Safety, tolerability, bioavailability, and biological activity of inhaled interferon-α2b in healthy adults: The IN2COVID phase 1 randomized trial

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Abstract

Aim: Interferons (IFNs) have been identified as a potential treatment alternative for Coronavirus Disease 19 (COVID-19). This study assessed the safety, tolerability, bioavailability, and biological activity of inhaled interferon-α2b (IFN-α2b) in healthy adults. Methods: A double-blind, randomized, phase 1 clinical trial was conducted with two cohorts of healthy subjects aged 18-50 years old. The first cohort received 2.5 MIU of inhaled IFN-α2b twice daily for 10 days (n=6) or placebo (n=3); the second cohort received 5.0 MIU of inhaled IFN-α2b in a similar scheme (n=6) or placebo (n=3). The first two doses were administered in an Emergency Department, then participants completed their treatment at home. Safety was measured through vital signs, new symptoms, and laboratory tests. Tolerability was measured as the participant´s treatment acceptability. Bioavailability and biological activity were measured from serum IFNα levels and real-time quantitative PCR of interferon-induced genes in blood before and after treatments. Results: Exposure to inhaled IFN-α2b at 2.5 MIU or 5 MIU doses did not produce significant changes in participant vital signs, or elicit new symptoms, and standard hematological and biochemical blood measurements were comparable to those recorded in individuals who received placebo. All adverse events were mild or moderate and did not require medical care. Participants reported very high tolerability. A dose-dependent mild increase in serum IFN-α concentrations and an increase in serum RNA expression of IFN-induced genes were observed after treatment. Conclusion: Inhaled IFN-α2b was safe, well-tolerated, and induced systemic biological activity in healthy subjects.

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Running title: Phase 1 trial of inhaled interferon-α2b

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The authors confirm that the principal investigators for this paper are Diego Garcia-Huidobro and Arturo Borzutzky and that they had direct clinical responsibility for patients.

Keywords: phase 1 trial, interferon, COVID-19

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What is already known about this subject:

- Whilst widespread vaccination has reduced the burden of COVID-19, the emergence of mutated viral strains that cause less severe disease has not ended the pandemic.
- Improving the immune response to SARS-CoV-2 infection is a therapeutic strategy for COVID-19.
- Interferon treatment for COVID-19 patients has been successful, however formal safety assessments have not been reported for all interferon types and administration routes.

What this study adds:

- Inhaled interferon-α2b is safe and well tolerated in healthy adults.
- Sustained use of inhaled interferon-α2b produces systemic effects that provide for an alternative administration route to treat medical conditions that require intramuscular or subcutaneous interferon treatment.

ABSTRACT

Aim: Interferons (IFNs) have been identified as a potential treatment alternative for Coronavirus Disease 19 (COVID-19). This study assessed the safety, tolerability, bioavailability, and biological activity of inhaled interferon-α2b (IFN-α2b) in healthy adults.

Methods: A double-blind, randomized, phase 1 clinical trial was conducted with two cohorts of healthy subjects aged 18-50 years old. The first cohort received 2.5 MIU of inhaled IFN-α2b twice-daily for 10 days (n=6) or placebo (n=3); the second cohort received 5.0 MIU of inhaled IFN-α2b in a similar scheme (n=6) or placebo (n=3). The first two doses were administered in an Emergency Department, then participants completed their treatment at home. Safety was measured through vital signs, new symptoms, and laboratory tests. Tolerability was measured as participants' treatment acceptability. Bioavailability and biological activity were measured from serum IFNα levels and real-time quantitative PCR of interferon-induced genes in blood before and after treatments.

Results: Exposure to inhaled IFN-α2b at 2.5 MIU or 5 MIU doses did not produce significant changes in participant vital signs, or elicit new symptoms, and standard hematological and biochemical blood measurements were comparable to those recorded in individuals who received placebo. All adverse events were mild or moderate and did not require medical care. Participants reported very high tolerability. A dose-dependent mild increase in serum IFN-α concentrations and an increase in serum RNA expression of IFN-induced genes were observed after treatment.

Conclusion: Inhaled IFN-α2b was safe, well-tolerated and induced systemic biological activity in healthy subjects.

INTRODUCTION

The Coronavirus Disease 19 (COVID-19) pandemic has been a major challenge for health systems worldwide.[1] Vaccination has effectively reduced disease incidence and severity,[2-3] but the emergence of new SARS-CoV-2 variants that are highly transmissible, despite causing less severe disease, continues to burden healthcare systems. To date, monoclonal antibody treatments with bebtelovimab,[4] tixagevimab/cilgavimab,[5] and sotrovimab,[6] and the antivirals nirmaterlvir and molnupiravir[7] have shown to reduce the risk of hospitalizations among patients with mild or moderate symptoms. Despite these positive developments, the evolution of the COVID-19 pandemic requires new treatment alternatives, especially for the early stages of disease. As the host immune response is critical for the clearance of virus, essential to blunt transmission, this is a key target for new therapy development.[8]

Interferons (IFNs) are naturally occurring cytokines exhibiting pleiotropic effects in response to viral infection, directly inhibiting viral replication and activating both the innate and adaptive immune responses.[9] Upon infection, viruses induce IFN-α/β production which stimulates cellular production of IFN-regulated proteins to inhibit viral replication and multiplication.[9] IFNs-α/β recruit and activate immune cell populations to sites of infection, resulting in viral elimination. All viruses, including SARS-CoV-2, encode in their genomes factors to inhibit an IFN response, blunting the natural host defense to viral invasion, via both passive and active mechanisms.[10] In particular, SARS-CoV-2 limits an IFN response mediated by non-structural proteins 3, 6 and 12, and open reading frames 7 and 9b.[11-13] Moreover, accumulating evidence has shown that genetic defects that affect the IFN response or the presence of anti-IFN antibodies, underlie a small but significant percentage of severe and/or fatal COVID cases.[14-16] Cognizant of the critical role IFNs have in clearing viral infections, treatment with exogenous IFN may override these inhibitory effects.

Despite considerable evidence that identifies IFNs as critical to the host response to viral infection, clinical use of IFNs has met with limited success. A recent narrative literature review identified 178 studies reporting the safe use of IFN-α in the treatment of COVID-19 patients.[17] Of these, 15 were clinical trials where IFN-α was administered by different routes: subcutaneous, inhaled, or as nasal drops. In general, most studies had positive outcomes, especially when used within five days of symptom onset, as late use was associated with clinical deterioration. However, another review, using a systematic approach, identified 11 randomized trials that examined the therapeutic benefits of IFN treatments, yet variability across trials confounded interpretation: participants with differing illness severity, the use of different IFN types, dosing differences, different routes of administration, and co-treatments.[18] In this context, about one half of the trials reported therapeutic benefit compared with control treatments, while other studies showed similar or worsening disease outcomes. A notable observation was that early treatment after symptom onset led to a greater likelihood of therapeutic benefit.

