High-Dose Inhaled Nitric Oxide in Acute Hypoxemic Respiratory Failure due to COVID-19: A Multicenter Phase 2 Trial

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AJRCCM Articles in Press. Published September 29, 2023 as 10.1164/rccm.202304-0637OC

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AJRCCM Articles in Press. Published September 29, 2023 as 10.1164/rccm.202304-0637OC

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AJRCCM Articles in Press. Published September 29, 2023 as 10.1164/rccm.202304-0637OC

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Sources of support:

Each institution provided funding for costs related to the trial. At Massachusetts General Hospital, the study was supported by internal funds from the Department of Anesthesia, Critical Care and Pain Medicine and by a grant provided by iNO Therapeutics, LLC, a subsidiary of Mallinckrodt Pharmaceuticals. This research was also supported in part by the Massachusetts Consortium for Pathogen Readiness (Dr. Li) and the National Institutes of Health UM1AI069412 award (Dr. Li) for virology studies. At the University of Alabama at Birmingham, the study was supported by internal funds from the Department of Medicine. At the Beth Israel Deaconess Medical Center, the study was supported by funding from the Department of Anesthesia Critical Care and Pain Medicine, and the gas was provided by iNO Therapeutics, LLC, a subsidiary of Mallinckrodt Pharmaceuticals. At LSU Health Shreveport, the study was supported by internal funds from the Department of Medicine. The part of the study conducted at Danderyd Hospital in

AJRCCM Articles in Press. Published September 29, 2023 as 10.1164/rccm.202304-0637OC

Stockholm, Sweden, was supported by Linde, who provided the nitric oxide and its delivery system, the INOmax. The funding source at each site had no role in the design, analysis, or decision to publish.

Running Head: High-dose iNO in respiratory failure

Descriptor number: 4.4 Clinical Trials in Critical Care Medicine

Total word count of the body of the manuscript: 3977 words. Abstract 250 words

"At a Glance Commentary": 198 words

Scientific Knowledge on the Subject: Prior clinical trials have shown that low-dose, 1 to 20 ppm of inhaled nitric oxide (NO) leads to short-term improvement in oxygenation in critically ill patients with acute lung injury. During the severe acute respiratory syndrome (SARS) outbreak of 2003, low-dose inhaled NO was shown to improve oxygenation. Subsequent laboratory studies have demonstrated that NO inhibited the in-vitro replication of SARS-CoV-2 in a dose-dependent manner. This phase II, multicenter, single-blind, randomized, controlled, parallel-arm trial hypothesized that high-dose (up to 80 ppm) NO would inhibit viral replication and cause sustained improvement in oxygenation in COVID-19 patients with acute hypoxemic respiratory failure.

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What This Study Adds to the Field: Compared with usual care, inhaled NO improved oxygenation at 48 hours. Administration of inhaled NO did not reduce mortality, length of mechanical ventilation, or duration of hospital stay. Participants treated with NO experienced a faster reduction of viral load in sputum and blood samples and had a reduced rate of sensory and motor neurologic symptoms. Finally, treatment with NO was well tolerated, and no serious adverse events were recorded. Further studies are required to characterize the antiviral properties of high-dose NO and determine the optimal dosage.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

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Abstract

Rationale and Objectives: The effects of high-dose inhaled nitric oxide on hypoxemia in COVID-19 acute respiratory failure are unknown.

Methods: Mechanically ventilated adults with COVID-19 pneumonia were enrolled in a phase II, multicenter, single-blind, randomized, controlled, parallel-arm trial. Participants in the intervention arm received inhaled nitric oxide at 80 parts-per-million (ppm) for 48h, compared with the control group receiving usual care (without placebo). The primary outcome was the change in arterial oxygenation (PaO_2/FiO_2) at 48h. The secondary outcomes included: time to reach a PaO_2/FiO_2>300 mmHg for at least 24h, the proportion of participants with a PaO_2/FiO_2>300 mmHg at 28 days, and survival at 28- and at 90-days.

Measurements and Main Results: 193 participants were included in the modified intention-to-treat analysis. The mean change in PaO_2/FiO_2 ratio at 48h was 28.3 mmHg in the intervention group and -1.4 mmHg in the control group (mean difference: 39.1 mmHg (95%CrI:18.1-60.3). The mean time to reach a PaO_2/FiO_2>300 mmHg in the interventional group was 8.7 days compared to 8.4 days for the control group (mean difference:0.44(95%CrI:-3.63 to 4.53)). At 28 days, the proportion of participants attaining a PaO_2/FiO_2>300 mmHg was 27.7% in the inhaled nitric oxide group and 17.2% in the controls (RR:2.03(95%CrI:1.11 to 3.86)). Duration of ventilation and mortality at 28 and 90 days did not differ. No serious adverse events were reported.

Conclusions: The use of high-dose inhaled nitric oxide resulted in an improvement of PaO_2/FiO_2 at 48h compared with usual care in adults with acute hypoxemic respiratory failure due to COVID-19.

AJRCCM Articles in Press. Published September 29, 2023 as 10.1164/rccm.202304-0637OC

Abstract word count: 250

Key words: (I) Respiration, Artificial; (II) Pneumonia; (III) Critical Illness; (IV) viremia; (V) COVID-19

AJRCCM Articles in Press. Published September 29, 2023 as 10.1164/rccm.202304-0637OC

Introduction

Inhaled nitric oxide (NO), a selective pulmonary vasodilator, was first approved by the United States Food and Drug Administration (FDA) in 1999 for the delivery of 20 parts per million (ppm) in newborns with hypoxemic respiratory failure with persistent pulmonary hypertension (1-3). Subsequently, the use of inhaled NO therapy was expanded to critically ill adult patients with hypoxemic respiratory failure and to postoperative cardiac patients (4, 5). The beneficial effects of inhaled NO therapy have been attributed to its ability to reduce intrapulmonary shunting (6), resulting in improved oxygenation for the first 24 hours of inhalation in mechanically-ventilated adult patients with severe acute respiratory distress syndrome (ARDS) (7-10). Despite its well-defined physiological effects and excellent safety profile, inhaled NO up to 20 ppm did not demonstrate efficacy in improving clinical outcomes among adults with ARDS in prior randomized trials (7, 10-13).

Numerous in vitro studies have shown that nitric oxide in solution has dose-dependent bactericidal properties (14, 15) and inhibits viral replication.(16, 17) Prior studies utilized low doses of inhaled NO to facilitate pulmonary vasodilation and improve oxygenation.(18) While the antiviral dose of inhaled NO has not been established, early application of high-dose inhaled nitric oxide (up to 300 ppm) has been shown to sustainably improve systemic oxygenation in non-intubated hospitalized adults and decrease the length of hospitalization in pregnant and pediatric patients with viral and bacterial pneumonia.(19, 20) However, the role of high-antiviral doses of inhaled NO in improving systemic oxygenation has not been assessed in critically ill COVID-19 patients requiring mechanical ventilation.

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Based on mounting evidence (14, 21-28), this study tested the hypothesis that a high concentration of inhaled NO administered early after the onset of infection, beyond what had been previously evaluated, might be beneficial in critically ill patients with acute hypoxemic respiratory failure due to coronavirus disease (COVID-19) pneumonia. This study was designed to evaluate the effect of inhaled NO on systemic oxygenation after 48 hours among critically ill and mechanically ventilated patients with COVID-19 in a phase II, multicenter, single-blind, randomized (1:1), controlled, parallel-arm trial.

Methods

Study Design and Participants

This was an investigator-initiated multicenter, single-blind, randomized (1:1), controlled, parallel-arm clinical trial conducted at four sites in the United States and one site in Sweden. The study enrolled adult patients with confirmed SARS-CoV-2 infection (using RT-PCR) admitted to the intensive care units who were intubated and mechanically ventilated. Detailed information on the study protocol, inclusion and exclusion criteria, randomization, masking, and consent procedures are available in the **Online Data Supplement**. This study was registered on ClinicalTrials.gov as NCT04306393 (Registered on March 12th, 2020). **Figure 1** describes patient enrollment and follow-up as per CONSORT recommendations.

Procedures

Participants in the treatment arm received inhaled NO at 80 ppm for the first 48 hours after enrollment. The gas was started immediately after randomization within the first 72 hours of mechanical ventilation. After the first 48 hours of treatment, the gas was reduced to 40 ppm and

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maintained at this concentration until severe hypoxemia resolved (PaO_2/FiO_2 >300 mmHg). The procedures for inhaled NO administration and weaning are described in the **Online Data Supplement.**

Outcomes

The primary outcome of this study was the change in arterial oxygenation (PaO_2/FiO_2) at 48 hours.

The secondary outcomes were all-cause mortality at 28 and 90 days, time to reach PaO_2/FiO_2 ratio above 300 mm Hg for at least 24 hours, and the proportion of participants attaining a PaO_2/FiO_2 ratio above 300 mm Hg in the two groups at 28 days. The safety outcomes for this clinical trial included methemoglobinemia defined as methemoglobin (MetHb) exceeding 5%, inhaled nitrogen dioxide >3 ppm, hemodynamic instability (rebound hypotension) during weaning, the occurrence of acute kidney injury by 28 days, or the initiation of renal replacement therapy by 90 days. Exploratory study outcomes included change in viral load (Log_{10} copies of SARS-CoV-2 RNA per mL) in plasma and sputum, duration of mechanical ventilation, use of venous-venous extracorporeal membrane oxygenator (VV-ECMO), and neurological signs and symptoms (motor and sensory) at 90 days. The 90-day follow-up procedures and the preparation of plasma and sputum samples for measurement are described in the **Online Data Supplement**. To describe oxygenation beyond PaO_2/FiO_2 ratio, saturation of oxygen (SaO_2), alveolar-arterial oxygenation gradient, and ventilatory ratio were analyzed and presented as exploratory outcomes.

Statistical Analysis

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Participants randomized to inhaled NO were hypothesized to have at least 20% greater improvement in PaO_2/FiO_2 at 48 hours after gas initiation compared with the usual care alone (29). Assuming a two-tailed alpha of 0.05, the enrollment of 182 participants would provide 90% power to detect an effect size of 38 mmHg PaO_2/FiO_2 change based on the effect estimates in a previous investigation in hypoxemic intubated and mechanically ventilated patients. Presuming a 10% dropout, the target sample size was 100 in each group (n=200 total). The target sample size was 100 in each group (n=200 total).

The baseline characteristics were summarized as the median and interquartile range for continuous data and counts and percentages for categorical data. Standardized mean difference (SMD) is reported to quantify the differences between the two study arms, with values greater than 0.20 suggesting a potential imbalance between groups.

The primary and secondary outcomes analysis was conducted using a Bayesian framework that estimates the treatment effect conditional on pre-specified variables defined a priori (age, age^2, sex, BMI, and APACHE II score). Additionally, a sensitivity analysis was conducted including the pre-specified covariates and variables with SMD>0.20 (race, study site, hypertension, diabetes, malignancy, and liver disease). (**Data Supplement Table E1**) To assess the primary outcome, the PaO_2/FiO_2 ratio at 48 hours was regressed on baseline PaO_2/FiO_2 ratio, randomized group assignment, and additional covariates, as specified above. Time to reach PaO_2/FiO_2 ratio above 300 mm Hg was evaluated using the Kaplan-Meier method. All study outcomes were analyzed in the modified intention-to-treat (mITT) population. All statistical analyses were performed using R 4.0.2 (R Core Team) with Bayesian estimation conducted in RStan. The

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statistical analysis and the detailed statistical analysis plan are described in the **Online Data Supplement**.

Results

Patient Characteristics

The study enrolled 200 participants with respiratory failure due to SARS-CoV-2 between March 2020 and May 2022 (**Figure 1**). Subject recruitment occurred between March 22nd, 2020, and May 21st, 2021, with the final follow-up on June 15th, 2022. The primary mITT analysis included 193 participants who met inclusion criteria and did not meet exclusion criteria. The study cohort had a median age of 62 (IQR 50–70) years and included 33.7% females; 51.8% identified as White and 29.5% as Hispanic. Baseline clinical and demographic characteristics were balanced between the study arms except for (SMD >0.20) APACHE II score, hypertension, diabetes, malignancy, liver disease, connective tissue disease, smoking history, race, and creatinine (**Table 1**). The baseline PaO_2/FiO_2 ratio was 177 [125, 241] mm Hg in the treatment arm and 195 [120, 235] mm Hg in the control arm. Ventilator settings and adjunctive therapies are presented in **Table 2** and **Table E2,** respectively. On December 2nd, 2020, the DSMB noted a difference in the primary outcome of the study between the two groups. However, the stopping rule (defined as a significant increase in mortality in the NO group) was not met. Thus, the trial continued to complete enrollment.

Primary Outcome

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The mean change in PaO_2/FiO_2 at 48 hours in the inhaled NO arm was 28.3 (89.3) mm Hg and -1.4 (68.9) mm Hg in the usual care arm. The change in PaO_2/FiO_2 from baseline to 48 hours was 39.1 (95% CrI: 18.1, 60.3) higher in the treatment arm compared with the usual care arm (**Table 3 and Figure 2**). Inhaled NO therapy had a 99.5% probability of increasing the PaO_2/FiO_2 at 48 hours. (**Figure 3**). The probability of inhaled NO therapy improving the PaO_2/FiO_2 at 48 hours using various thresholds has been described in **Table E3**.

Secondary Outcomes

The mean time to reach a sustained PaO_2/FiO_2 ratio above 300 mm Hg in survivors was 8.7 (5.0) days in the treatment group versus 8.4 (6.5) days in the usual care arm. Among survivors, the probability that inhaled NO therapy would decrease the time to PaO_2/FiO_2 ratio above 300 mm Hg was 42.9% compared with the usual care arm (mean difference: 0.44; 95% CrI -3.63–4.53). At 28 days, the overall proportion of participants with a PaO_2/FiO_2 ratio above 300 mm Hg was 27.7% in the inhaled NO group and 17.2% in the controls, respectively. There was a 98.1% probability that inhaled NO therapy would increase the chance of attaining a PaO_2/FiO_2 ratio above 300 mm Hg (Risk Ratio [RR] 2.03; 95% CrI 1.11–3.86; **Table 3** and **Figure E1** of the **Online Data Supplement**). In the inhaled NO arm, the proportion of deaths within 28 days and 90 days was 28.7% and 34.0%, respectively. In the usual care arm, the proportion of deaths within 28 days and 90 days was 27.3% and 32.3%, respectively. Participants randomized to the inhaled NO group had a 71.9% and 71.4% probability of having a lower risk of death at 28 days and 90 days, respectively, compared to usual care alone. [RR for 28 days mortality: 0.85; 95% CrI 0.50–1.46); RR for 90 days mortality: 0.87; 95% CrI 0.52–1.43].

