of patients with moderate COVID-19 as a proof of concept of its antiviral efficacy.

In a previous study, combination of intravenous and inhaled aprotinin with 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, the authors hypothesized that SARS-CoV-2 clearance was due to the effect of favipiravir, but the improved recovery from the infection was most likely due to the actions of aprotinin.

The effect of intravenous camostat mesylate, a TMPRSS2 protease inhibitor (one of the host proteases that aprotinin can also inhibit along with human trypsin, plasmin, fibrinogen, kallikrein, chemotrypsin, activated protein C, thrombin and neutrophil elastase) was reported to decrease SARS-CoV-2 viral entry into lung cells in vitro. However, in a clinical trial, there was no improvement in the clinical course, duration of disease, oxygen therapy or mortality. This is because camostat mesylate does not target the full spectrum of proteases used by the coronavirus, it loses efficacy and does not prevent thrombo-inflammation. In contrast, broad-spectrum host protease inhibitors such as aprotinin have shown greater in vitro efficacy in inhibiting SARS-CoV-2 activation, entry and replication in epithelial cell lines compared with the selective ones. In addition, aprotinin by inhibiting kallikreins regulates coagulation by affecting factor XII, plasminogen and PAR-1 thrombin receptors. The explanation for these actions is that SARS-CoV-2 capsid proteins cause activation

![Figure 1](image-url)
of kallikreins that trigger neutrophilia,\(^{11}\) participating in the formation of neutrophil extracellular networks and vascular microthrombus formation.\(^{12}\) Other anti-inflammatory actions of aprotinin include inhibition of (1) mediator release (e.g. interferon alpha); (2) granulocyte and monocyte adhesion molecule expression; (3) nitric oxide synthase; (4) tracheobronchial secretion; or (5) plasminogen preventing activation of complement proteins such as C3a and C5a.\(^7\) These mechanisms may account aprotinin for the viral load lowering in our patients and make it a candidate drug for treating SARS-CoV-2 infections.

Overall, aprotinin treatment was well tolerated in our clinical trial, and our findings may reflect immunomodulatory/anti-inflammatory effects that could be either secondary to the reduction in viral load or aprotinin-mediated and might participate in the rapid discharge and shorten treatment in patients who had aprotinin + standard of care.

### 5 CONCLUSION

Our study displayed the potential of aprotinin as a repurposed drug that can achieve therapeutic concentrations at the target site upon inhaled administration for the treatment of mild-to-moderate COVID-19 inpatients. Hence, it may also have a particular potential to prevent severe COVID-19 disease when applied early after diagnosis or even as a prophylactic drug in people likely exposed to contagion.

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### CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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### TABLE 1

Comparison between patients treated with placebo vs. patients treated with aprotinin

|                      | Placebo    | Aprotinin  | p-value |
|----------------------|------------|------------|---------|
| Age (years)          | 8          | 10         |         |
| Sex (woman)          | 8          | 10         |         |
| BMI (Kg/m²)          | 7          | 9          |         |
| Treatment time (days)| 8          | 10         |         |
| Admission time (days)| 8          | 10         |         |
| Viral load measurement (post-time)| 8          | 10         |         |
| Viral load (log₁₀ copies/ml) change| 8          | 10         |         |
| aTTP (s) change      | 7          | 9          |         |
| Leukocytes (10⁹/ml) change| 7          | 9          |         |
| Lymphocytes (10⁹/ml) change| 7          | 9          |         |
| C-reactive protein (mg/dl) change| 7          | 9          |         |
| Fibrinogen (mg/dl) change| 7          | 9          |         |
| Segmented (10⁵/ml) change| 7          | 9          |         |
| Platelets (10⁵/ml) change| 7          | 9          |         |
| D-dimer (ng/ml) change| 6          | 9          |         |
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APPENDIX A

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