The adverse effects of IFN treatment when administered either by subcutaneous or intramuscular routes include fever, chills, generalized aches and pains, headache, anorexia, and fatigue.[19,20] Even though inhaled IFNs have been frequently used in some parts of the world, and Chinese National Guidelines recommend it as part of the standard COVID-19 treatment,[21] formal safety assessments have not been reported for all inhaled IFN types. Studies using nebulized IFN-β, IFN-λ, and IFN-κ have reported high treatment tolerability, no systemic effects, and the induction of antiviral biomarkers in participants' sputum.[22-26] However, to the best of our knowledge, no formal safety evaluations including bronchoconstriction assessments or acute allergic reactions to inhaled IFN-α2b have been reported. Thus, the purpose of this study is to report findings for the phase 1 trial evaluating the safety, tolerability, bioavailability, and systemic biological activity of inhaled IFN-α2b among healthy adults. We hypothesize that exogenous use of 2.5 and 5.0 MIU of IFN-α2b

twice per day for 10 days will be safe, tolerable, and will not induce systemic effects compared to placebo treatment in study participants.

METHODS

Study design

We conducted a prospective double-blind, placebo-controlled clinical trial in two consecutive cohorts (Figure 1). Each cohort included 9 subjects who were randomly allocated to inhaled IFN-α2b (AP-003, Altum Pharmaceuticals, Inc.) or placebo at a 2:1 ratio. In the first cohort, participants allocated to AP-003 received 2.5 MIU of AP-003 nebulized twice daily for 10 days. After receiving approval by the Data Safety Monitoring Board (DSMB), in the second cohort, participants allocated to AP-003 received 5.0 MIU of AP-003 nebulized twice daily for 10 days. Participants were followed for 11 days after randomization. All procedures were approved by the Pontificia Universidad Católica de Chile Institutional Review Board (IRB). The trial was registered in ClinicalTrials.gov (NCT04988217).

Setting

The study was conducted in Santiago, Chile. The first two treatment doses were administered at the Emergency Department of one of the hospitals of the Red de Salud UC Christus, the largest university healthcare network in the country. Remaining doses were self-administered at the participants' homes.

Participants and screening

Male subjects aged 18-50 years were invited to participate in the study. All were screened by a medical doctor at one of the hospitals of the Red de Salud UC Christus. The evaluation included a complete medical history, recording vital signs, a physical examination, complete blood count, blood urea nitrogen, creatinine, alanine and aspartate aminotransferases,

total and direct bilirubin, chest X-ray, electrocardiogram, a complete spirometry, and a SARS-CoV-2 Polymerase Chain Reaction (PCR) evaluation. Enrolled participants were required to be in a good state of health, determined by medical history, physical exam, and normal laboratory tests at screening, able to provide informed consent for participation and able and willing to comply with the study schedule and procedures. Participants were excluded if they had active SARS-CoV-2 infection, required continuous positive airway pressure (CPAP) for sleep apnea, had a pre-existing pulmonary disease or any serious acute concomitant illness that, in the opinion of the investigator, interfered with evaluation of safety of AP-003, or put the participant at risk of harm from study participation, were currently receiving an investigational agent, had participated in another study of an investigational agent within 30 days of enrollment, were legally incompetent and unable to understand the study's purpose, significance, and consequences, and make decisions accordingly, or if they had known hypersensitivity to IFNα or any component of the study drug or placebo.

Recruitment, Randomization, and Follow-up

Participants were recruited through flyers and posters. Once participants signed the informed consent document, and eligibility was confirmed, they were randomly assigned to treatment or placebo at a 2:1 ratio. Randomization was conducted centrally using QMinim (http://rct.mui.ac.ir/q/).[27] All participants received daily follow-up calls during the treatment administration. An in-person visit was scheduled after 10 days of treatment.

Interventions

Cohort A received 2.5 MIU (1mL) of AP-003 (IFNα2b) or placebo via nebulizer twice daily for 10 days. Cohort B received 5.0 MIU (2mL) of AP-003 or placebo using the same administration route and time. Placebo was identical to AP-003 with the exception that there was no active medication.

All participants received the first two doses under medical supervision at the Emergency Department of one of the hospitals of the UC Christus health network. Then, participants received a cooler box with treatment vials and a data logger to monitor their temperature. Participants were asked to store the medications inside their refrigerator, between 2 and 8°C, and 30 minutes before administration place the selected vial at room temperature. All treatments were administered using the PARI BOY® Classic nebulizer (PARI, Starnberg, Germany) through a face mask. Participants added 1 mL of NaCl 0.9% to the inhalation solution. The first treatment dose was administered by the research team. Then, participants were instructed on how to use the nebulizer by following an instruction manual and an educational video. The second dose was self-administered but supervised by the research team. Then, all treatment administrations were self-administered at the participant's home. Participants were required to report the time and duration of each treatment administration and clean the nebulizer as per the manufacturer's specifications.

All participants received 24/7 contact information of the study team and were instructed to report any new symptom. In addition, all participants were trained in how to recognize bronchospasm, and symptoms and signs of anaphylaxis. All participants received a standard epinephrine pen with administration instructions if needed and were instructed to attend the closest emergency department in case of severe anaphylaxis and then contact the research team to report the event.

Outcomes

The primary outcome was the safety and tolerability of AP-003. Safety was measured by subject incidence of treatment-emergent adverse events. Tolerability was assessed by participant´s report of treatment acceptability. Secondary outcomes included pharmacokinetics of nebulized AP-003 and the evaluation of IFN-induced blood biomarkers after treatment with AP-003.

Data Collection

Safety

As the first two treatment doses were administered at an emergency department, participants were continuously monitored by a medical doctor during and after each treatment administration. During this time, participants underwent serial assessments of heart rate (HR), blood pressure, temperature, respiratory rate (RR), pulse oxygen saturation (SpO_2), and peak expiratory flow (PEF). The MightySat® Rx Fingertip Pulse Oximeter (Masimo, CA) was used for SpO_2; and the Peak Expiratory Flow Mini-Wright® Standard (Clement Clarke, UK) for PEF. The participants were discharged by the medical doctor after two hours. Then, a trained clinician conducted a daily telemedicine participant assessment, evaluating storage conditions of the allocated treatment, treatment administration, vital signs, and any new symptoms. Before and after each treatment administration, participants recorded their vital signs including temperature, HR, RR, SpO_2, and PEF. In addition, participants completed, on a daily basis, a 23-item checklist to report any new symptom, possible side effect, and the use of any concomitant medication. In instances that a measured parameter was outside the normal range or participants experienced a new symptom, the investigators conducted a safety evaluation and decided if it was considered a clinically significant event. In this decision, the other measures, the overall condition of the participant, and the results of the previous assessments were considered. Adverse events (AEs) were classified according to their severity (mild, moderate, severe, and life threatening), serious (yes, no), the need for medical treatment (yes, no), the action taken regarding the treatment (none, treatment stopped, or treatment interrupted), outcome (resolved no sequelae, resolved with sequelae, and other) and their relationship to the treatment (not related, unlikely, possibly related, probably related, confirmed).

Laboratory safety was completed at screening and at the end of the 10-day treatment. Blood samples were taken for hematology and chemistry analyses. Local reference ranges

were used to determine whether laboratory values were within normal range. In instances that a measured parameter was outside the normal range, the investigators decided whether it was considered a clinically significant abnormality. In this decision, the other parameters, the overall condition of the participant and the results of the screening evaluation were considered.

Treatment tolerability

Tolerability was defined according to the most recent recommendations.[28] At the final assessment, participants responded to two questions assessing treatment tolerability using a 0-100 mm visual analogue scale (not tolerable at all-completely tolerable). The first question assessed participant´s acceptability towards a nebulized treatment, and the second one towards a twice daily 10-day nebulized treatment.