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The posterior probability curves of the secondary outcomes with inhaled NO therapy have been depicted in **Figure 3.**

Safety and Adverse Outcomes

High-dose inhaled NO therapy was well tolerated, with no serious adverse events related to inhaled NO reported (**Table 4**). The median duration of inhaled NO therapy of ≥ 20 ppm was 10.8 days (IQR 5.1–16.3). MetHb exceeded the threshold of 5% eight times during the 1,282 inhaled NO-treatment days. In 5 of these events, a dose reduction of inhaled NO by 50% was required to achieve an appropriate reduction in MetHb under 5%. The inhaled nitrogen dioxide reached 3 ppm on one occasion and rapidly decreased upon reduction of inhaled NO from 80 ppm to 40 ppm. No events of hemodynamic instability or rebound pulmonary hypertension were reported during the inhaled NO treatment and subsequent weaning of inhaled NO. Nitric oxide did not increase the risk for acute kidney injury (RR 0.82; 95% CrI 0.39–1.70) or the need for renal replacement therapy (RR 1.65; 95% CrI 0.78–3.56).

Exploratory Outcomes

For quantitative SARS-CoV-2 viral load testing, plasma was collected serially for two weeks from 37 patients (17 in the treatment group and 20 in the control group) for a total of 145 samples (68 from participants enrolled in the treatment group and 77 from participants enrolled in the control group). Patient characteristics are listed in the **Online Data Supplement Table E4.** Sputum was collected for up to seven weeks from 37 participants (17 in the treatment group and 20 in the control group) for a total of 82 samples (38 from participants in the treatment group and 44 from participants in the control group). The median viral loads in the first plasma samples

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obtained after randomization did not differ between study arms: 2.6 $\log_{10}$ RNA copies/mL (IQR 2.2–3.4) in the treatment group vs. 2.8 $\log_{10}$ RNA copies/mL (IQR 1.8–3.7) in the control group. Similarly, the median viral loads when comparing the first sputum samples obtained after randomization was similar in the study groups: 7.6 $\log_{10}$ RNA copies/mL (IQR 6.0–9.0) in the treatment group vs. 6.9 $\log_{10}$ RNA copies/mL (IQR 5.9–8.0) in the control group. Over time, there was a steeper decline in plasma viral load (Change per unit time:-0.21, 95% CrI: -0.25 – -0.17; Group differences:-0.30, 95% CrI: -1.00–0.42) in patients enrolled in the inhaled NO arm compared with those in the control arm (Time x Group Estimate:-0.04, 95% CrI: -0.12 – 0.04; **Figure 4**). Similarly, among the subset of patients from whom sputum samples were taken, there was a greater decline in viral load over time (Change per unit time:-0.13, 95% CrI: -0.16 – -0.11; Group differences: 0.29, 95% CrI: -0.87 – 1.44) in the treatment arm compared with the control arm (Time x Group Estimate:-0.04, 95% CrI: -0.09 – 0.01).

In the exploratory analysis, while the duration of mechanical ventilation and the use of VV-ECMO were not different between the two groups, the frequency of neurological signs and symptoms in the inhaled NO group at 90 days was lower compared with the usual care group, 4.2% and 17.2% respectively (RR 0.17(95%CrI:0.04 to 0.62); **Table 5**). Compared to usual care, participants in the treatment group with inhaled NO demonstrated fewer sensory symptoms (0% vs. 14.1%, RR 0.01; 95% CrI 0.00–0.12; **Table 5**). Detailed motor and sensory findings from notes by a physician caring for the patient are listed in **Table E5 (Online Data Supplement).** Change in the SaO_2, alveolar-arterial oxygenation gradient, and ventilatory index at 48 hours with inhaled NO therapy are presented in **Table 5** and a subgroup analysis stratified for PaO_2/FiO_2 is presented in Table E6.

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Discussion

This investigator-initiated, phase II, multicenter, single-blind, randomized, controlled, parallel-arm trial showed that high-dose inhaled NO improved systemic oxygenation in mechanically ventilated critically ill participants with acute hypoxemic respiratory failure due to COVID-19 pneumonia. The median PaO_2/FiO_2 ratio increased from 177 (125, 241) mm Hg to 200 (157, 239) mm Hg in the treatment but decreased from 195 (120, 235) mm Hg to 183 (122, 235) mm Hg in the control arm. Compared with the usual care group, a larger proportion of participants in the inhaled NO group reached PaO_2/FiO_2 >300 mmHg for at least 24 hours at 28 days, but the time to attain the level of oxygenation was similar. Further, while there was no difference in mortality or other exploratory clinical outcomes, participants who received inhaled NO had a lower occurrence of sensory symptoms than those who received usual care alone 90 days post-randomization.

Prior evidence from a meta-analysis combining 4 RCTs demonstrated that inhaled NO therapy in ARDS patients was associated with an increased risk of AKI (30). However, this large contemporary RCT of critically ill mechanically ventilated ARDS patients showed that the incidence of AKI was high but similar in both arms of the study. Thus, we cannot conclude on whether NO reduce or increase the risk of AKI and reduce or increase the need for kidney replacement therapy. The high incidence of AKI may be secondary to ARDS and COVID-19 infection. The increased risk of AKI due to COVID-19 has been attributed to direct cytotoxicity, microvascular thrombosis, and endothelial dysfunction.(31) Inhaled NO therapy was not associated with an increased risk of any adverse events, including AKI and the need for RRT.

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However, the present trial does not eliminate the possibility that NO therapy could be potentially nephrotoxic due to the relatively small number of participants. Future larger trials are needed to evaluate the renal toxicity of high-dose of early administration of inhaled NO. All participants in the treatment group tolerated the administration and weaning of inhaled NO. Though there were eight events of MetHb exceeding 5% and one with inhaled nitrogen dioxide >3 ppm, reduction of inhaled NO led to the resolution of these abnormalities.

In multiple randomized clinical trials conducted over two decades ago (7, 8, 10-13), inhaled NO between 0.01 to 20 ppm was shown to improve systemic oxygenation in adult ARDS patients, presumably due to decreased intrapulmonary shunting (3). However, the previous studies showed that oxygenation improved at 24 hours but not at 48-72 hours after initiation of inhaled NO therapy. In contrast, in the current trial, a sustained improvement in systemic oxygenation was noted in the NO group at least up to 28 days after initiation of inhaled NO in patients with respiratory failure due to COVID-19 pneumonia. The reasons for this observed discordance may include the implementation of protective lung ventilation in this trial, the depletion of NO synthesis due to widespread injury of the endothelium caused by the viral infection, and the antiviral effects of high-dose NO. Furthermore, a homogenous population of patients with acute hypoxic respiratory failure due to COVID-19 was included in the current investigation instead of the numerous heterogeneous etiologies of ARDS in prior investigations.

To date, randomized trials on inhaled NO preceded the implementation of the 2000 ARDS Network (ARDSnet) ventilatory strategies for acute hypoxemic respiratory failure (32), and used high tidal volume and high airway pressure, which likely induced lung injury. A large,

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randomized trial demonstrated that such a ventilatory approach itself results in lung injury leading to death (32). In contrast, patients enrolled in this trial received low tidal volume, and low airway pressure ventilation according to the ARDSnet tables for mechanical ventilation. The avoidance of injurious ventilation in this trial may have unmasked beneficial effects of inhaled NO and markedly prolonged the improvement in oxygenation compared to the pre-ARDSnet NO trials. This is reminiscent of the trial results on prone positioning (33-35) and ECMO (36, 37) in patients with respiratory failure.

At the pathophysiological level, COVID-19 pneumonia is characterized by severe endothelial injury with widespread thrombosis and microangiopathy of the pulmonary vessels (37), resulting in profound perfusion abnormalities seen in dual-energy CT imaging studies (38). In an autopsy study, Villalba et al. compared histological parenchymal and vascular alterations of patients deceased for respiratory failure due to COVID-19 pneumonia to those for other etiologies. Lungs of COVID-19 patients showed increased pulmonary congestion and aberrant alveolar-septal congestion.(38) Administration of inhaled NO might replete the NO deficiency observed in COVID-19 patients (39). Bypassing the dysfunctional endothelium, inhaled NO may directly alleviate intrapulmonary shunting and improve pulmonary blood flow resulting in sustained improvements in oxygenation. Moreover, the observed improved ventilatory ratio indicates a reduction of alveolar dead space, possibly due to the anti-platelet or anti-leukocyte adhesion properties of NO (39, 40).

In a subset of participants with daily sputum and plasma sampling for quantitative SARS-CoV-2 viral load estimation, the use of inhaled NO was associated with faster clearance of viremia and a

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more rapid viral load reduction in the sputum. This antiviral property of NO may have contributed to the sustained improvement in systemic oxygenation observed in this trial. Since SARS-CoV-2 viral load is associated with increased disease severity and mortality (41, 42), faster reduction of viral load by inhaled NO is expected to decrease the disease severity of pneumonia and improve oxygenation. Previous *in vitro* studies showed that the antiviral or antibacterial effects of NO are dose-dependent. For example, laboratory studies showed that NO directly inhibits SARS-CoV-2 replication by nitrosating viral membrane proteins and hindering SARS-CoV-2 viral protease in a dose-response manner (16). A recent phase III randomized trial showed that, compared to a placebo, repeated NO nasal spray administrations reduced SARS-CoV-2 viral load from the nasal cavity (43). Antiviral activity of NO has also been demonstrated against influenza, coxsackie, and SARS-CoV-1 (17, 23, 44). Although the concentrations of inhaled NO that exert anti-microbial effects are unknown, studies have shown that high-dose inhaled NO (up to 300 ppm) is well-tolerated and improves respiratory function in hospitalized adults and decreases the length of hospitalization in pregnant patients and pediatric patients with viral and bacterial pneumonia (19-22, 45).

Experimental evidence in animals and recent human studies suggests that SARS-CoV-2 infection causes neuroinflammation and neuronal damage (46, 47). Furthermore, accumulating evidence points to an increased risk of long-term neurologic disorders in people who had COVID-19 (48). In the current study, participants receiving inhaled NO had reduced rates of sensory findings at 90 days. Inhaled NO has been shown to elicit systemic anti-inflammatory and anti-thrombotic responses, which may explain our findings (49). Further studies are needed to investigate the mechanisms and effects of inhaled NO on neurological outcomes, as persistent neurological

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deficits are a major driver of healthcare burden in survivors of ARDS and severe COVID-19 infection (50, 51).

This study presents some limitations that warrant discussion. Firstly, this study was a relatively small phase II trial that was not powered to test whether NO exposure reduces mortality. Nevertheless, the positive findings and the pragmatic design of this multicenter study pave the way for larger and more extensive phase III clinical trials evaluating the effects of high-dose inhaled NO on mortality. Secondly, healthcare providers were not blind, and the control (usual care) group lacked a placebo intervention. This was done to protect healthcare workers from an increased risk of COVID-19 exposure. The trial started in March 2020, when no vaccines were available and disconnection of respiratory tubing from the ventilator could expose healthcare workers to contaminated respiratory equipment and aerosolization. Thus, in agreement with the investigational review board at our institutions, the trial was designed without a placebo and with an absence of blinding. Similarly, baseline levels of right heart dysfunction measured by transthoracic echocardiography were not obtained to minimize healthcare workers' exposure to COVID-19. A third limitation of this study is that the trial enrolled exclusively critically ill participants with COVID-19 pneumonia, limiting the generalizability of the results to other causes of acute hypoxemic respiratory failure. Differently from most critical care trials in respiratory failure and, specifically, from prior inhaled NO trials, this investigation included participants with a singular etiology of hypoxemic respiratory failure (i.e., COVID-19 pneumonia). Enrolling such a well-defined population allowed us to avoid heterogeneity from other mechanisms of respiratory failure. It enabled the characterization of the effects of inhaled NO in this specific patient population. Future studies are required to evaluate the benefits of

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inhaled NO therapy in other patient populations. Fourthly, the time from the onset of first symptoms of COVID-19 to the time of intubation and the use (and duration) of non-invasive ventilation and high flow were not recorded and the protocol of the study allowed intensivists to implement the local guideline recommendations on COVID-19 ARDS to care for the patients enrolled in the study. Hence, the trial protocol did not mandate the optimization of PEEP prior to enrollment or the use of recruitment maneuvers. Fifthly, the formation of methemoglobin during inhaled nitric oxide treatment might decrease the oxygen-carrying capacity, which may offset the improvement in the PaO_2/FiO_2 ratio.(52) To address this concern, this study also measured changes in oxygen saturation (SaO_2) from the arterial blood samples at 48 hours, which was similar to usual care. Sixthly, this study did not investigate a concentration higher than 80 ppm of inhaled NO. Other studies have shown that up to 300 ppm of inhaled NO is safe and decreases the length of stay in patients with viral pneumonia. This was observed in both COVID-19 (19) and Respiratory Syncytial Virus pneumonia (20). The role of high-dose NO as a therapeutic for respiratory infections needs further investigation. Finally, the impact of inhaled NO on the length of ICU and hospital stay could not be accurately evaluated in this study. During the pandemic, ICUs and hospital floors underwent major modifications. Many regular hospital floors were transformed into ICUs to allow caring for intubated and mechanically ventilated patients. Patients were discharged directly from the ICU to their homes, while others were discharged to improvised facilities where patients were allowed to recover until they tested negative for COVID-19. The above conditions made it impossible to compare ICU and hospital stay between groups.

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Conclusions

In mechanically ventilated critically ill participants with acute hypoxemic respiratory failure due to COVID-19 pneumonia, high-dose inhaled NO at 80 ppm for the first 48 hours of mechanical ventilation improved PaO_2/FiO_2 compared with the use of usual care alone. The treatment with inhaled NO did not reduce mortality, or duration of mechanical ventilation, but exploratory results suggest that participants with inhaled NO had a steeper reduction in plasma viral load and reduced rates of sensory neurologic symptoms and signs at 90 days. Finally, treatment with inhaled NO was well tolerated, and no serious adverse events related to the intervention were reported. Overall, the findings highlight the importance of planning future dose-response investigations into the anti-microbial and clinical properties of high-dose inhaled NO therapy in adults with acute hypoxemic respiratory failure.

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Acknowledgments: The authors would like to thank the participants, site staff, site investigators, and the entire Nitric Oxide Study Team. This work is dedicated to the recently deceased Prof. Warren M. Zapol, MD, Prof. Robert M. Kacmarek, PhD, and Prof. Goran Hedenstierna, MD, members of the initial steering committee of the present trial and instrumental in the design of the trial itself. The authors thank Prof. Zapol, Director of the Anesthesia Center for Critical Care Research, who pioneered the work on inhaled nitric oxide more than 30 years ago at the Massachusetts General Hospital in Boston. Prof. Zapol and colleagues presented the first physiological results on inhaled nitric oxide in newborns with persistent pulmonary hypertension in 1992 (Roberts et al. Inhaled nitric oxide in persistent pulmonary hypertension of the newborn. Lancet 1992;239:818–9). Over the past decades, Prof. Zapol taught and discovered ground-breaking therapeutic implications of inhaled nitric oxide and provided intellectual insight during the first phases of the present work. The authors thank Prof. Kacmarek, Director of the Respiratory Care Department at Massachusetts General Hospital. Prof. Kacmarek was pivotal in performing the first studies on inhaled nitric oxide, teaching our respiratory care personnel, and creating and coordinating the nitric oxide delivery in the present work. The authors thank Prof. Hedenstierna, Director of the Laboratory of Clinical Physiology at Uppsala University in Sweden. Prof. Hedenstierna and colleagues first documented the benefits of high-dose inhaled nitric oxide in a series of patients during the SARS pandemic in China and performed *in-vitro* studies demonstrating dose-dependent antiviral properties of nitric oxide donors.

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Figure Legends:

Figure 1. Consolidated Standards of Reporting Trials (CONSORT) Diagram

Figure 2. Systemic Oxygenation at Baseline, 24 and 48 Hours

This figure depicts the mean change in the PaO_2/FiO_2 ratio from baseline to 24 and 48 hours from the time of randomization. The treatment group (N=94) and the control group (N=99) have been depicted in red and blue, respectively. The data are represented as mean (point) and standard error of the mean (error bars).

Figure 3: **Posterior Probability Curves for the Association of Study Outcomes with Inhaled Nitric Oxide Therapy**

Figure 4. Blood and Sputum Viral Count

Panels A and B show predicted Log_{10} of SARS-CoV-2 viral load by PCR over time in blood (Panel A) and sputum (Panel B). Panel A: treatment group n=17 patients (68 samples) in red, and control group n=20 patients (77 samples) in blue. Panel B: treatment group n=17 patients (38 samples) in red, and control group n=20 patients (44 samples) in blue.

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	Treatment Group (94)	Control Group (99)	SMD
Age, Median [IQR]	64 [53.0, 70.0]	62 [50.0, 69.5]	0.142
Gender, No. (%) Female	31 (33.0)	34 (34.3)	0.029
Race, No. (%)			0.282
American Indian / Alaska Native	1 (1.1)	0 (0.0)	
Asian	6 (6.4)	7 (7.1)	
Black / African American	23 (24.5)	20 (20.2)	
Other	14 (14.9)	11 (11.1)	
Unknown	1 (1.1)	0 (0.0)	
White	49 (52.1)	61 (61.6)	
Hispanic or Latino ethnicity, No. (%)	27 (28.7)	30 (30.3)	0.035
BMI, kg/m^2 Median [IQR]	31.0 [26.9, 35.8]	30.2 [26.8, 35.4]	<0.001
Smoking History, No. (%)			0.202
Current Smoker	4 (4.3)	6 (6.1)	
Former Smoker	25 (26.6)	30 (30.3)	
Never Smoked	49 (52.1)	42 (42.4)	
Unknown	16 (17.0)	21 (21.2)	
Hypertension, (No. %)	63 (67.0)	46 (46.5)	0.424
History of Myocardial Infarction, No. (%)	13 (13.8)	11 (11.1)	0.082
Diabetes, No. (%)	39 (41.5)	29 (29.3)	0.257
Cerebrovascular Disease, No. (%)	5 (5.3)	8 (8.1)	0.111
Chronic Kidney Disease, No. (%)	10 (10.6)	8 (8.1)	0.088
COPD, No. (%)	4 (4.3)	8 (8.1)	0.160
Connective Tissue Disease, No. (%)	6 (6.4)	1 (1.0)	0.288
Dementia, No. (%)	4 (4.3)	3 (3.0)	0.065
Hemiplegia, No. (%)	4 (4.3)	0 (0.0)	0.065
Immune Deficiency, No. (%)	5 (5.3)	3 (3.0)	0.115
Liver Disease, No. (%)	8 (8.5)	0 (0.0)	0.431
History of Malignancy, No. (%)	7 (7.4)	0 (0.0)	0.401
History of Peptic Ulcer, No. (%)	3 (3.2)	4 (4.0)	0.045
ARDS Class, No. (%)			
COVID-19, PaO_2/FiO_2 300-400 mmHg	9 (9.6)	5 (5.1)	0.170
Mild ARDS, PaO_2/FiO_2 200-300 mmHg	28 (29.8)	36 (36.4)	0.140
Moderate ARDS, PaO_2/FiO_2 100-200 mmHg	43 (45.7)	37 (37.4)	0.170
Severe ARDS, PaO_2/FiO_2 <100 mmHg	14 (14.9)	21 (21.2)	0.165
APACHE II Score, Mean (SD)	24.6 (7.7)	21.1 (6.5)	0.499

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SOFA Score, Median [IQR]	8.5 [7, 11]	8 [7, 10]	0.100
Compliance, ml/cmH_2O Mean (SD)	36.7 (18.7)	36.8 (15.6)	0.004
PEEP (cmH_2O), Median [IQR]	12 [10,14]	12 [10,14]	0.019
Tidal Volume (mL/Kg), mean (SD)	4.7 (1.5)	4.7 (1.4)	0.009
PaO_2/FiO_2 ratio, mmHg	177 [125, 241]	195 [120, 235]	0.066
FiO_2, Mean (SD)	0.59 (0.21)	0.61 (0.21)	0.110
$PaCO_2$ (mmHg), mean (SD)	42 [37,47]	43 [39,50]	0.228
Minute Ventilation (L/min), Median [IQR]	8.7 [7.3, 10.5]	8.5 [7.2, 9.7]	0.186
Creatinine, mg/dL, Median [IQR]	1.08 [0.84, 1.96]	0.99 [0.72, 1.42]	0.261
D-Dimer	2492 [1414, 5377]	1815 [1014, 5018]	0.200

Table 1. Baseline demographics and clinical characteristics of all the enrolled and randomized intubated patients with COVID-19 who were included in the modified intention-to-treat analysis. Tidal Volume has been calculated for the ideal body weight (mL/Kg). COPD = chronic obstructive pulmonary disease. ARDS = acute respiratory distress syndrome. Patients with COVID-19 pneumonia and impaired PaO_2/FiO_2 but with PaO_2/FiO_2 between 300-400 mmHg are listed as "COVID-19 with PaO_2/FiO_2 300-400 mmHg". Patients with ARDS are classified as mild PaO_2/FiO_2 200-300 mmHg, moderate PaO_2/FiO_2 100-200 mmHg and severe PaO_2/FiO_2 <100 mmHg according to the Berlin Definition.

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	Baseline		24 Hours		48 Hours	
Variables	**Treatment Group**	**Control Group**	**Treatment Group**	**Control Group**	**Treatment Group**	**Control Group**
PaO_2/FiO_2 Ratio, mm Hg	177 (125, 241)	195 (120, 235)	196 (150-252)	188 (130-263)	200 (157-239)	183 (122-235)
FiO_2, %	51.5 (40-70)	60 (42.5-75)	45 (36-57)	50 (40-60)	45 (38-60)	50 (40-60)
PEEP, cm H_2O	12 (10-14)	12 (10-14)	12 (10-14)	12 (10-14)	12 (10-13)	12 (10-14)
Plateau Pressure, cm H_2O	24 (21-28)	23 (21-27)	24 (21-26)	23 (20-26)	24 (21-26)	24 (20-26)
Respiratory system compliance, ml/cm H_2O	31.7 (24.1-37.5)	32.0 (27.0-40.0)	32.0 (25.5-41.9)	32.0 (27.0-41.0)	31.0 (25.0-38.0)	35.0 (27.0-42.0)
Respiratory Rate, breaths per min	22 (20-25)	22 (18-25)	22 (20-25)	23 (19-26)	22 (18-26)	24 (20-27)
Tidal Volume/IBW, ml/kg	6.0 (6-6.8)	6.0 (5.6-6.7)	6.1 (5.6-6.6)	6.0 (5.4-6.5)	6.0 (5.5-6.5)	5.9 (5.5-6.5)
Minute Ventilation, L/min	8.8 (7.1-10.1)	8.4 (7.2-9.9)	8.4 (7.5-10.1)	8.7 (7.3-9.9)	8.6 (7.0-10.2)	8.7 (7.4-10.4)
Use of neuromuscular blockade	53 (56%)	45 (45%)	46 (49%)	39 (39%)	46 (49%)	30 (30%)
Lifting sedation	0 (0%)	4 (4%)	0 (0%)	3 (3%)	1 (1%)	3 (3%)

Table 2. Ventilator Settings at Baseline, 24 Hours, and 48 Hours.
Median (IQR) or n (%) are presented.

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	Treatment Group (94)	Control Group (99)	Difference or Risk Ratio (95% CrI)
Primary Endpoint: Change in PaO_2/FiO_2 ratio at 48 hours, mmHg			
Overall Population	28.3 (89.3)	-1.4 (68.9)	39.1 (18.1, 60.3)
Stratified by Baseline PaO_2/FiO_2 ratio			
<100 mm Hg	85.9 (72.1)	31.0 (44.6)	50.6 (5.1, 95.6)
100-200 mm Hg	34.6 (74.1)	10.2 (53.2)	32.5 (1.9, 63.1)
≥200 mm Hg	-0.6 (101.9)	-28.5 (80.9)	27.6 (-16.5, 72.3)
Secondary Endpoints			
Mortality within 28 days	27 (28.7%)	27 (27.3%)	RR: 0.85 (0.50 to 1.46)
Mortality within 90 days	32 (34.0%)	32 (32.3%)	RR: 0.87 (0.52 to 1.43)
Time to PaO_2/FiO_2 ratio above 300 mm Hg, days*	8.7 (5.0)	8.4 (6.5)	0.44 (-3.63 to 4.53)
Patients reaching PaO_2/FiO_2 ratio above 300 mm Hg*	33 [35.1%]	21 [21.2%]	RR: 2.03 (1.11 to 3.86)

Table 3. Primary and Secondary Outcomes in the final analysis population

Data are mean (SD) or n (%) *Measured over 28 days after randomization in survivors with baseline PaO_2/FiO_2 <300 mm Hg, as pre-specified. (67 patients Treatment Group and 72 Control Group)

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	Treatment Group (94)	Control Group (99)	Difference or Risk Ratio (95% CrI)
Safety Outcomes			
Acute Kidney Injury	65 (69.1%)	69 (69.7%)	RR 0.82 (0.39 to 1.70)
Class 1	17 (18.1%)	20 (20.2%)	
Class 2	11 (11.7%)	22 (22.2%)	
Class 3	37 (39.3%)	27 (27.2%)	
RRT	33 (35.1%)	22 (22.2%)	RR 1.65 (0.78 to 3.56)
Haemodynamic instability during weaning	0 (0%)		
MetHb above 5%			
Events / Treatment days overall	8/1282		
Events / Treatment days at 80 ppm	7/292		
Events requiring dose reduction	5		
MetHb highest daily level (%)			
Overall	1.4 [0.7-1.5]		
At 80 ppm	2.2 [1.5-3.0]		
NO_2 above 3 ppm			
Events / Treatment days overall	1/1282		
Events / Treatment days at 80 ppm	1/292		
Events requiring dose reduction	1		
NO_2 highest daily level (ppm)			
Overall	0.8 [0.0-1.0]		
At 80 ppm	1.0 [1.0-1.8]		

Table 4. Safety Outcomes in the final analysis population assigned to treatment

Data are median [IQR] or n. CrI = credible interval. MtHb = methemoglobin. ppm = parts-per-million. RRT = Renal Replacement Therapy.

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	Treatment Group (94)	Control Group (99)	Difference or Risk Ratio (95% CrI)
Exploratory Outcomes			
Requirement for VV-ECMO	4 (4.2%)	5 (5.0%)	RR 0.70 (0.14 to 3.39)
Neurological Signs and Symptoms (day 90)*	4 (4.2%)	17 (17.2%)	RR 0.17 (0.04 to 0.62)
Motor	4 (4.2%)	12 (12.1%)	RR 0.36 (0.08 to 1.42)
Sensory	0 (0.0%)	14 (14.1%)	RR 0.01 (0.00 to 0.12)
Ventilator Time (hours)	447.1 (225.4)	448.6 (962.4)	33.73 (-187.52 to 254.18)
			Mean Difference (95% CrI)
Change in A-a gradient at 48 hours			-28.8 (-59.8, 1.0)
Change in SaO_2 at 48 hours			0.12 (0.00, 0.24)
Change in Ventilatory Ratio at 48 hours			-0.10 (-0.30, 0.09)

Table 5. Exploratory Outcomes in the final analysis population

Data are mean (SD) or n. CrI = credibile interval. VV-ECMO = veno-venous extracorporeal membrane oxygenation.

* Measured at 90 days after randomization in survivors, 62 patients in Treatment Group and 67 in Control Group.