Biospecimen collection, processing, and storage

Peripheral blood (22-26 mL) was collected at baseline, 30, 60, 120 minutes, 12 hours, and 11 days by an experienced staff, kept on ice, and transported to the laboratory. Blood samples were centrifuged; serum and PAXGene (PreAnalytiX, BD Biosciences) RNA blood samples were frozen at -80°C.

Laboratory Processing

Bioanalytical methods and IFN-α serum concentrations

Hematological determinations were made using an automatic cell counter and biochemistry was conducted using standard methodologies within a single clinical laboratory. Serum concentrations of IFN-α were measured by ELISA, using VeriKine-HS Human IFN-α All Subtype ELISA Kit (#41115 PBL Assay Science, Piscataway, NJ), following the manufacturer's instructions. Plate readings were performed using a microplate reader (BioteK, Epoch), to determine the absorbance at 450nm.

IFN-α gene signature

Type I IFN signature gene analysis was determined by real-time quantitative PCR (RT-qPCR) from patients' whole blood collected in PAXgene Blood RNA tubes (PreAnalytiX, BD Biosciences). RNA was extracted from the PAXgene Blood RNA kit (Qiagen) according to the manufacturer's protocol. Total RNA was reverse-transcribed to complementary DNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-qPCR analysis was carried out on the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems) using TaqMan Gene Expression Assays for IFIT1, IFI27, ISG15, RSAD2, MX1, IFI44L, SIGLEC1 and INFB (Life Technologies).

Study Monitoring

A Contract Research Organization (CRO) oversaw the execution of the study. They conducted strict vigilance of the clinical trial ensuring compliance with good clinical practice and adherence to the approved study protocol.

An independent DSMB was convened twice to evaluate the participants' clinical and laboratory data after all participants in cohort A and cohort B completed the 11-day follow-up (Figure 1). The cohort B study was initiated only after obtaining written approval from the DSMB ensuring that the 2.5 MIU twice daily administration for 10 days was safe and tolerable.

Data Analysis

Data were analyzed using STATA v 14.2 following the recommendations for phase 1 trials.[29] Participants' health, demographic and tolerability data were summarized using descriptive statistics, including means, standard deviations (SD), and proportions. Within treatment arms Fisher's exact, Chi^2, and paired t-tests were used to compare pre and post change in vital signs with treatment administration. Fisher's exact, Chi^2, and independent sample t-tests were used to compare categorical and continuous data between participants in

each treatment group and placebo (AP-003 2.5 MIU vs placebo, and AP-003 5.0 MIU vs placebo). IFN gene signature data were normalized to the housekeeping gene GAPDH expression. Fold-change values were determined from normalized cycle threshold (CT) values from the IFN treatment cohort compared with normalized CT values from the placebo cohort, using the 2^-ΔΔCT method.[30] Figures were prepared using GraphPad Prism version 9.0.0 for macOS Monterrey 12.0.1 (GraphPad Software, San Diego, USA, www.graphpad.com). Resulting p-values < 0.05 were considered statistically significant.

RESULTS

A total of 20 participants enrolled in the study. Two participants were excluded at screening, one due to severe allergic rhinitis that could preclude correct inhalation of the nebulized drug, and one due to altered liver function tests on baseline laboratory evaluation. All randomized subjects completed the 11-day follow-up (Figure 1).

A description of randomized subjects by treatment allocation is presented in Table 1. Overall, participants were 28.6±10.3 years old (range 18.8 - 51.0), had a body mass index of 25.8±4.6 (range 19.8 - 37.6), and had baseline PEF values of 552±96 (range 350 - 720). There were no statistically significant differences in participant characteristics among treatment allocations.

Safety

Adverse events

All participants completed the observed administration of the first two treatment doses in the emergency department without adverse reactions. In total over the 11-day period, 58 AEs were observed (Table 2), including mildly decreased levels of SpO_2 (92-95%; n=13, 22.4%), mild bradycardia (n=10, 17.2%), mild tachycardia (n=6, 10.3%), rhinorrhea (n=6, 10.3%), mild tachypnea (n=5, 8.6%), headache (n=5, 8.6%), sore throat (n=5, 8.6%), epistaxis (n=2, 3.4%),

dizziness (n=2, 3.4%), mild hypotension (n=1, 1.7%), cough (n=1, 1.7%), nasal dryness (n=1, 1.7%), and loose stools (n=1, 1.7%). None of these AEs were serious AEs (SAEs) or required treatment discontinuation, and all resolved without sequelae. Twenty-nine AEs (50%) were possibly or likely to be related to treatment use, however no statistically significant differences between IFN-α2b treatment and placebo were observed in these assessments. No participant had allergic reactions to the nebulized treatment or used the epinephrine pen.

Vital signs

Vital signs before and after each nebulization by treatment arm are presented in Figure 2. Participants receiving AP-003 2.5 MIU presented a small but statistically significant change in SpO_2 and PEF (-0.5% p=0.001, and -14L/min, p<0.001, respectively) between before and after each nebulization, which was not observed with AP-003 5 MIU. No other statistically significant change in participants' physiology were observed before and after nebulization.

When comparing treatment groups, participants receiving AP-003 2.5 MIU had a statistically significant difference compared to participants receiving placebo on SpO_2 (-0.6%, p=0.001) and PEF (-15.5 L/min, p=0.007). There were no other statistically significant differences in vital signs between treatment groups as all other resulting p-values for independent sample t-test comparisons between AP-003 groups and placebo were not statistically significant.

The impact on participant vital signs over the trial is presented in Figure 3. Overall, participant PEF increased over time from 552 L/min at screening, to 643 L/min at day 11 (p<0.001). All other vital signs remained unchanged over time. All between-treatment group p-values for independent sample t-tests were non-significant, demonstrating that the study drug did not impact a participant's physiology over time.

Laboratory

Participant laboratory values at screening and after treatment are presented in Table 3. Participants in the placebo and AP-003 2.5 MIU groups, but not AP-003 5 MIU group, had a

statistically significant reduction in their hemoglobin levels (p=0.034 and p=0.045, respectively). There were no additional differences within groups (paired t-tests comparisons in laboratory values before and after complete), or between groups.

IFN Bioavailability

Figure 4 describes serum IFN-α concentrations over the study period by treatment arm. Paired t-tests for each treatment group reported that there were no statistically significant changes from baseline after 30, 60, 120 minutes and 12 hours after the first nebulization. After completing the treatment, participants allocated to the AP-003 5.0 MIU group exhibited a statistically significant increase in IFN-α concentration from baseline to Day 11 (7.46±5.15 IU increase, p=0.016). There were no statistically significant increases in serum IFN-α levels in participants allocated to AP-003 2.5 MIU (p=0.513).

There were no statistically significant differences in serum IFN-α concentration between AP-003 groups and placebo at 30, 60, 120 minutes and 12 hours after the first nebulization. After treatment, on Day 11, participants in the AP-003 5.0 MIU group had higher levels of circulating serum IFN-α (7.27±5.38 IU increase, p=0.006), which was not observed in the AP-003 2.5 MIU treatment group (p=0.302).

IFN Gene Signature

Figures 5 A-H present fold changes for IFIT1, IFI27, ISG15, RSAD2, MX1, IFI44L, SIGLEC1 and IFNB at baseline, 60, 120 minutes, 12 hours, and 11 days after randomization. For participants in both AP-003 groups, most genes had increased expression levels at Day 11 compared to placebo. Except for the expression of SIGLEC1 at 12 hours post treatment (p=0.044), there were no statistically significant differences in the gene signatures between allocation groups.