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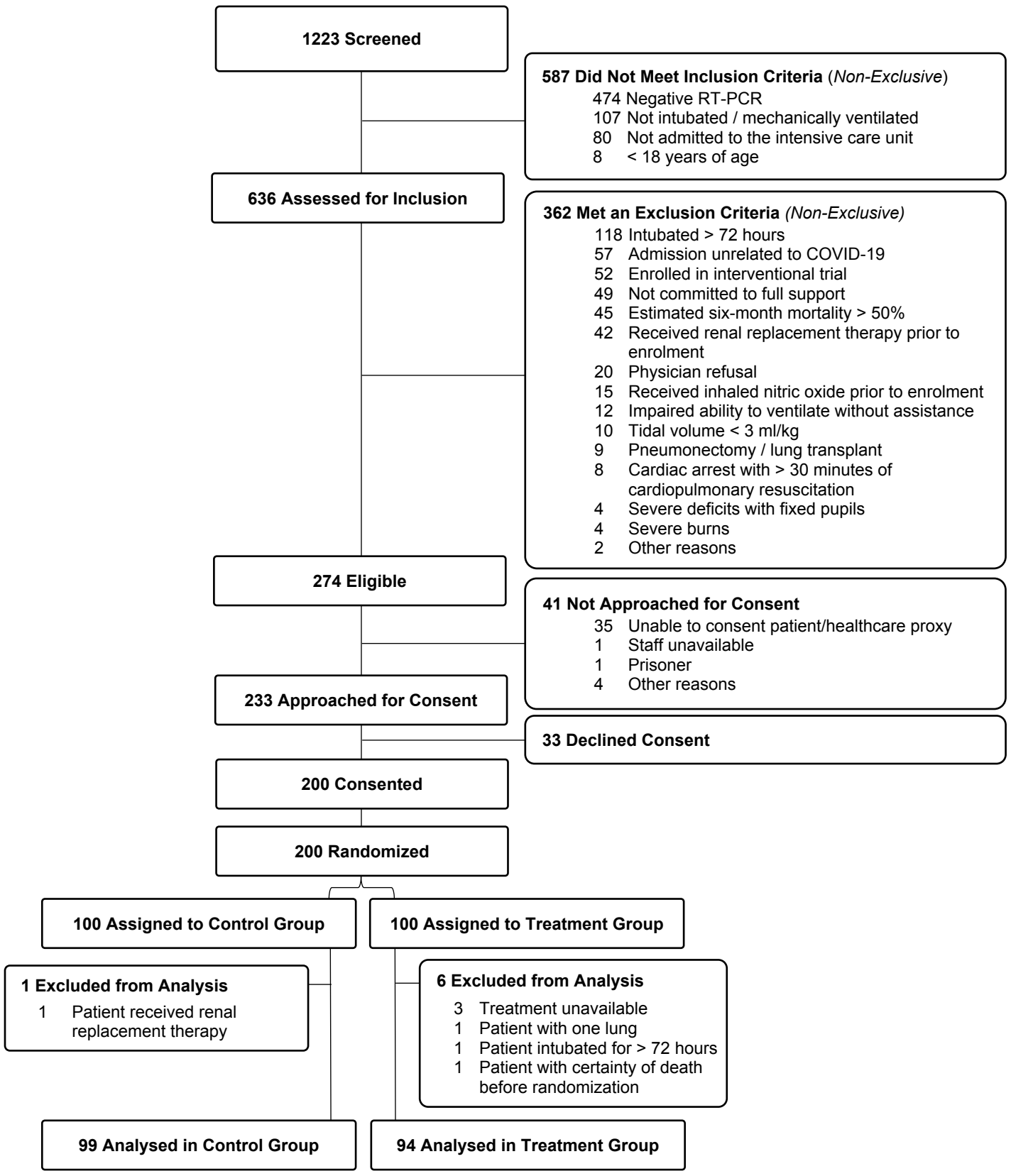

Figure 1.

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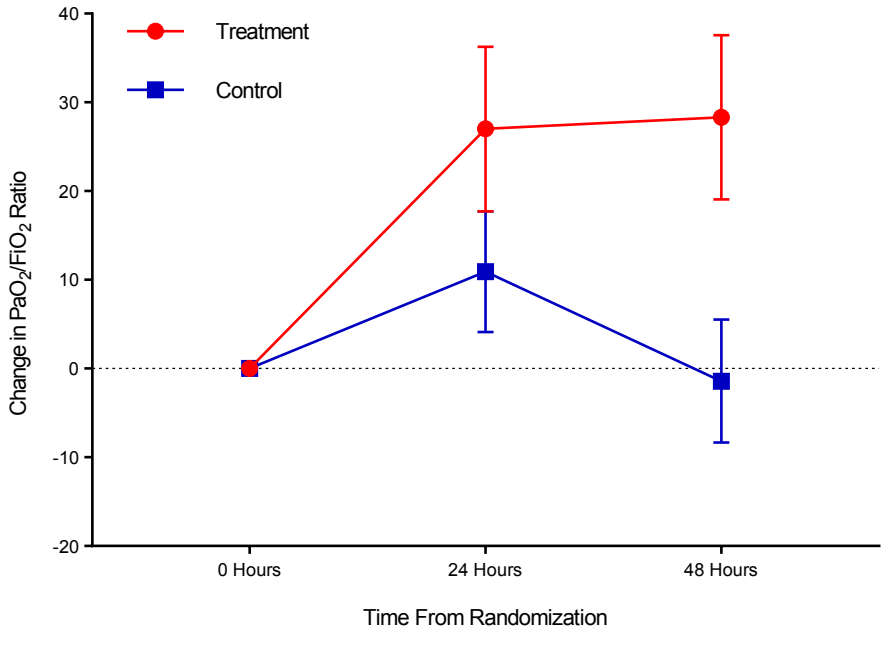

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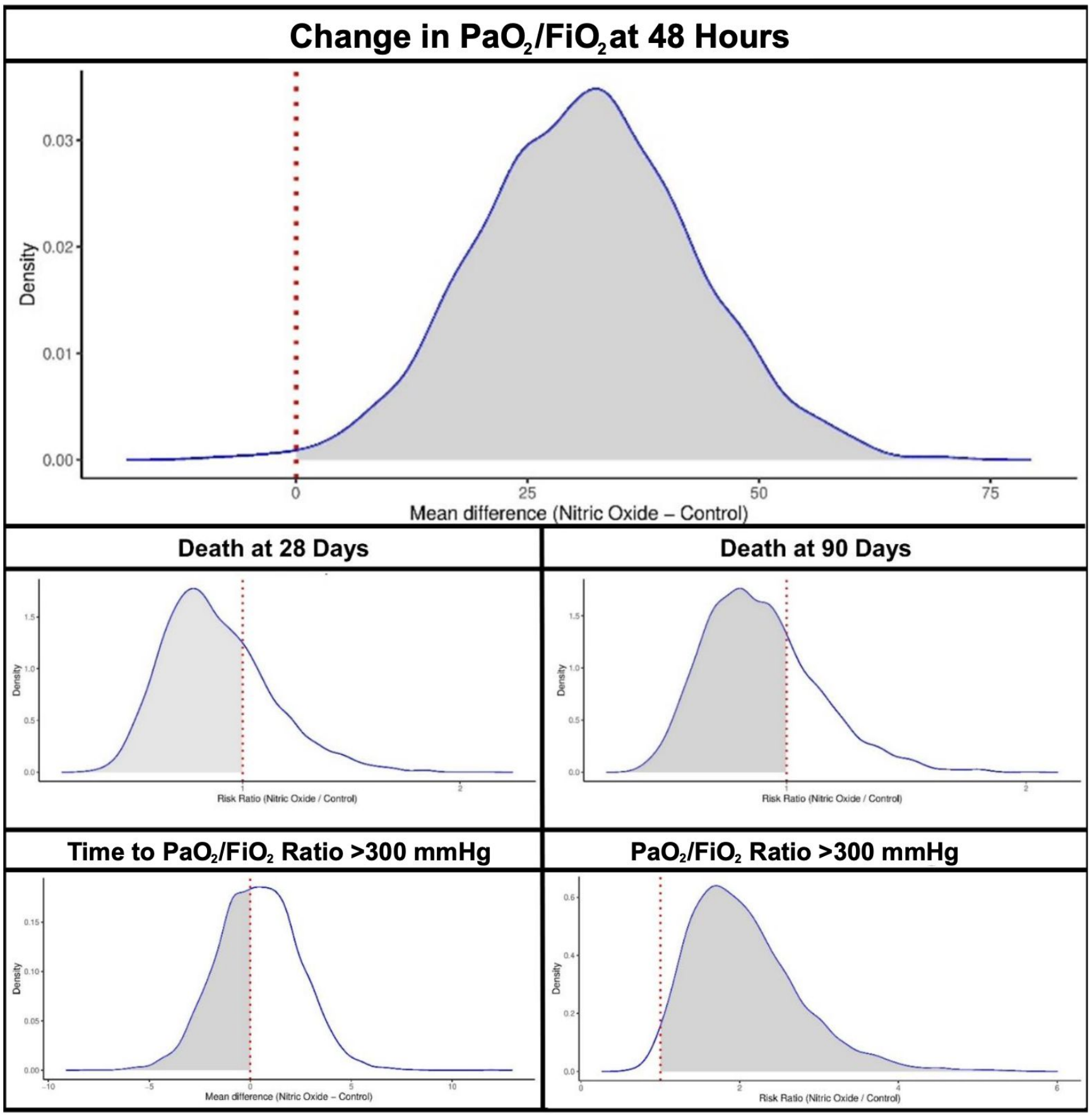

Figure 3.

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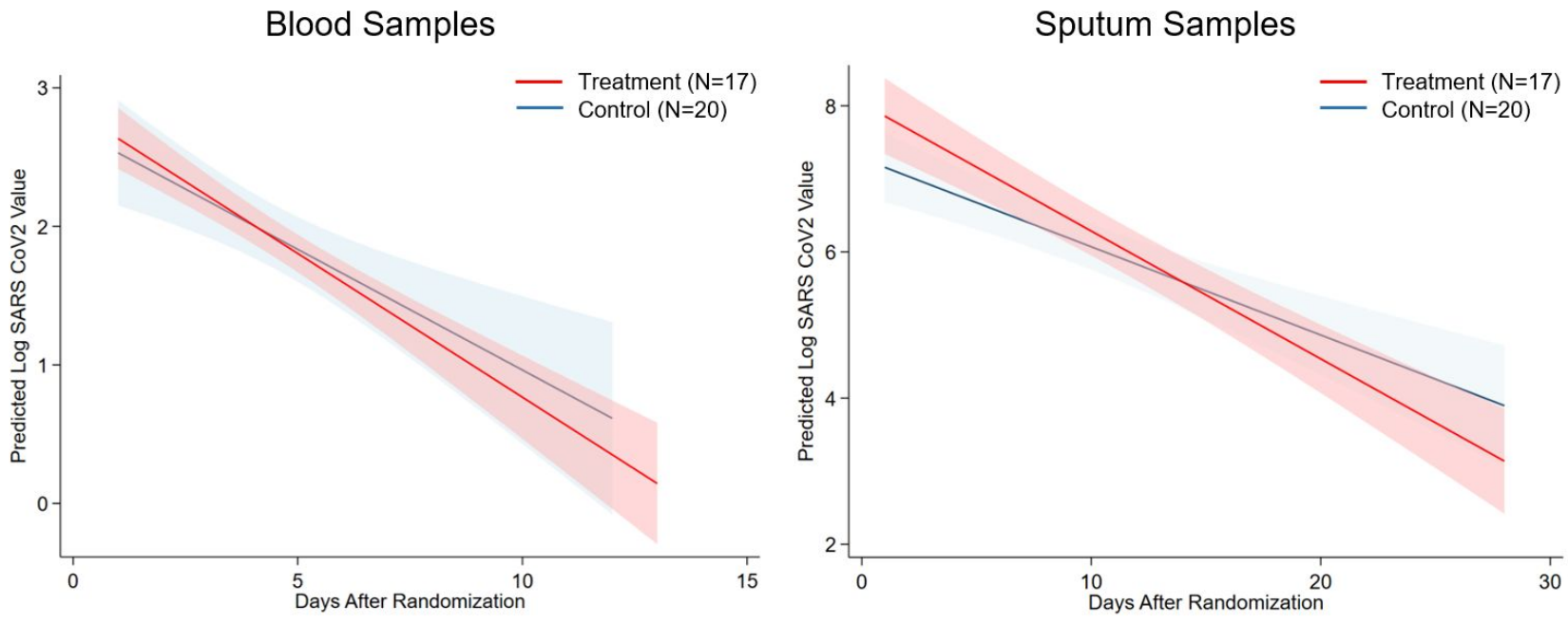

Figure 4.

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High-Dose Inhaled Nitric Oxide in Acute Hypoxemic Respiratory Failure due to COVID-19: A Multicenter Phase 2 Trial

Raffaele Di Fenza, Naman S Shetty, Stefano Gianni, Vibhu Parcha, Valentina Giammatteo, Bijan Safaee Fakhr, Daniel Tornberg, Olof Wall, Piotr Harbut, Peggy S Lai, Jonathan Z Li, Sabrina Paganoni, Stefano Cenci, Ariel L Mueller, Timothy T Houle, Oluwaseun Akeju, Edward A Bittner, Somnath Bose, Louie K Scott, Ryan W Carroll, Fumito Ichinose, Magnus Hedenstierna, Pankaj Arora and Lorenzo Berra, on behalf of the Nitric Oxide Investigators.

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ONLINE DATA SUPPLEMENT

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STUDY METHODS

Study Design

This was an investigator-initiated multicenter, single-blinded, randomized (1:1), controlled, parallel-arm clinical trial conducted at five sites in the United States (Massachusetts General Hospital, University of Alabama at Birmingham, Louisiana State University, and Beth Israel Deaconess Medical Center) and Sweden (Danderyd Hospital). All participants or their medically authorized representatives provided written informed consent, and the study was approved by the Institutional Review Boards at each of the respective study sites. The detailed study protocol and the statistical analysis plan are included in this Online Data Supplement document. This study was registered on ClinicalTrials.gov as NCT04306393 (Registered on March 12th, 2020).

Participants

The study enrolled adult patients with SARS CoV-2 infection (confirmed diagnosis using RT-PCR) admitted to the intensive care units and were intubated and mechanically ventilated. Individuals were excluded if they had been intubated for >72 hours or the physician of record opposed the enrollment due to safety concerns. In July 2020, exclusion criteria were expanded to additionally exclude those who: 1) were enrolled in another intervention study, 2) had a prior medical history of lung malignancy, pneumonectomy, or lung transplant, 3) were receiving tidal volume support <3 ml/kg of ideal body weight at the screening to assure NO delivery, 4) had severe burns (>40% of total body surface area), 5) had experienced cardiac arrest with cardiopulmonary resuscitation for longer than 30 minutes, 6) had a presumed severe deficit in cerebral function with fixed dilated pupil, 7) were receiving renal replacement therapy at the time of screening, 8) had a history of malignancy or other irreversible disease/conditions with an estimated six-month mortality > 50%, 9) had received inhaled nitric oxide gas before screening, or 10) were admitted to the hospital for reasons unrelated to COVID-19. Expansion of the exclusion criteria occurred during the first months of the trial to ensure a uniform patient population among centers during the evolving pandemic. Figure 1 describes patient enrolment and follow-up as per CONSORT recommendations (Appendix, p 42).