Treatment Tolerability

Participant ratings of treatment tolerability are reported in Figure 6. All participants reported high tolerability of the nebulized treatment, with no statistically significant difference between treatment groups and placebo (p=0.883 and p=0.220 for independent sample t-tests between AP-003 2.5 MIU and placebo, and AP-003 5.0 MIU and placebo, respectively). Using the treatment twice a day during 10-days revealed similar tolerability between groups (p=0.664 for both independent sample t-tests between AP-003 2.5 MIU and placebo and AP-003 5.0 MIU and placebo).

DISCUSSION

This study reports that inhaled IFN-α2b is safe and tolerable in healthy adult participants. Most AEs were mild, mainly transient changes in vital signs after participants received the nebulized treatment, that did not warrant treatment discontinuation. After review of safety data in cohorts A and B, the DSMB and the IRB agreed that all AEs were minor, approving the use of IFN-α2b at the dose of 5.0 MIU in a phase 2 trial with COVID-19 patients. A dose of 5.0 MIU is expected to have clinical impact on COVID-19 patients, as this dose is recommended in Chinese clinical guidelines[21] and findings from certain trials have used this dose with promising results.[31] The phase 2 double-blind, placebo-controlled, randomized clinical trial is currently underway in Chile, anticipating findings that will confirm the safety of this treatment and evaluate the therapeutic efficacy of AP-003 in COVID-19 patients.

Accumulating evidence identifies COVID-19 as a disease that is not restricted to the respiratory tract, but that SARS-CoV-2 infection of the respiratory tract may lead to gastrointestinal, cardiovascular, cardiac, neurological and immune system involvement.[32] Type I IFN receptors are ubiquitously expressed on all cell types, in contrast to type III, IFN-λ receptors, that have a more restricted expression. The data suggest that IFN-λ functions predominantly to restrict infection at anatomical barrier sites. Although both type I and type III IFNs exhibit broad spectrum antiviral activity, for an infection with the potential to involve

multiple organs and the immune system, type I IFNs – IFNs α/β – may have the greatest effects. Given the extensive experience with IFN-α2 as an antiviral, in contrast to the clinical application of IFN-β in multiple sclerosis, the use of IFN-α2 for viral infections seems more judicious.

Although unexpected, a dose-dependent effect of inhaled AP-003 was observed in IFN-α2b concentrations, that over time, produced systemic expression of IFN-induced genes. However, extent of systemic circulation of the inhaled IFNα2b was much smaller than the concentration observed with other administration routes.[33-35] The maximum concentrations obtained through intramuscular or subcutaneous routes were approximately 18 to 116 IU/mL and occurred 3 to 12 hours after administration. Serum concentrations with these routes of administration were undetectable after 16 hours of use. Even though, these observed circulating levels might not achieve a therapeutic level for hepatitis B and C, lymphoma, melanoma, for which the use of IFN-α2b injections have been approved,[33,36,37] this finding opens the possibility of a tolerable new administration route for various medical conditions, avoiding uncomfortable side effects such as flu-like symptoms, and injection site inflammatory reactions. Future studies will evaluate the safety, tolerability, and serum concentrations achieved with higher doses or prolonged treatment duration of nebulized AP-003.

There continues to be an urgent need to develop new treatments for COVID-19 and other emerging viral respiratory diseases. Even though there are several pathophysiological mechanisms by which IFN-α2b could enhance a patient´s immune response to SARS-CoV-2 infection, future trials need to evaluate the impact of AP-003 on COVID-19 clinical outcomes. This is an obvious limitation of all phase 1 clinical trials that include healthy participants, but a required first step towards new therapy development. Therefore, future studies in patients with COVID-19 need to be conducted. A second limitation relates to the location of treatment delivery. As treatments were administered at the homes of study participants, the research team could not oversee all study procedures. To minimize this risk, participants had logs to report the completion and accuracy of each procedure (e.g., refrigerated storage of the medication, proper

use, and cleaning of the nebulizer, etc.). Serum IFN dose-response findings and related IFN gene-signature profiles suggest that participants followed the study procedures and used their allocated treatment.

By enhancing the early immune response to SARS-CoV-2 infection, AP-003 may limit viral replication and interrupt the progression of the infection, potentially resulting in reduced hospitalizations, COVID-19-related death, and long-COVID. In addition, limiting viral replication, may impact viral shedding and, therefore, transmission in community settings. This study reports that AP-003 5.0 MIU nebulized twice-daily for 10 days is safe, tolerable, becomes bioavailable, and has a systemic biological effect, suggesting a potential therapeutic benefit for COVID-19 patients.

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AUTHOR CONTRIBUTIONS

All authors contributed to the study design and/or implementation. The study protocol was designed by Arturo Borzutzky, Carolina Iturriaga, Diego Garcia-Huidobro, Eleanor Fish, and José A. Castro-Rodríguez. Material preparation was performed by Carolina Iturriaga, Paula Fajuri, Marcela Urzúa, and Arturo Borzutzky. Participant recruitment and data collection procedures were supervised by Paula Fajuri and Marcela Urzúa. Laboratory samples and

analyses were processed by Guillermo Perez-Mateluna and Juan Pablo Fraga. Javiera de la Cruz and Cecilia Poli conducted the interferon gene signature analyses. Nicolas Severino oversaw the drug storage and administration. Data analyses were conducted by Diego Garcia-Huidobro, Javiera de la Cruz, and Arturo Borzutzky. The first draft of the manuscript was written by Diego Garcia-Huidobro and all authors commented and reviewed subsequent versions of the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

All authors have no conflicts of interest to disclose.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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Table 1: Baseline characteristics of healthy volunteers

	All	Placebo	AP-003 2.5 MIU	p-value	AP-003 5.0 MIU	p-value
Demographics						
Age, years (SD)	28.6 (10.3)	27.6 (11.0)	28.3 (10.1)	0.919	29.9 (11.4)	0.731
Race, Caucasian (%)	14 (77.8%)	5 (83%)	6 (100%)	1.0	3 (50%)	0.545
Country of birth, Chile (%)	18 (100%)	18 (100%)	18 (100%)	---	18 (100%)	---
Occupation, student (%)	9 (50%)	4 (66.7%)	3 (50%)	1.0	2 (33.3%)	0.567
Medical History						
Pre-existing medical conditions, %	0 (%)	0 (%)	0 (%)	---	0 (%)	---
Smoking status, %						
Current smoker	3 (16.7%)	1 (16.7%)	1 (16.7%)	1.0	1 (16.7%)	1.0
Past smoker	4 (26.7%)	2 (40.0%)	1 (20.0%)	1.0	1 (20%)	1.0
e-cigarette user	1 (5.6%)	0 (0%)	1 (16.7%)	1.0	0 (0%)	1.0
Past e-cigarette user	1 (5.9%)	0 (0%)	1 (20.0%)	0.455	0 (0%)	1.0
Current marihuana use, %	3 (16.7%)	2 (33.3%)	0 (0%)	0.455	1 (16.7%)	1.0
Physical exam						
Body Mass Index	25.8 (4.6)	26.6 (6.2)	25.7 (5.4)	0.811	25.2 (2.1)	0.614
Abnormal physical exam	1 (5.6%)	0 (0%)	1 (16.7%)	1.0	0 (0%)	1.0