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Randomization and Masking

After informed consent was obtained, eligible participants were randomized on the same day using a centralized, secure computer platform stratified by site, age (≤ or >60 years-old), and sex. Because of the uncertainty of site enrolment due to the variable nature of the pandemic, a small, fixed block size of two was used for the first 20 allocations at each site. Future assignments were conducted using randomized permuted blocks of size 2 and 4 to respective study arms. All assignments were generated using a 1:1 ratio using a randomization scheme prepared by the study statistician. Allocation concealment was accomplished using a centralized computer system. The study interventions included either the institutional usual care alone (control arm) or the institutional usual care with the addition of high-dose inhaled nitric oxide (treatment arm). Usual care was delivered according to each institution's protocols (including ventilation strategies and use and dosage of antivirals and antimicrobials, anti-inflammatory agents including steroids, inhaled nitric oxide as a rescue therapy at 5-20 ppm when PaO_2/FiO_2<100 mmHg, inotropic-vasopressor agents and initiation of extracorporeal membrane oxygenator). Enrolment of participants occurred at the onset of the pandemic and before vaccines were available. In order to minimize aerosolization procedures and associated possible contamination and infection of healthcare professionals from the break of the ventilator circuit per recommendations of the World Health Organization, no placebo was used.16 Masking was only possible for participants and/or their legally authorized representative, as patients were unconscious and no one other than medical personnel was allowed in the participant's room. Healthcare professionals and study team members assessing outcomes were not blinded to the treatment.

Procedures

Participants in the treatment arm received inhaled nitric oxide at 80 ppm for the first 48 hours after enrolment. The gas was started immediately after randomization within the first 72 hours of mechanical ventilation. After the first 48 hours of treatment, the gas was reduced to 40 ppm and maintained at this concentration until severe hypoxemia resolved (PaO_2/FiO_2>300 mmHg). The gas administration was synchronized with the ventilator and delivered through an injector module at constant concentration throughout the respiratory cycle into the inspiratory limb of the patient's breathing circuit. Weaning from

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inhaled nitric oxide was initiated when PaO_2/FiO_2>300 mmHg was recorded for a duration of greater than 24 hours. Gradual weaning by 50% every four hours was employed with cautious evaluation for rebound hypoxemia and acute hypotension. The previous dose was used when rebound hypoxemia or acute hypotension occurred. The detailed inhaled nitric oxide weaning protocol is described in the Appendix (pp 13-14).

Participants from all centers were evaluated by the research staff up to 90 days after study completion. In participating U.S. centers, alive participants were contacted on day 90 for a follow-up phone call. In addition, data were retrieved from physicians' notes to determine outcomes after hospital discharge (e.g., hospital readmission, admission to a rehabilitative facility, death) and to investigate functional status. The phone interviews were conducted by research staff through a structured questionnaire provided in the Appendix (pp 22-31). During the follow-up phone calls, participants were asked if they were experiencing any sensory symptoms or motor deficits. No phone calls were performed in Sweden, and data were retrieved only from the medical records (following the same format as the U.S. questionnaire). Electromyography and imaging (magnetic resonance imaging or computed tomography) reports were collected from medical records for participants reporting sensory or motor symptoms. For the final analysis, participants from any center were considered positive for neurological findings if sensory symptoms and/or motor deficits were confirmed and reported by a physician caring for the patient, such as a neurologist or physiatrist, in the medical charts.

Outcomes

The primary outcome of this study was the change in arterial oxygenation (PaO_2/FiO_2) at 48 hours. The secondary outcomes were all-cause mortality at 28 and 90 days, time to reach normoxaemia (defined by a PaO_2/FiO_2 >300 mmHg for at least 24 hours among survivors), and the proportion of normoxaemic participants in the two groups at 28 days. The safety outcomes for this clinical trial included

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methaemoglobinaemia defined as methaemoglobin (MetHb) exceeding 5%, inhaled nitrogen dioxide >3 ppm, haemodynamic instability (rebound hypotension) during weaning, the occurrence of acute kidney injury by 28-days, or the initiation of renal replacement therapy by 90 days. Exploratory study outcomes included change in viral load (Log10 copies of SARS-CoV-2 per mL) in plasma and sputum, duration of mechanical ventilation, use of venous-venous extracorporeal membrane oxygenator (VV-ECMO), and neurological signs and symptoms (motor and sensory) at 90 days.

Plasma and Sputum Preparation

Starting June 24th, 2020, at Massachusetts General Hospital, plasma and sputum (endotracheal aspirate in intubated participants, spontaneously expectorated in extubated participants) were collected prospectively. Biological samples of those participants who consented were stored at -80 ºC for quantification of the SARS-CoV-2 viral load. There was no post-hoc selection of participants for this analysis. Quantification of viral load in blood and sputum was obtained as previously described.17 Briefly, samples were centrifuged at 21,000 g for 2 hours at 4 ºC. The supernatant was removed, and Trizol-LS was added to the samples and vortexed briefly. The samples were incubated at 4 ºC for 15 minutes and subsequently treated with chloroform. Samples were vortexed briefly and then centrifuged at 21,000 g for 15 minutes at 4 ºC. The resulting supernatant containing RNA was then concentrated using isopropanol precipitation, and SARS-CoV-2 RNA was measured by RT-qPCR.

Statistical Analysis

Participants randomized to inhaled nitric oxide were hypothesized to have at least 20% greater improvement in PaO_2/FiO_2 at 48 hours after gas initiation compared with the usual care alone. Assuming a two-tailed alpha of 0.05, the enrolment of 182 participants would provide 90% power to detect an effect size of 38 mmHg PaO_2/FiO_2 change based on the effect estimates in a previous investigation in hypoxemic intubated and mechanically ventilated patients.18 Presuming a 10% dropout, the target sample size was 100 in each group (n=200 total). The original sample size estimates were derived using a frequentist approach to the

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analysis. However, due to reporting requirements posed by the FDA and IRB and the need for rapid learning in the context of the COVID-19 crisis, the analysis plan was revised (April 2020) to employ Bayesian estimation, which allowed for sequential group analyses. Prior probability distributions were specified for the intercept, the individual predictors, and the error term(s) (i.e., sigma for linear regression). Traditionally, weakly informative priors specified as ~ usual (0, 2.5 x SD of the outcome) were used. For the appropriateness of the choice of priors, a prior predictive check was conducted by simulating the distributions. Generally, the predicted values from the priors appeared reasonable, as the predicted values resided in a diverse range of plausible values for the outcome.

The baseline characteristics were summarized as the median and interquartile range for continuous data and counts and percentages for categorical data. Standardized mean difference (SMD) is reported to quantify the differences between the two study arms, with values greater than 0.20 suggesting a potential imbalance between groups. All study outcomes were analyzed using Bayesian estimation of generalized linear models. Bayesian estimation utilized in this trial allowed interpretation of the posterior probability distributions for respective effects. The primary and secondary outcomes analysis was conducted using a Bayesian framework that estimates the treatment effect conditional on several pre-specified additional variables included in the model (defined a priori: age, age^2, sex, BMI, and APACHE II score and variables with SMD>0.20). The PaO_2/FiO_2PaO2/FiO2 ratio was modeled for the primary outcome using a normal distribution and identity link function. The PaO2/FiO2 ratio at 48 hours was regressed on baseline (i.e., enrollment) PaO2/FiO2 ratio, randomized group assignment, and additional covariates, as specified above. Secondary endpoints were conducted using outcome distributions and link functions appropriate to the outcome (e.g., normal, gamma, negative binomial) using the same covariates (i.e., age, age^2, sex, BMI, and APACHE II score and variables with SMD>0.20) and baseline PaO2/FiO2 ratio. The primary and secondary study outcomes are reported as adjusted effect estimates and 95% credible interval (CrI).

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To assess the change in viral load in 149 plasma samples from 33 patients and 86 sputum samples from 22 patients, a Bayesian linear mixed-effect model was used to determine the effect of high dose inhaled nitric oxide on viral load (log10 ΔCT [threshold cycle]) over time. This model included fixed effects of the treatment arm, time from randomization, and an interaction term (arm × time), using a repeated measures covariance structure (first-order autoregressive structure). A random intercept at the level of the participant was used in these models.

All study outcomes were analyzed in the modified intention-to-treat population, excluding participants for whom respiratory treatment was unavailable at the study site or who did not meet the secondary review of the inclusion/exclusion criteria within 24 hours of enrolment. A total of seven patients were excluded after randomization for the modified intention-to-treat analysis (mITT, Figure 1). Due to the ongoing pandemic, relatives and family members were not allowed to come to the hospital. This limited the information on the medical history of critically ill patients entering the intensive care unit, rendering a mandatory secondary review of inclusion/exclusion criteria after randomization. Additionally, the pandemic impacted the supply of nitric oxide gas tanks at the respective study sites, which was sometimes intermittent.

All statistical analyses were performed using R 4.0.2 (R Core Team) with Bayesian estimation conducted in RStan. Bayesian posterior distributions are summarized using posterior means and two-sided 0.95 CrI. No formal adjustments were made to the inferences that would be appropriate for statistical significance thresholds using frequentist adaptive designs. Full details of the statistical analysis plan – including prior probability definitions, full model details, and stopping rules – are outlined in Appendix (p 32-41). The trial was overseen by an independent data safety monitoring board (DSMB) which evaluated unmasked interim data for futility, efficacy, and safety. The DSMB met to review interim data analysis after every 25 patients were enrolled. The study continued until the target recruitment was reached. The only stopping rule for the trial was defined as a significant increase of mortality with nitric oxide gas.

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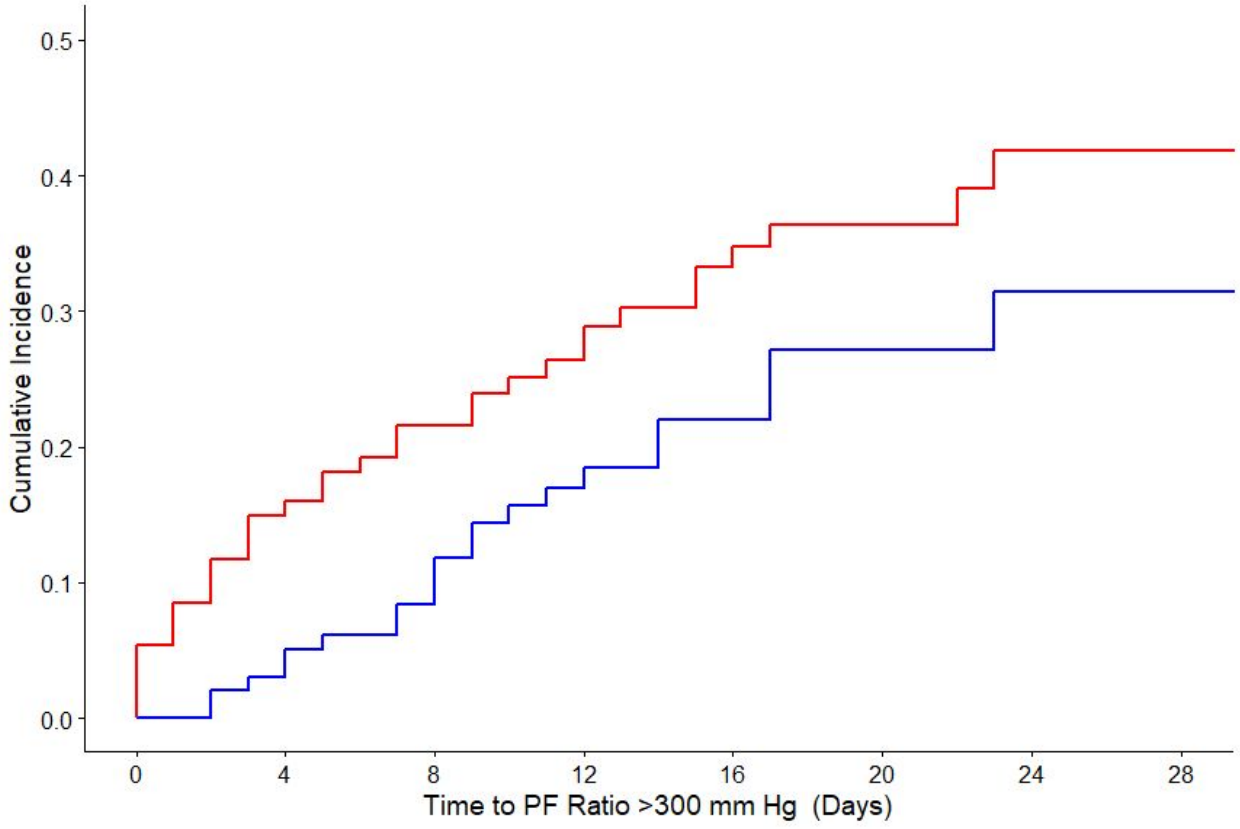
SUPPLEMENTARY FIGURE

Figure E1. This figure depicts the daily cumulative incidence of hypoxemia-free patients from the time of randomization, measured as daily PaO_2/FiO_2 ratio above 300 mmHg for at least 24 hours. The treatment group (N=94) and the control group (N=99) have been depicted in red and blue, respectively. The data are represented as fractions of 1.