Table 2: Adverse events (AEs) by treatment group

	All	**Placebo**	**AP-003 2.5 MIU**	**p-value***	**AP-003 5.0 MIU**	**p-value†**
Participants with AEs, No (%)	14 (77.8%)	6 (66.7%)	3 (33.3%)	0.35	5 (55.6%)	1.0
Any AEs, n	58	22	20		16	
Severity, Mild (%)	54 (93.1%)	21 (95.5%)	19 (95.0%)	1.0	14 (87.5%)	0.56
Required medical care, No (%)	0 (0%)	0 (0%)	(0%)	---	(0%)	---
Required medical treatment, No (%)	3 (5.2%)	0 (0%)	2 (10.0%)	0.22	1 (6.3%)	0.42
Action taken, Treatment discontinuation (%)	0 (0%)	0 (0%)	(0%)	---	(0%)	---
Outcome, Resolved no sequelae (%)	58 (100%)	22 (100%)	20 (100%)	---	16 (100%)	---
Relationship with AP-003, Possible or Likely (%)	29 (50.0%)	9 (40.9%)	13 (65.0%)	0.14	7 (43.8%)	1.0
Serious AE, No (%)	0 (0%)	0 (0%)	0 (0%)	---	0 (0%)	---

* AP-003 2.5 MIU vs. placebo, † AP-003 5.0 MIU vs. placebo

Table 3: Laboratory values before and after treatment by group

	Placebo			AP-003 2.5 MIU			AP-003 5.0 MIU		
	Before	**After**	**p-value**	**Before**	**After**	**p-value**	**Before**	**After**	**p-value**
Hematology									
Hematocrit, % (DS)	46.8 (2.5)	45.6 (1.8)	0.056	45.4 (1.7)	44.8 (1.7)	0.303	45.7 (1.3)	45.6 (2.1)	0.878
Hemoglobin, g/dL (DS)	16.2 (0.9)	15.6 (0.8)	0.031	15.7 (0.4)	15.4 (0.5)	0.045	15.6 (0.4)	15.3 (0.6)	0.124
Leukocyte count, 10^3/µL (DS)	6.1 (1.0)	6.0 (0.6)	0.757	6.3 (1.0)	6.1 (1.1)	0.578	7.2 (2.9)	6.9 (1.4)	0.711
Neutrophil count, 10^3/µL (DS)	3.4 (0.7)	3.1 (0.5)	0.047	3.6 (0.7)	3.5 (0.8)	0.671	4.0 (2.7)	4.1 (1.1)	0.924
Lymphocyte count, 10^3/µL (DS)	1.9 (0.3)	2.0 (0.4)	0.717	2.0 (0.6)	1.9 (0.4)	0.594	2.3 (0.9)	1.9 (0.5)	0.388
Eosinophil count, 10^3/µL (DS)	0.2 (0.1)	0.3 (0.3)	0.355	0.1 (0.1)	0.2 (0.1)	0.227	0.1 (0.1)	0.2 (0.1)	0.119
Platelet count, 10^3/µL (DS)	212 (46)	225 (38)	0.304	244 (52)	253 (52)	0.450	238 (40)	241 (38)	0.728
Erythrocyte Sedimentation Rate, mm/hr (DS)	4.0 (2.4)	4.0 (2.4)	1.0	3.0 (2.4)	5.0 (3.8)	0.394	6.2 (3.9)	4.5 (2.3)	0.267
Chemistry									
Blood urea nitrogen (BUN), mg/dL (DS)	13.3 (2.6)	13.5 (2.3)	0.872	17.0 (5.0)	16.2 (4.9)	0.363	13.5 (4.0)	14.2 (2.5)	0.516
Creatinine, mg/dL (DS)	1.0 (0.2)	1.0 (0.2)	0.956	1.0 (0.2)	0.9 (0.2)	0.022	1.0 (0.1)	1.0 (0.1)	1.0
Alanine aminotransferase (ALT), U/L (DS)	42.5 (35.4)	34.3 (21.1)	0.292	26.8 (14.3)	25.0 (14.7)	0.445	24.3 (10.7)	20.7 (10.6)	0.407
Aspartate aminotransferase (AST), U/L (DS)	33.8 (17.8)	27.3 (9.7)	0.221	24.7 (5.3)	22.5 (4.5)	0.071	26.2 (7.9)	22.3 (5.8)	0.204
Total bilirubin, mg/dL (DS)	0.7 (0.1)	0.7 (0.4)	0.975	0.6 (0.2)	0.6 (0.3)	0.656	0.8 (0.5)	0.7 (0.2)	0.742

Figure 1: Diagram of the phase 1 of the IN^{2}COVID trial.

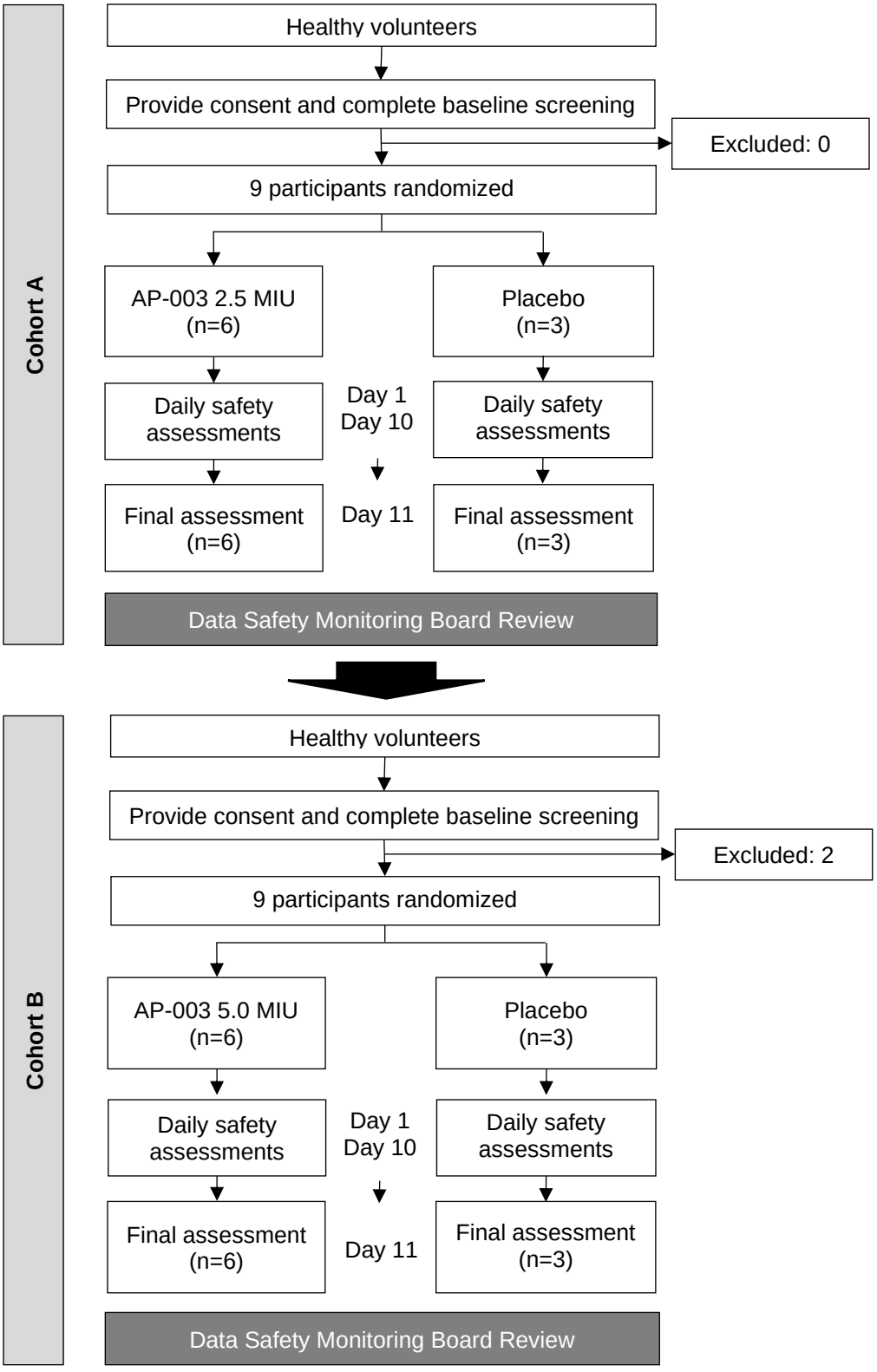


Figure 2: Vital signs before and after each nebulization, by treatment group. A. Heart rate; B. Respiratory rate; C. Pulse oxygen saturation; D. Temperature; E. Peak expiratory flow.
Note: *=p<0.05 between AP-003 2.5 MIU before and after nebulization, #=p<0.05 between AP-003 2.5 MIU and placebo groups.

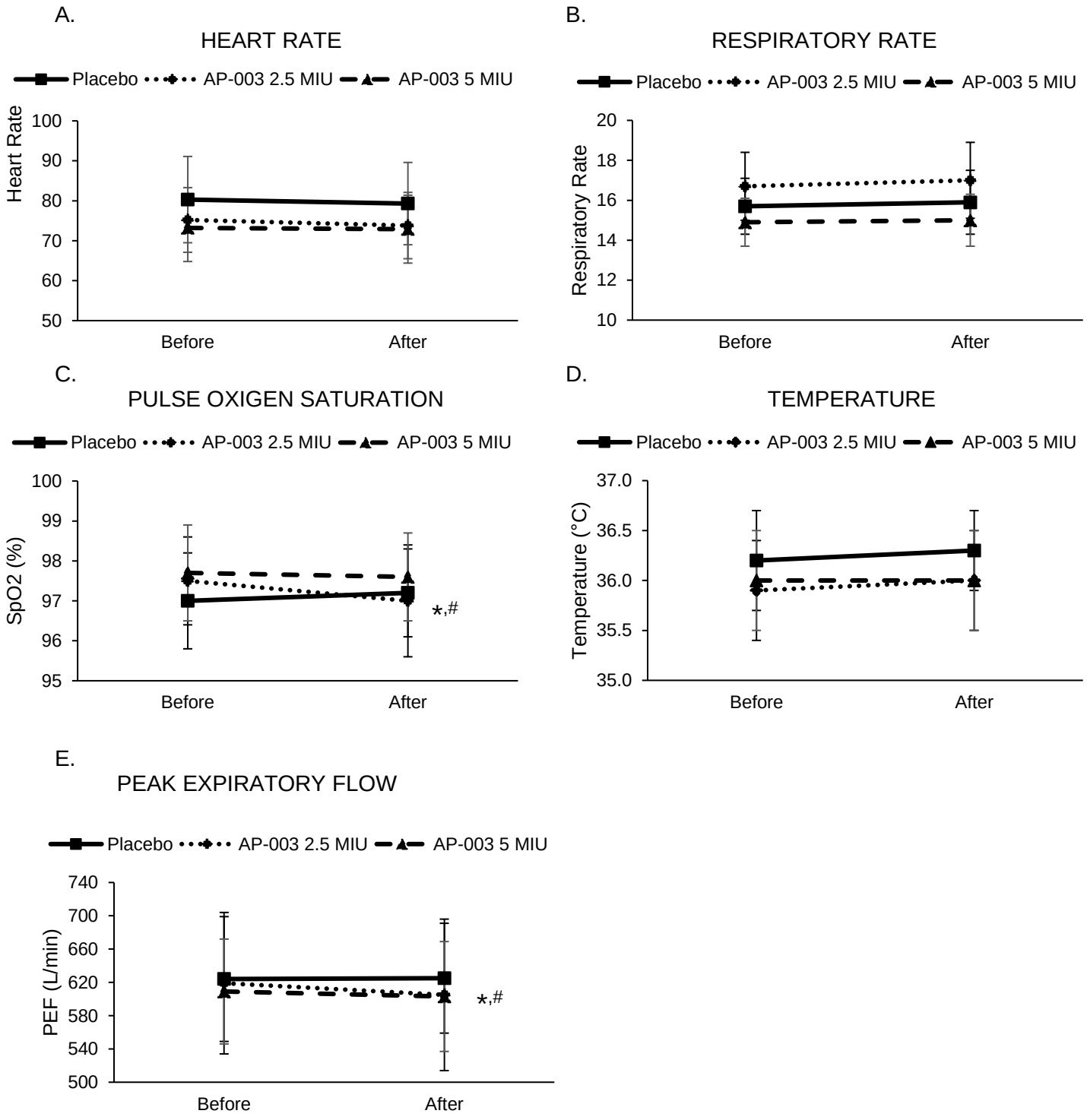


Figure 3: Vital signs over study period, by treatment group. A. Heart rate; B. Respiratory rate; C. Pulse oxygen saturation; D. Temperature; E. Peak expiratory flow.