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SUPPLEMENTARY TABLE

Table E1. Sensitivity Analysis of Association of Study Outcomes with Inhaled Nitric Oxide Therapy

	Treatment Group (94)	**Control Group (99)**	**Difference or Risk Ratio (95% CrI)**
			Sensitivity
Primary Endpoint			
Change in PaO_2/FiO_2 ratio at 48 hours, mmHg	28.3 (89.3)	-1.4 (68.9)	32.9 (12.4, 52.8)
Secondary Endpoints			
Mortality within 28 days	27 (28.7%)	27 (27.3%)	RR: 0.74 (0.39 to 1.40)
Mortality within 90 days	32 (34.0%)	32 (32.3%)	RR: 0.77 (0.44 to 1.32)
Time to Normoxemia, days*	8.7 (5.0)	8.4 (6.5)	0.67 (-2.68-4.09)
Patients reaching PaO_2/FiO_2>300	26 [27.7%]	17 [17.2%]	RR: 1.65 (0.79 to 3.39)

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Table E2. Adjunctive Treatment during ICU Stay

	Total (n=193)	Treatment group (n=94)	Control group (n=99)
ECMO	8 (4%)	4 (4%)	4 (4%)
Lopinavir	0 (0%)	0 (0%)	0 (0%)
Remdesivir	73 (38%)	37 (39%)	36% (36%)
Chloroquine	30 (16%)	13 (14%)	17 (17%)
Antibiotic agents	193 (100%)	94 (100%)	99 (100%)
N-Acetylcysteine	21 (11%)	10 (11%)	11 (11%)
Corticosteroids	109 (56%)	56 (60%)	53 (54%)
Tocilizumab	1 (0.5%)	1 (1%)	0 (0%)
Favipiravir	1 (0.5%)	1 (1%)	0 (0%)
Convalescent plasma	16 (8%)	9 (10%)	7 (7%)

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Table E3. Posterior Probability of Improving Study Outcomes with Inhaled Nitric Oxide Therapy

Outcome	Probability
Mean Difference in Change of PaO_2/FiO_2 ratio at 48 hours >0	99.5%
Mean Difference in Change of PaO_2/FiO_2 ratio at 48 hours >5	98.7%
Mean Difference in Change of PaO_2/FiO_2 ratio at 48 hours >10	96.6%
Mean Difference in Change of PaO_2/FiO_2 ratio at 48 hours >20	82.6%
Mean Difference in Time to PaO_2/FiO_2, >300	42.9%
Mortality at 90 Days (RR< 1)	71.4%
Mortality at 28 Days (RR< 1)	71.9%
PaO_2/FiO_2>300 (RR> 1)	98.1%

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Table E4. Baseline demographics and clinical characteristics of the enrolled and randomized patients with COVID-19 from whom plasma and sputum samples were collected for quantitative viral load testing. Tidal Volume has been calculated for the ideal body weight (mL/Kg). COPD = chronic obstructive pulmonary disease. AHRF = acute hypoxemic respiratory failure. ARDS = acute respiratory distress syndrome. Patients with COVID-19 pneumonia and impaired PaO_2/FiO_2 but with PaO_2/FiO_2 between 300-400 mmHg are listed as "COVID-19 with PaO_2/FiO_2 300-400 mmHg". Patients with ARDS are classified as mild PaO_2/FiO_2 200-300 mmHg, moderate PaO_2/FiO_2 100-200 mmHg and severe PaO_2/FiO_2 <100 mmHg according to the Berlin Definition.

	Treatment Group (17)	**Control Group (20)**	**SMD**
Age, Median [IQR]	64 [62.0, 75.0]	66.5 [59.8, 71.8]	0.204
Gender, No. (%) Female	7 (41.2)	8 (40.0)	0.023
Race, No. (%)			0.582
Asian	1 (5.9)	3 (15.0)	
Black / African American	2 (11.8)	1 (5.0)	
Other	6 (35.3)	3 (15.0)	
Unknown	1 (5.9)	0 (0.0)	
White	7 (41.2)	13 (65.0)	
Hispanic or Latino ethnicity, No. (%)	7 (41.2)	5 (25.0)	0.339
BMI, kg/m^2 Median [IQR]	31.9 [29.2, 36.3]	31.1 [27.0, 35.1]	0.033
Smoking History, No. (%)			0.394
Current Smoker	0 (0.0)	1 (5.0)	
Former Smoker	6 (35.3)	8 (40.0)	
Never Smoked	9 (52.9)	8 (40.0)	
Unknown	2 (11.8)	3 (15.0)	

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Hypertension, (No. %)	12 (70.6)	10 (50.0)	0.419
History of Myocardial Infarction, No. (%)	5 (29.4)	2 (10.0)	0.489
Diabetes, No. (%)	8 (47.1)	8 (40.0)	0.139
Cerebrovascular Disease, No. (%)	1 (5.9)	4 (20.0)	0.419
Chronic Kidney Disease, No. (%)	5 (29.4)	1 (5.0)	0.664
COPD, No. (%)	0 (0.0)	3 (15.0)	0.579
Connective Tissue Disease, No. (%)	3 (17.6)	0 (0.0)	0.635
Immune Deficiency, No. (%)	2 (11.8)	0 (0.0)	0.501
Liver Disease, No. (%)	2 (11.8)	0 (0.0)	0.501
ARDS Class, No. (%)			
COVID-19, PaO_2/FiO_2 300-400 mmHg	0 (0.0)	1 (5.0)	0.316
Mild ARDS, PaO_2/FiO_2 200-300 mmHg	8 (47.1)	12 (60.0)	0.254
Moderate ARDS, PaO_2/FiO_2 100-200 mmHg	6 (35.3)	7 (35.0)	0.006
Severe ARDS, PaO_2/FiO_2 <100 mmHg	3 (17.6)	0 (0.0)	0.635
APACHE II Score, Mean (SD)	25.8 (6.3)	19.9 (5.0)	0.499
SOFA Score, Median [IQR]	9 [8, 11]	9 [6.8, 10.3]	0.361
Compliance, ml/cmH_2O Mean (SD)	36.7 (8.1)	31.5 (10.2)	0.566
PEEP (cmH_2O), Median [IQR]	12 [10,13]	12 [10, 14.3]	0.378
Tidal Volume (mL/Kg), mean (SD)	5.8 (0.7)	6.2 (0.7)	0.603
PaO_2/FiO_2 ratio, mmHg	196 (143-224)	228 (190-253)	0.618
FiO_2, Mean (SD)	0.64 (0.23)	0.54 (0.16)	0.545
$PaCO_2$ (mmHg), mean (SD)	42 (37, 44)	43 (40,49)	0.614

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Minute Ventilation (l/min), Median [IQR]	7.8 [6.7, 9.0]	8.1 [6.5, 8.9]	0.089
Creatinine, mg/dL, Median [IQR]	1.13 [0.86, 1.73]	1.02 [0.80, 1.47]	0.207
D-Dimer	1844 [1082, 3992]	1080 [831, 2254]	0.098

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Table E5. Clinical description of neurological signs/symptoms and supportive imaging/diagnostic test results reported at 90 days since randomization in the modified intention-to treat cohort. CT: Computed Tomography; EMG: Electromyography; MRI: Magnetic Resonance Imaging; PT: Physical Therapy

Patient #	Clinical Description	Supportive Diagnostic Test Results
Treatment Group		
iNO 1	Bilateral hand weakness; right shoulder and arm weakness	None
iNO 2	Bilateral lower extremity weakness (left > right)	None
iNO 3	Left upper extremity and bilateral lower extremities weakness	EMG: evidence for subacute to chronic left upper trunk plexopathy, evidence for left ulnar neuropathy at the elbow, as can be seen in cubital tunnel syndrome.
iNO 4	Bilateral lower extremity weakness	Head CT: hemorrhagic conversion of right parieto-occipital and right cerebellar infarctions
Control Group		
C 1	Right ulnar sensorimotor neuropathy	EMG: Moderate motor and sensory damage to the median nerve at the wrist level and distal ulnar motor nerve damage in the hand.
C 2	Bilateral lower extremities neuropathic pain	None
C 3	Right shoulder neuropathic pain	None
C 4	Right lateral cutaneous nerve sensory neuropathy	None
C 5	Left-sided weakness (upper extremity > lower extremity)	Head CT: negative for acute intracranial hemorrhage or territorial infarction
C 6	Right shoulder weakness	MRI brain: Middle cerebral artery stroke during hospital admission. Punctate embolic appearing infarct in left precentral gyrus, (silent) right cerebellar hemisphere infarct.
C 7	Left ulnar sensorimotor neuropathy	None
C 8	Left foot drop	None
C 9	Lateral left thigh hypoesthesia	None

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C 10	Complete left foot paralysis; left lower extremity with reduced tone; left foot allodynia to touch; left upper leg and calf hyperesthesia to pinprick; left foot and ankle no vibration sensation; left foot no proprioception; left foot and lateral calf no temperature sensation; left foot and lateral calf no pinprick sensation; right upper extremity with positive Hoffman sign	EMG: evidence for severe, subacute-chronic left sciatic neuropathy.
C 11	Left foot drop; weakness in the left lower extremity; hypersensitivity to left lower extremity; right hand weakness	None
C 12	Right shoulder weakness and paresthesia; upper extremity and hand weakness; numbness of fourth and fifth fingers; left foot	EMG: evidence for diffuse right brachial plexopathy, with greatest involvement of the right lower trunk/medial cord. Evidence for generalized sensory predominant axonal polyneuropathy in all 4 extremities.
C 13	Lower extremities severe bilateral weakness (no anti-gravity movement of the left lower limb); no improvement despite physical therapy	None
C 14	Left hand weakness and paresthesias in the left forearm	EMG: evidence for severe left-sided ulnar neuropathy; evidence for right-sided ulnar neuropathy
C 15	Right foot drop and hypoesthesial right>left hip adductor weakness; right upper extremity and feet paresthesia; right-sided neck pain	MRI spine: no definitive signal abnormality
C 16	Right lower extremity weakness and pain	None
C 17	Left lower extremity paresthesias	None

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Table E6. Change in the PaO_2/FiO_2 Ratio at 48 hours with inhaled nitric oxide stratified by baseline PaO_2/FiO_2 ratio

	Sample Size		Mean Difference (95% CrI)
	Treatment (n=94)	**Controls (n=99)**	
<100 mm Hg	14 (14.9%)	21 (21.2%)	50.6 (5.1, 95.6)
100-200 mm Hg	43 (45.7%)	37 (37.4%)	32.5 (1.9, 63.1)
≥200 mm Hg	37 (39.4%)	41 (41.4%)	27.6 (-16.5, 72.3)

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STUDY PROTOCOL AND FOLLOW-UP QUESTIONNAIRE

High-Dose Inhaled Nitric Oxide Gas Therapy in Mechanically Ventilated Patients with Severe Acute Respiratory Syndrome in COVID-19.

Dates of study protocol to Massachusetts General Hospital Investigational Review Board:
Approval Created: February 28, 2020
Approved: March 18, 2020

Clinicaltrials.gov Protocol Number: NCT04306393

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  University of Alabama, Birmingham, AL, USA (Pankaj Arora, MD)
  Louisiana State University, Shreveport LA, USA (L Keith Scott, MD)
  Beth Israel Deaconess Medical Center, Boston, MA, USA (Somnath Bose, MD)
  Danderyds Sjukhuis, Danderyd, Stockolm, Sweden (Magnus Hedenstierna, MD)

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Synopsis and Study Scheme

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Title Inhaled Nitric Oxide Gas Therapy in Mechanically Ventilated Patients Affected by Severe Acute Respiratory Syndrome in COVID-19

Principal Investigator Lorenzo Berra, MD

Study Objective (s) To determine whether inhaled nitric oxide leads to a prompt rescue from severe hypoxemia in patients with severe COVID-19
To determine whether inhaled nitric oxide decreases severity of disease and mortality in patients with severe COVID-19

Study Design Multicenter randomized clinical trial

Study Population Screening and recruitment will only be performed in critical patients that are admitted in participating intensive care units.

Main selection criteria

Inclusion criteria
(1) Adult subjects ≥ 18 years-old
(2) ICU admission
(3) Intubation and mechanical ventilation
(4) Confirmed diagnosis of SARS-CoV2 by positive rt-PCR

Exclusion criteria
(1) Subjects intubated > 72 hours from initiation of the treatment gas
(2) Subjects enrolled in another interventional research study
(3) Past medical history of lung malignancy or pneumonectomy or lung transplant
(4) Tidal volume < 3 cc/Kg of IBW at the time of enrollment
(5) Severe burns (>40% total body surface area)
(6) Cardiac arrest with CPR for longer than 30 minutes
(7) Presumed severe deficit in cerebral function with fixed dilated pupil
(8) Receiving renal replacement therapy at the time of enrollment
(9) Impaired ability to ventilate without assistance (e.g. C5 or higher spinal cord injury, ALS, GBS, myasthenia gravis)
(10) History of malignancy or other irreversible disease/conditions with 6-month mortality >50%
(11) Patients not committed to full support at the time of enrollment
(12) Physician of record opposed to enrolling the patient due to perceived safety concerns or any condition that does not allow the protocol to be followed safely
(13) Patients who received inhaled nitric oxide prior to enrollment
(14) Admission unrelated to the COVID-19 disease

Total expected number of subjects 200

Coordinating Center Massachusetts General Hospital, Boston, MA, USA

Study Drug or Intervention Inhaled Nitric Oxide gas at 80 parts per million

Evaluation criteria Oxygenation evaluated as PaO_2/FiO_2 ratio

Primary Endpoints Difference in oxygenation at 48 hours between the two groups

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Secondary Endpoints	Time to reach normoxemia for at least 24 hours, defined by a $PaO_2/FiO_2 \geq 300$ mmHg Proportion of normoxemic patients in the two groups during the first 28 days after enrollment. Survival at 28 days and 90 days from enrollment.
Exploratory Endpoints	Measurement of oxygenation in the two groups daily until day 28 or hospital discharge. Proportions of patients needing renal replacement therapy during the first 28 days in the two groups. Proportions of patients needing mechanical support of circulation (i.e., ECMO, intra-aortic balloon pump, VADs) during the first 28 days in the two groups. Average number of days free from vasopressors during the first 28 days in the two groups Average number of ventilator-free day at 28 days in the two groups
Safety Endpoints	Methemoglobin levels to be kept under 5% Nitrogen dioxide (NO_2) levels to be kept under 2 ppm
Stopping Rule	The study will only be stopped if the interim analysis detects a significant increase of mortality with nitric oxide gas.
Statistical Considerations	Data analysis will be based on the intention-to-treat principle. For patients dying during the first 48 hours of treatment, the last available blood gas analysis will be used to assess the primary outcome. The sample size is based on the primary outcome. We hypothesize that NO gas therapy leads to an improvement of oxygenation due to amelioration of ventilation perfusion matching in patients affected with SARS-CoV2. A difference of 20% in PaO_2/FiO_2 ratio between the two groups at 48 hours from enrollment is clinically relevant. A previous study in ARDS patients ventilated according to standard of treatment (ARDSnet table) reported a PaO_2/FiO_2 ratio at 72 hours of 190 + 71 mmHg [1]. We hypothesize that iNO gas may increase the PaO_2/FiO_2 ratio by at least 20% in the treatment group. Assuming an alpha of 0.05 and a beta (power) of 0.9, we calculated with a two-sample means test a need for N1=91 and N2=91 patients. A 10% of dropouts after ICU discharge is foreseen and sample size is thereby increased to N=200 patients. Estimated sample sizes where calculated using Stata 14.1 software.
Duration of Study Period	March 10, 2020 – March 10, 2021 for patient enrollment March 10, 2021 – March 10, 2022 for data analysis