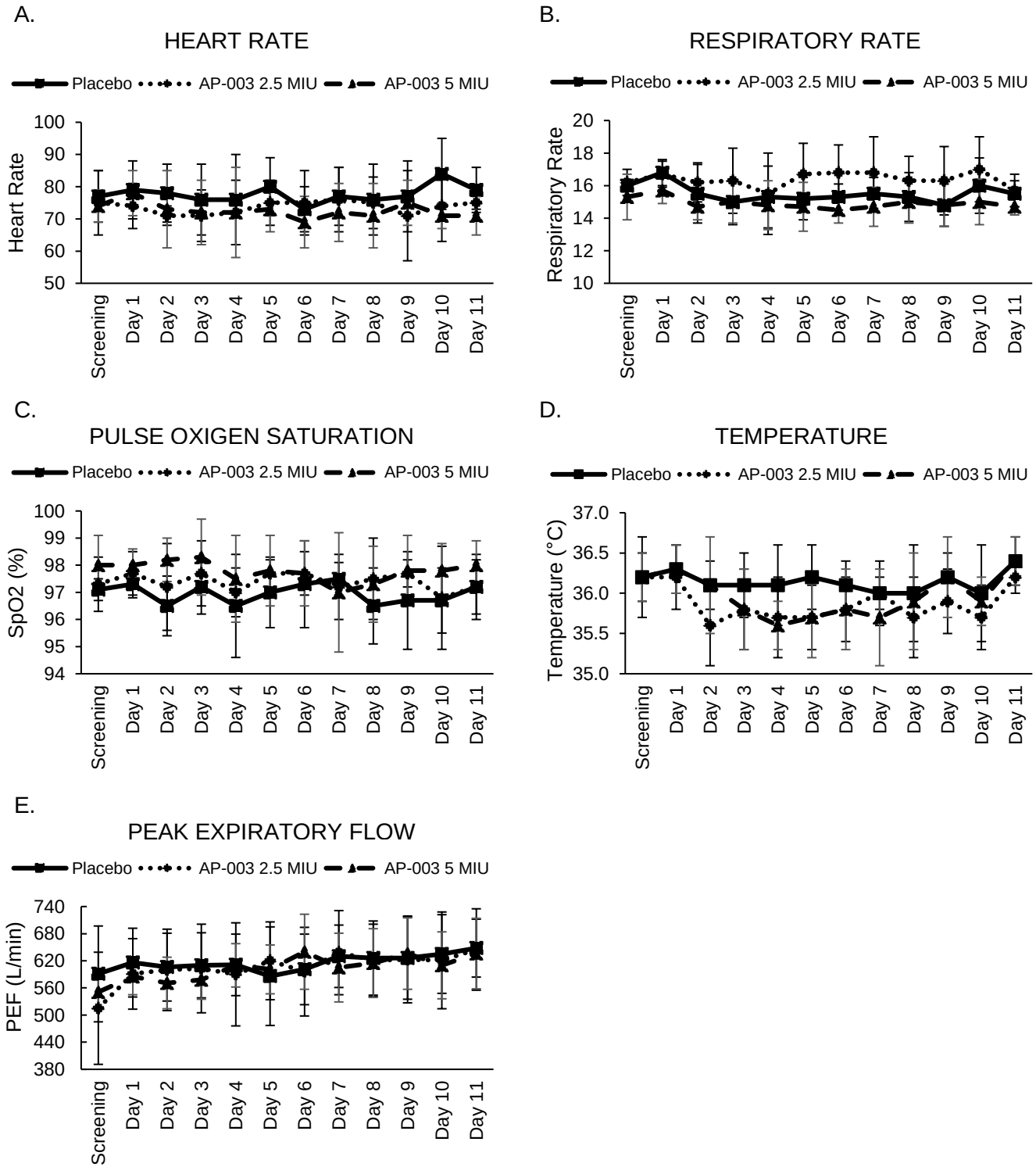


Figure 4: IFN-α serum concentration at baseline, 30, 60, 120 minutes, 12 hours, and 11 days from baseline.
Note: [&]=p<0.05 between AP-003 5.0 MIU at Day 11 and Baseline, [^]=p<0.05 between AP-003 5.0 MIU and placebo at Day 11.

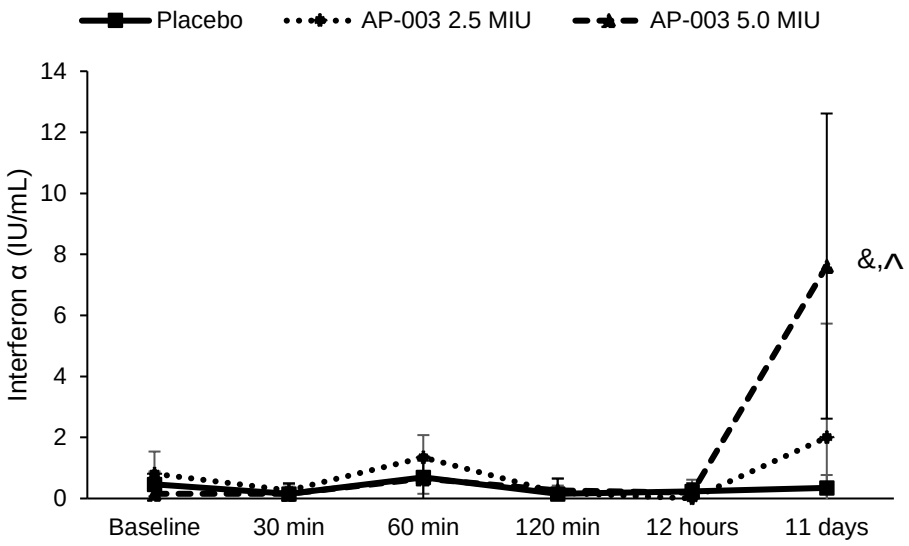
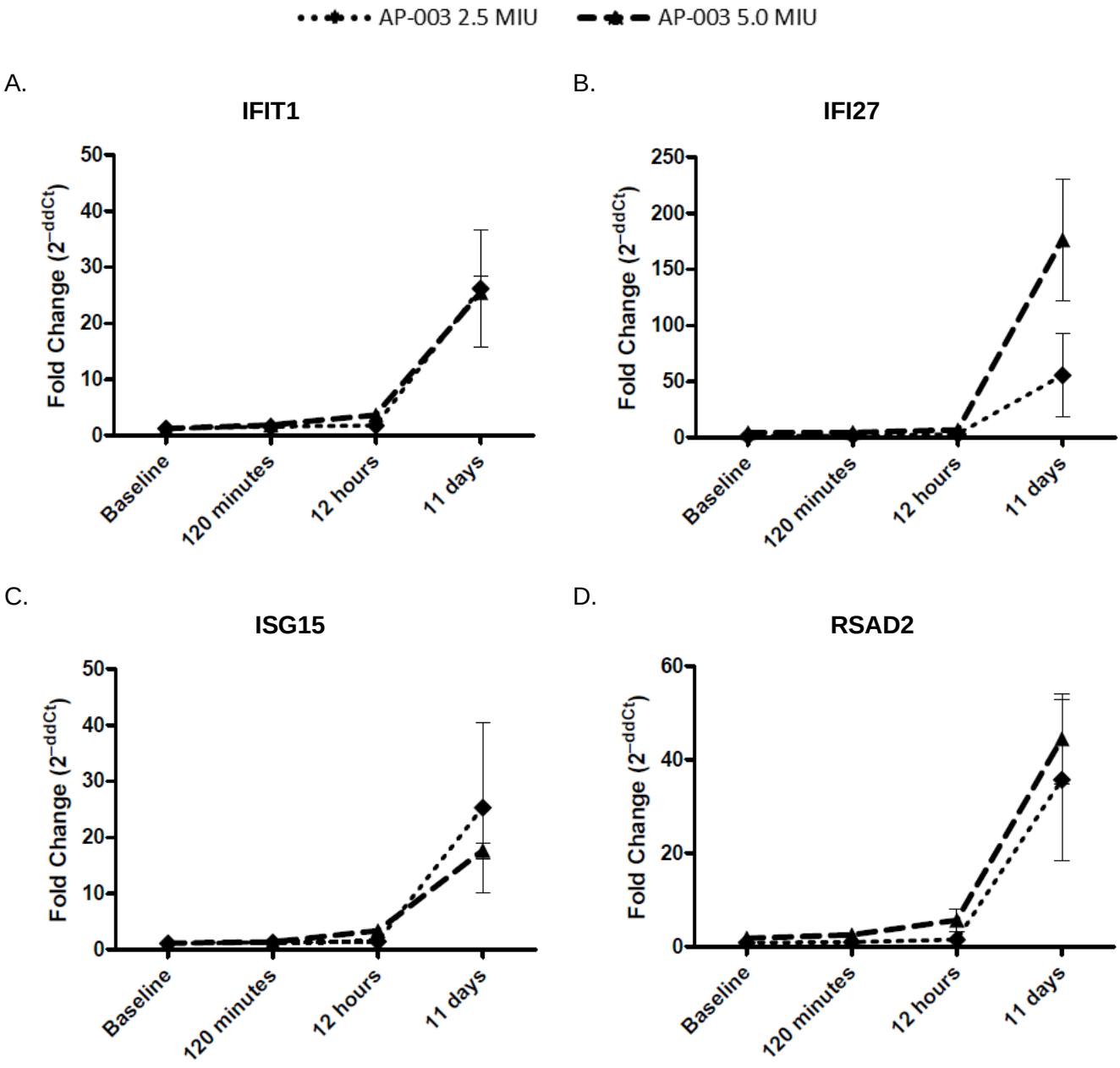
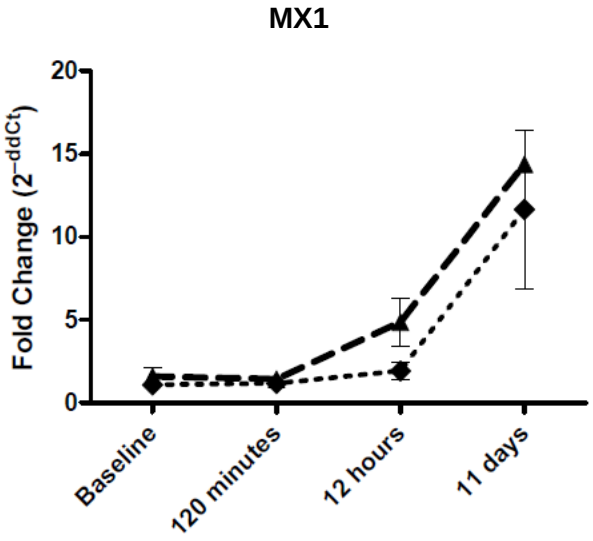