Figure 1 Study schema

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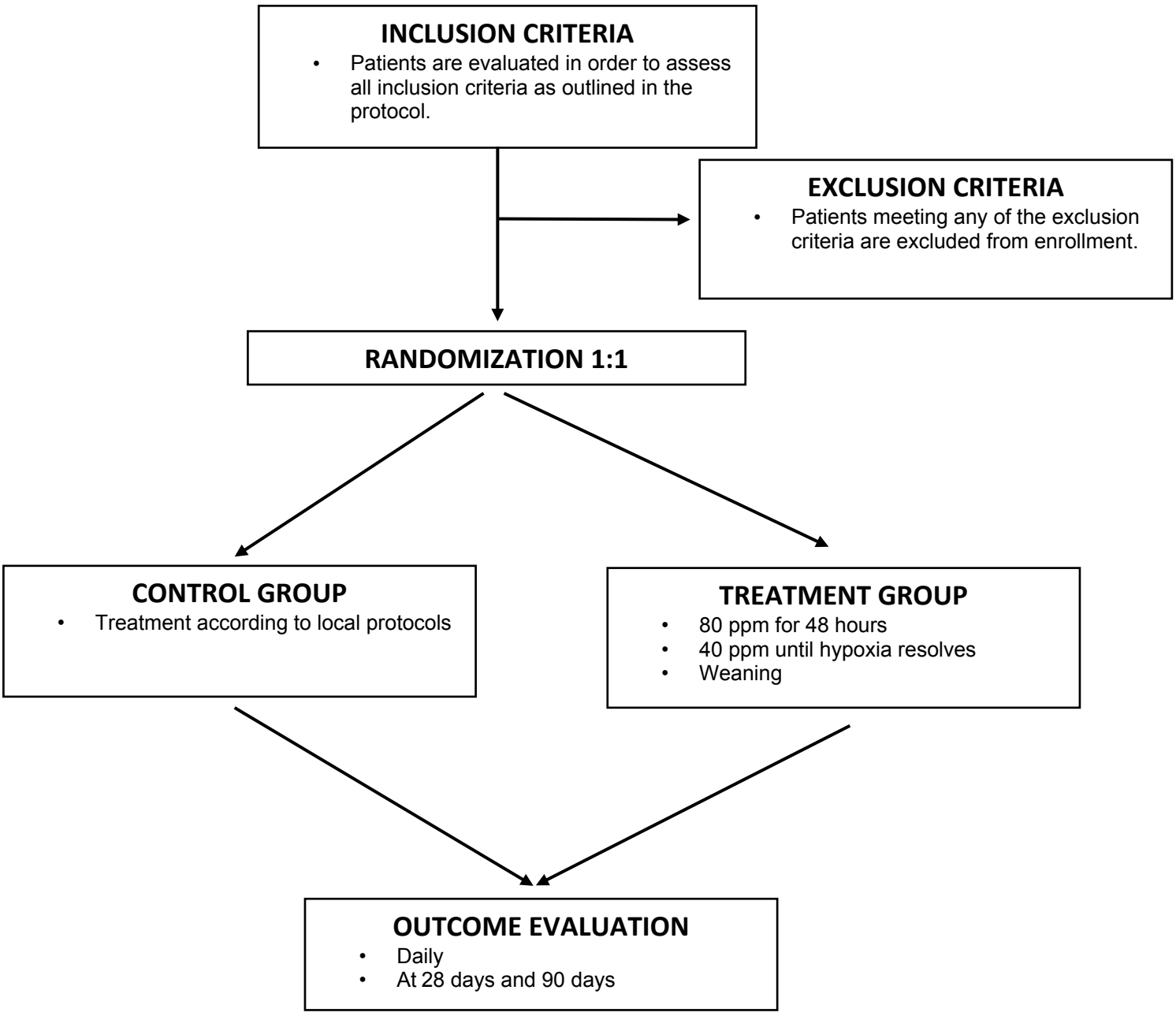

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I. BACKGROUND AND SIGNIFICANCE

Historical Background

The first pneumonia cases caused by a novel coronavirus-related infection (COVID-19) were identified in Wuhan, China in early December 2019 [2]. The genomic sequence of this new pathogen and a diagnostic real-time reverse transcriptase polymerase (rt-PCR) chain assay were published in January 2020. High-throughput sequencing of the bronchoalveolar samples from these patients has revealed the existence of this novel betacoronavirus that has been named Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) as both the virus and the clinical manifestations resemble that of a previous outbreak in 2003 (SARS-CoV) [3]. SARS-CoV-2 has so far infected more than 110,000 people worldwide with more than 4,000 deaths (3.6% of diagnosed patients). The World Health Organization has declared the SARS-CoV-2 infection as a public health emergency of international concern. [4]. The majority of the cases has been reported in China, but significant outbreaks are taking place in South Korea, Japan, Europe, Iran and more recently in USA [5]. In the human host, SARS-CoV-2 infection (COVID-19) may be asymptomatic or may cause a syndrome ranging from common cold to a severe pneumonia with acute respiratory syndrome and need of mechanical ventilation in intensive care unit (ICU). In a retrospective Chinese study on 138 consecutive patients admitted with COVID-19, the median time from clinical onset to hospital admission was 7 days, 26% of patients were admitted to the ICU and 61% of them met clinical criteria for acute respiratory distress syndrome (ARDS) [6]. Another retrospective Chinese study on critically ill patients with COVID-19 pneumonia showed that 67% of patients met ARDS criteria, with a mortality of 61.5% at 28 days. Reported casualties in the ICU are characterized of various profiles of multiorgan failure (81% of deceased patients had ARDS, 37.5% had AKI, 28% had cardiac injury and 28% had liver failure) [7]. Autoptic findings in a published clinical case showed features resembling those of coronavirus-related infections such as Severe Acute Respiratory Distress Syndrome (SARS) and Middle Eastern Respiratory Syndrome (MERS), including bilateral diffuse alveolar damage with fibro-myxoid exudates, desquamation of pneumocytes and hyaline membrane formation. Findings in cardiac and hepatic tissues may suggest the contribution of a viral infection as well[8].

To-date, no definitive treatment has shown an increase in survival and a decrease in the need for ventilatory support in patients with severe acute respiratory syndrome due to COVID-19. In responding to this epidemic, the treatment strategies should be safe, can be quickly pass through regulatory reviews and can be used on a massive scale at low cost.

Previous Clinical and Pre-clinical Studies Leading Up and Sustaining the Proposed Research

In clinical settings, nitric oxide (NO) gas has been approved by the US Federal Drug Administration for the treatment of pulmonary hypertension of the newborn in the presence of hypoxic respiratory failure. However, NO gas has been advocated as a rescue treatment in adults with hypoxic ARDS [9].

In 2004, during the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) outbreak, Chen et al. reported the use of inhaled NO gas (iNO) in six patients with severe symptoms. Treatment with iNO reversed pulmonary hypertension, improved remarkably severe hypoxemia and shortened the length of ventilatory support as compared to matched control patients with SARS-CoV [10]. In a subsequent *in-vitro* study, nitric oxide (NO) donors (e.g. S-nitroso-N-acetylpenicillamine) greatly increased the survival rate of SARS-CoV-infected eukaryotic cells, suggesting direct antiviral effects of NO [11]. Coronavirus responsible for SARS-CoV shares most of the genome of COVID-19 virus indicating the potential effectiveness of iNO therapy in these patients.

Rationale and Hypothesis

COVID-19 is highly contagious and responsible for thousands of casualties and is now spreading to many countries. The combined effect of the high transmission and the reported high incidence of severe disease in symptomatic patients poses a threat to healthcare systems involved in the outbreaks in different ways,

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including cumulative casualties, increased need for hospital and ICU beds causing work overload for all the healthcare staff and often forcing hospitals to shut down all elective surgical activity, high social and economic costs, dramatically reduction of the productivity by the rigorous quarantine requirements and strategies for curtailing disease spreading, which made economic pain that disrupts supply chains and stock markets.

Due to similarities with the coronavirus responsible for SARS and COVID-19, we hypothesize that in addition to improving the oxygenation of the severe cases, iNO gas retains potent antiviral activity against 2019-nCoV responsible for SARS-CoV-2. This study is designed to assess whether continuous delivery of the maximum approved dose of iNO as rescue therapy increases oxygenation and improves survival in patients with SARS-CoV-2.

We hypothesize that: 1) NO improves ventilation/perfusion mismatching (V/Q) and increases oxygenation in patients with severe respiratory syndrome in COVID-19; 2) Due to genetic similarities with the coronavirus responsible for SARS, we hypothesize that iNO retains potent antiviral activity against SARS-CoV2.

II. SPECIFIC AIMS

The proposed multicenter randomized parallel-arm controlled clinical trial will assess the efficacy of continuous inhalation of iNO at 80 ppm for 48 hours, followed by 40 ppm until the patient is no longer hypoxic, as determined by a $PaO_2/FiO_2 > 300$ mmHg.

Aim 1
To assess the change in oxygenation

Aim 2
To assess an improvement in survival, organ damage and weaning from mechanical ventilation in SARS-CoV-2 patients. This would indicate an effect that cannot be addressed only the amelioration of V/Q mismatching in the course of a disease characterized by pneumonia and, in most severe cases, multiorgan failure.

Aim 3
To assess the time at which a patient is negative for SARS-CoV2, tested by RT-PCR, reflective of a possible antiviral effect

III. SUBJECT SELECTION

a. Inclusion and Exclusion Criteria

Inclusion criteria
1. Adult patients, ≥ 18-year-old
2. Patients admitted to the ICU
3. Patients who are intubated and mechanically ventilated;
4. Confirmed diagnosis of SARS-CoV2 by positive rt-PCR;

Exclusion criteria
1. Patients intubated for more than 72 hours from initiation of the treatment gas
2. Subjects enrolled in another interventional research study

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3. Past medical history of lung malignancy or pneumonectomy or lung transplant
4. Tidal volume < 3 cc/Kg of IBW at the time of randomization
5. Severe burns (>40% total body surface area)
6. Cardiac arrest with CPR for longer than 30 minutes
7. Presumed severe deficit in cerebral function with fixed dilated pupil
8. Receiving renal replacement therapy at the time of enrollment
9. Impaired ability to ventilate without assistance (e.g. C5 or higher spinal cord injury, ALS, GBS, and myasthenia gravis)
10. History of malignancy or other irreversible disease/conditions with 6-month mortality >50%
11. Patients not committed to full support at the time of enrollment
12. Patients who received inhaled nitric oxide prior to enrollment
13. Admission unrelated to the COVID-19 disease
14. Physician of record opposed to enrolling the patient due to perceived safety concerns or any condition that does not allow the protocol to be followed safely

Source of Subjects and Recruitment Methods

Given the rapid spread of the disease characterized by severe refractory hypoxemia, its high mortality and the absence of a targeted therapy, we propose this protocol to all interested centers treating SARS-CoV-2 cases. This study targets a population of critically ill patients. Screening for inclusion criteria will be performed exclusively on patients admitted to participating ICUs.

IV. SUBJECT SELECTION

At ICU admission, the clinicians will screen patients for eligibility after inclusion criteria are met and within 72 hours. No patient in the targeted population is capable of taking part in the consent process. Therefore, a study physician will approach the patient's surrogate (court-appointed guardian, healthcare proxy or attorney, spouse, adult child or other close family member) in case eligibility criteria are met. If the patient's surrogate agrees, an investigator will be contacted to discuss further details of the study.

Procedure for Obtaining Informed Consent

Given the nature of the study, it is both appropriate and necessary to enroll subjects incapable of providing consent due to coronavirus infection. Given that this research involves greater than minimal risk but presents the prospect of direct benefit to individual subjects, informed consent for participation may be obtained from a legally authorized representative (surrogate) for those who are incapable of providing informed consent.

The following categories of surrogates (listed in general order of preference) may provide consent in writing on behalf of potential subjects incapable of providing informed consent:

(1) court appointed guardian with specific authority to consent to participation in research or authority to make healthcare decisions for a class of diagnostic and therapeutic decisions inclusive of this research protocol;
(2) healthcare proxy/person with durable power of attorney with specific authority for making healthcare decisions inclusive of this research protocol;
(3) spouse, adult child, or other close family member who knows the subject well and has been involved in their care.

If the surrogate agrees to discuss the study with the investigators, an investigator of this protocol will go through the details of the study. The consent process will utilize a written informed consent form but will include obtaining verbal consent, consistent with current infection control procedures in the hospitalized population of subjects affected by SARS-CoV2 infections. A witness will be present when consent is obtained, and the consent process will be documented in the study record.

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Assent of subjects will be a requirement for participation in the research unless the subject is incapable of giving assent due to his/her medical condition. If the individual objects to participation, s/he should not be enrolled. When surrogate consent is relied upon, the Investigator must ensure that the surrogate understands that his or her decisions should be based on "substituted judgment." This means that the decision reflects a potential subject's own views when s/he had the capacity to express them. If a potential subject did not previously express a view on the matter, the surrogate should make the decision based on the potential subject's best interests.

Consent can be withdrawn at any time. The investigators will access Personal Medical Information (PMI) for study purposes.

Eligible subjects not participating in this study will receive standard care without any repercussions.
We will ensure that the surrogate understands that his/her decision reflects a potential subject's own views when she/he had the capacity to express them. Partners Human Research Committee (PHRC) order of preference will be followed, and investigators will document the relationship of the surrogate to the subject in the appropriate research record.