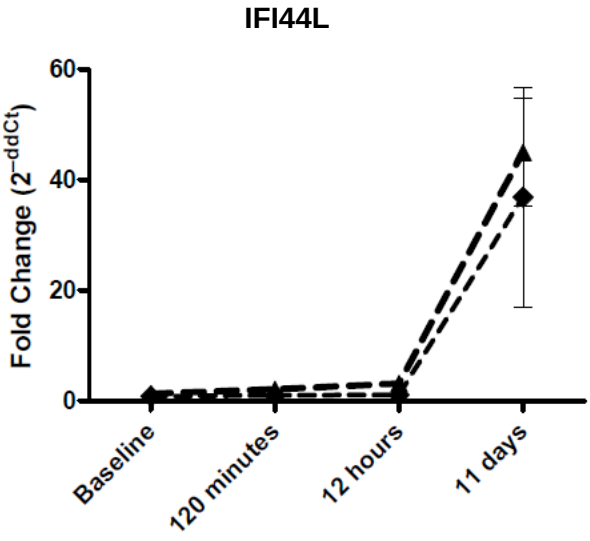
Figure 5: Fold change for gene signature at baseline, 120 minutes, 12 hours, and 11 days after randomization: A: Gene IFIT1; B: Gene IFI27; C: Gene ISG15; D: Gene RSAD2; E: Gene MX1; F: Gene IFI44L; G: Gene SIGLEC1; and H: Gene IFNB.
Note: ‡=p<0.05 between AP-003 2.5 MIU and AP-003 5.0 MIU.



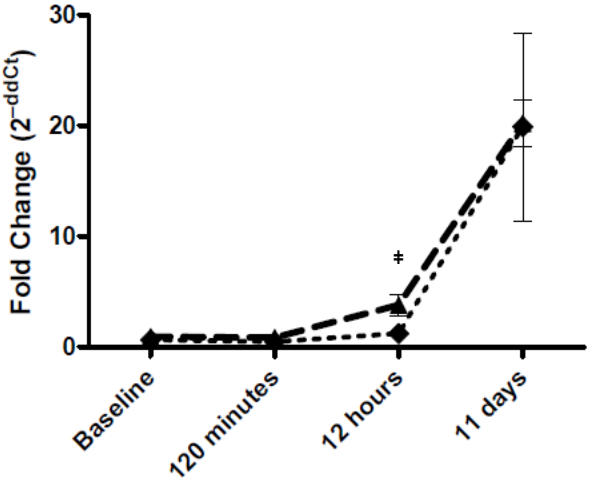
E.


MX1
Fold Change (2^{-ddCt})
20
15
10
5
0
Baseline
120 minutes
12 hours
11 days

F.


IFI44L
Fold Change (2^{-ddCt})
60
40
20
0
Baseline
120 minutes
12 hours
11 days

G.


SIGLEC1
Fold Change (2^{-ddCt})
30
20
10
0
‡
Baseline
120 minutes
12 hours
11 days

H.

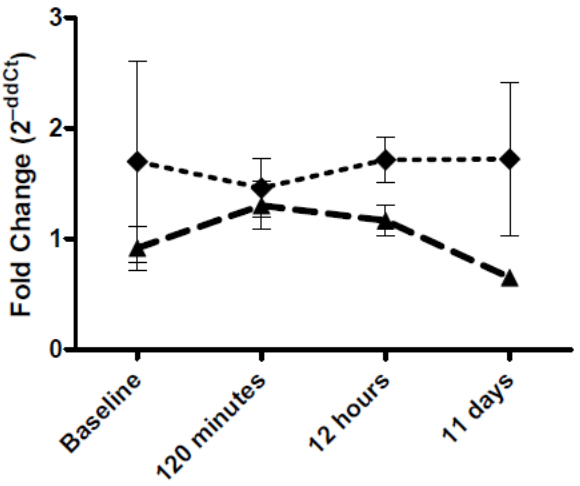
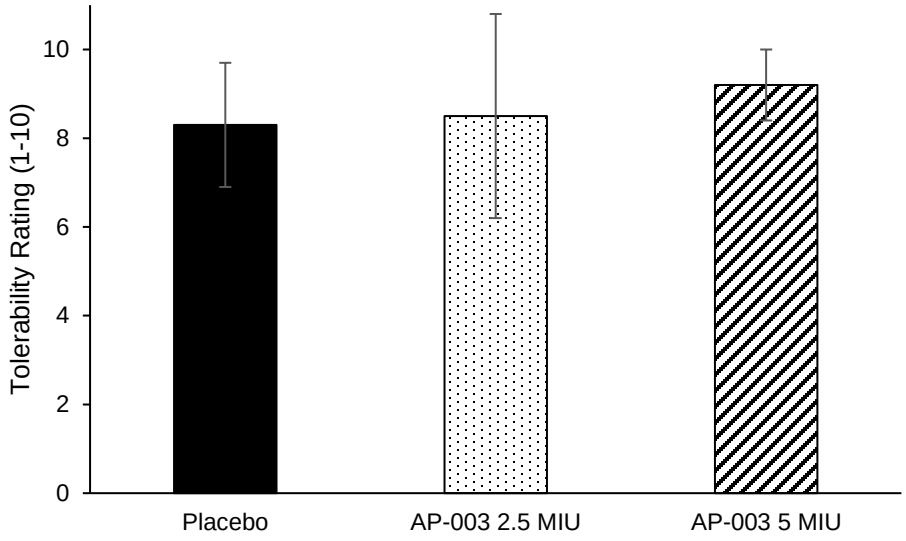

IFNB
Fold Change (2^{-ddCt})
3
2
1
0
Baseline
120 minutes
12 hours
11 days

Figure 6: Treatment tolerability by allocation group. A. Tolerability of receiving a nebulized treatment; B. Tolerability of a twice-daily 10-day nebulized treatment.

A.



B.