Of note, during the conduct of the study new information suggests that remdesivir may speed up a patient's recovery and discharge from the hospital and can be used in the hospital for severe COVID-19 infection. Although receipt of remdesivir does not preclude patients from participating in the trial, information about this drug has been incorporated into the consent form as a means to notify participants of this new information. Thus, newly enrolled participants will be made aware of this information from the consent form. For patients that were previously enrolled we will provide the patient/legally authorized representative with an addendum in person or via email. If it is not possible to send this information electronically, a paper copy will be mailed to the subject's home address. This addendum is available both in English and in Spanish.

Treatment Assignment and Randomization

Randomization will occur through random allocation sequence generated by a computerized random generation program on a web-based platform provided by REDCap. Parallel allocation to treatment or control group will occur with a 1:1 ratio. Randomization will be stratified by age (> 60 years), sex, and center in order to accurately randomize across risk strata. Randomization will be performed centrally by MGH for all sites.

Patients will be randomly allocated to the treatment group with NO or to the control group in which no treatment will be delivered. The intervention will consist in the administration of NO via a non-invasive ventilation circuit. A tank delivering the gas will be connected to a certain point in the circuit. In the control group the patient will receive only air and supplemental oxygen coming from the ventilator.

Blinding

The study will be blinded only to participating subjects.

Clinical Sites Participating in Study

This is a multicenter clinical trial in which Massachusetts General Hospital is going to be the coordinating center. A number of centers, both within the United States and internationally, are expected to participate. Each added site will be communicated to Partners Human Research Committee (PHRC) with an amendment as soon as it is approved by the peripheral center's local IRB and before that center starts to recruit patients. The study team can provide the peripheral centers with the documents from PHRC as templates. However, each document provided by the peripheral center must comply with its local IRB. Unique credentials to REDCap for each peripheral center will be provided only after the peripheral center has been fully recognized and activated by both local IRB and PHRC.

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V. STUDY PROCEDURES

The study was reviewed and approved by the MGH Anesthetist-in-Chief:

- Oluwaseun Johnson-Akeju, MD, investigator

The study was reviewed and approved by the leadership of the Respiratory Care service that will be in charge for delivering the treatment:

- Lorenzo Berra, MD, Medical Director, investigator
- Robert Kacmarek, RRT, PhD, Director, investigator

Study Visits and Parameters to be Measured

Time of treatment administration is variable and depends on when the patients recover from hypoxia (defined by a $PaO_2/FiO_2 > 300$mmHg), while outcomes will be evaluated daily until 28 days. Vitals, blood gas analysis, ventilator settings and vasoactive drug administration will be recorded. The last follow-up will be performed at 90 days from inclusion. If a patient is not in the hospital at day 28 or 90, members of the study team may contact participants via phone to assess mortality status. During this phone call, we will assess measures of function including the PROMIS Questionnaire of Global Health and Katz Activities of Daily Living Score. Activities of daily living will also be reported by the patient or their LAR at the time of enrollment.

Drugs to be Used

Inhaled NO will be delivered continuously via the ventilator circuit at 80 ppm for 48 hours. After 48 hours, this will be reduced to 40 ppm. Inhaled NO will be continuously administered at 40 ppm until the subject is no longer hypoxemic. No longer hypoxemic will be defined as the time in which they reach a sustained $PaO_2/FiO_2 \geq 300$ mmHg for at least 24 hours after the dose was reduced. At that time, the study drug will be weaned. To do this safely, the study gas will be reduced every 4 hours in a stepwise fashion starting from 40 ppm to 20, 10, 5, 3, 2 and 1 ppm. In the case of hypoxemia (SpO2< 92%) or acute hypotension (systolic blood pressure < 90 mmHg) during weaning, iNO should be increased to the prior higher concentration for 4 hours.

Devices to be Used

Methemoglobin will be continuously monitored non-invasively with a dedicated pulse oximeter system (Spmet, Masimo, Irvine, California). NO delivery will be decreased if the blood methemoglobin level exceeds 5%.

Nitric Oxide Weaning

Based on concerns for resource utilization of patients with markedly improved oxygenation, below we have outlined specific procedures in which weaning may be safely initiated if necessary.

The Standard Per Protocol Weaning

Inhaled NO will be delivered continuously via the ventilator circuit at 80 ppm for 48 hours.

- After 48 hours this will be reduced to 40 ppm.
    - If $PaO_2/FiO_2 \geq 300$ mmHg for at least 24 hours, we will commence weaning of NO
    - After 4 hours wean NO from 40 ppm to 20 ppm
    - After 4 hours wean NO to 10 ppm
    - After 4 hours wean NO to 5 ppm
    - After 4 hours wean NO to 3 ppm
    - After 4 hours wean NO to 2 ppm
    - After 4 hours wean NO to 1 ppm
    - After 4 hours cease NO

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- In the case of hypoxemia (SpO2< 92%) or acute systemic hypotension (systolic blood pressure < 90 mmHg) during weaning, iNO should be increased to the prior and higher concentration for 4 hours.

*Fast Weaning from Nitric Oxide Gas **After** the First 48 Hours of Intubation*
Inhaled NO will be delivered continuously via the ventilator circuit at 80 ppm for 48 hours.

- After 48 hours of breathing NO at 80 ppm, or at any point during the weaning protocol described above, but after 48 hours of 80 ppm of NO, weaning NO according to local NO weaning protocols may commence, if:
    - The ICU team believes that extubation will occur within a 48-hour period, and
    - The local Principal Investigator needs to be informed, and
    - Sustained (more than 24 hours) $PaO_2/FiO_2 \geq 200$ mmHg, and
    - Hemodynamics do not require infusion of more than 10 micrograms of norepinephrine or 2 inotropic-vasopressors to achieve a systolic blood pressure > 100 mmHg and mean arterial pressure > 60 mmHg
- In the case of hypoxemia (SpO_2< 92%) or acute hypotension (systolic blood pressure < 90 mmHg) during weaning, iNO should be increased to the prior higher concentration.

*EXTRA-Fast Weaning from Nitric Oxide Gas **Within** the First 48 Hours of Intubation*
If the ICU team believes that a newly intubated patient will need to be extubated within the next 12 hours, weaning NO according to a local NO weaning protocol may commence after:

- The local Principal Investigator is informed, and
- The Patient is on spontaneous ventilation with a $PaO_2/FiO_2 \geq 300$ mmHg, PEEP<10 cm H_2O and $FiO_2 \leq 50\%$, and
- Hemodynamic treatment does not require infusion of more than 10 micrograms/min of norepinephrine or 2 inotropic-vasopressors to achieve a systolic blood pressure > 100 mmHg and mean arterial pressure > 60 mmHg

In the case of hypoxemia (SpO_2< 92%) or acute hypotension (systolic blood pressure < 90 mmHg) during weaning, iNO should be increased to 80 ppm and 48 hours of ventilation at 80ppm and then commence NO weaning as per protocol or by the fast-weaning protocol.

Re-initiation of NO Gas Therapy After NO Discontinuation
NO should be re-initiated at 40 ppm and weaned according to weaning protocols if the patient meets any of the following criteria:

- P/F < 200 mmHg, or
- Requirement of paralysis, or
- Requirement for pronation.

<u>Echocardiograms</u>
To assess cardiac function, we will perform a transthoracic echocardiogram in consented patients. These point of care ultrasounds will be performed by members of the study team who will otherwise be in the room. Between use the machine will be appropriately cleaned in accordance with local infection control policies for patients with COVID-19.

Patients in both groups will receive an assessment at baseline. To assess whether there are changes over time, we will perform repeat echocardiographic evaluations each time the dose is changed in the nitric oxide group. Given that this data will be used in an exploratory analysis to assess cardiac function, we will not consider it a protocol deviation if the patient refuses a particular exam, or if the study staff are not

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available to perform the exam. Occurrence of an echocardiogram exam and its associated indices (ex. right ventricular systolic pressure) will be recorded in the study case report form.

Data to be Collected

Data collection at baseline includes:

- Age
- Gender
- Height
- Weight
- Race/ethnicity
- Comorbidities
- Time elapsed since intubation
- Apache II Score

Data to be collected daily until day 28 includes:

- Survival (alive – not alive)
- Blood Gas analysis (pO2, pCO2, pH, HCO3)
- Ventilation (type of ventilation, FiO2 %, plateau pressure, PEEP, tidal volume, respiratory rate)
- Hemodynamics (cardiac index, invasive arterial pressure, pulmonary pressure, central venous pressure)
- Vasopressor requirement
- Supportive interventions (need for mechanical support of circulation, failure to wean from mechanical ventilation, renal replacement therapy, use of veno-venous/-arterial extracorporeal membrane oxygenation [ECMO])
- Study drug administration characteristics
- Occurrence of adverse events

Specimen Collection and Processing

In an exploratory analysis we will collect several biospecimens from patients in both groups. These samples will be used for an exploratory analysis to evaluate both viral PCR and the microbiome of enrolled participants. Timing of the specimen collection is detailed below:

MICROBIOME	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 7**	**Day 14 (± 3d)**	**Day 21 (± 3d)**	**Day 28 (± 3d)**	**TOTAL**
Nasal/oropharyngeal Swab	X					X	X	X	X	
Saliva	X					X	X	X	X	
Sputum	X					X	X	X	X	
Stool	X					X	X	X	X	
Blood in Heparin	8 mL					8 mL	8 mL	8 mL	8 mL	**40 mL**
Viral PCR	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**TOTAL**
Sputum	X	X	X	X	X	X				
Blood in EDTA	2.0 mL	2.0 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL				**10 mL**

Saliva or nasal/oropharyngeal swabs to be collected at the time of study enrollment and weekly thereafter until hospital discharge. Swabs will be used for SARS-Cov-2 viral load testing or microbiome assessment. For intubated patients, oral secretions suctioned as part of general care will be collected. If the patient is

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no longer intubated at the time of collection, participants will be given a specimen collection cup and asked to spit into the cup. Sputum will be collected in intubated participants by suctioning their endotracheal or tracheostomy tubes. Intubated patients are routinely suctioned as part of their routine pulmonary clinical care. Non-intubated participants will be asked to spontaneously expectorate into a specimen cup along the same approach as specimens are collected as part of routine clinical testing. Stool will also be collected at the time of study enrollment and weekly thereafter until hospital discharge in order to assess the microbiome. Intubated patients will have stool collected during routine cleaning care or if they have pre-existing rectal tubes inserted, via their rectal tube. Non-intubated participants will be given a "toilet hat" type disposable toilet accessory and a specimen collection cup for stool collection. Blood will also be collected in enrolled patients. Blood will be used for assessment of immune markers and cytokine levels. If patients have an indwelling vascular device (such as a PICC line, central or arterial line), blood will be collected using an aseptic technique. If patients do not have an indwelling vascular device, we will do our best to coordinate blood draw as part of a blood draw for routine clinical care. No samples will be collected after a patient is discharged from the hospital. Further, no individual results will be returned to participants, nor will results from the specimen collection become part of a participant's medical record.

Blood, sputum, and stool will be initially processed in Warren 411/412, which is a BSL2+ certified laboratory for processing diverse types of COVID samples under Biosafety Protocol # 2020B000110, Mechanisms of Immune Dysregulation in COVID-19 disease. Plasma and PBMCs will be obtained from whole blood for subsequent flow cytometry assessment of immune profiles and multiplex cytokine analysis. Subsequently samples may be transferred to Charlestown Navy Yard, Building 149, Room 142 for BSL-2 work. Sputum and stool will be aliquoted and frozen at -80C until transport by an approved courier for BSL2+ samples to Dr. Jonathan Li's laboratory for viral load testing. An approved SARS-CoV-2 deactivating agent such as Zymo DNA/RNA shield will be added to aliquoted sputum and stool, then transported to Dr. Lai's CNY-149 lab for DNA extraction and subsequent shotgun metagenomics sequencing.

Of note, the decision to participate in the biospecimen collection will be an optional component of study participation. Patients will be able to participate in the main trial regardless of whether or not they consent to specimen collection. Further, patients (or their LARs) will be given the opportunity to consent to specific types of specimen collection. Given that this data will be used in an exploratory analysis to assess viral PCR and the patient's microbiome, we will not consider it a protocol deviation if the patient refuses a particular specimen collection, or if the study staff are not available to perform the collection/processing. Occurrence of specimen collection will be recorded in the study case report form. For patients in whom the study staff were not available to perform collection or processing of blood samples, blood samples may be obtained on study participants through the Partners Biobank. These samples will be used for assessment of immune markers and cytokine levels. Data obtained from blood samples through the Partners Biobank will be merged with data obtained from blood samples collected by study personnel.

VI. BIOSTATISTICAL ANALYSIS

Study Endpoints

The **primary endpoint** is the measure of the difference in oxygenation between the treatment and the control group at 48 hours. Oxygenation will be measured in terms of PaO_2/FiO_2 ratio.

Secondary endpoints are:

1. Time to reach normoxemia for at least 24 hours, defined by a $PaO_2/FiO_2 > 300$ mmHg among those with severe acute respiratory distress syndrome.
2. Proportion of normoxemic patients in the two groups during the first 28 days after enrollment.
3. Survival at 28 days and 90 days from enrollment.

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Exploratory endpoints are:

1. Measurement of oxygenation in the two groups daily until day 28 or ICU discharge, whichever comes first
2. Proportions of patients needing renal replacement therapy during the first 28 days
3. Proportions of patients needing mechanical support of circulation (i.e., ECMO, intra-aortic balloon pump, VADs) during the first 28 days
4. Average number of days free from vasopressors during the first 28 days
5. Average number of ventilator-free day at 28 days
6. Average time to SARS-CoV-2 RT-PCR negative in upper respiratory tract specimen assessed within the first 28 days
7. Average number of ICU-free days at 28 days
8. Hospital length of stay (LOS)

Statistical Methods

Data analysis will be based on the intention-to-treat principle. For patients dying during the first 48 hours of treatment, the last available blood gas analysis will be used to assess the primary outcome. For the proposed trial, we anticipate that no participants will miss the primary evaluation.

Demographic and clinical characteristics will be presented as number (percentage), mean (SD), or median (interquartile range [IQR]). Comparisons between groups will be made using the $\chi 2$ test or Fisher's exact test for categorical variables and T-test or Wilcoxon's rank-sum test for continuous variables as appropriate. Effect sizes will be described with the probability of more favorable outcome (probabilistic index) and 95% CI will be calculated. The primary endpoint will be compared using T-test or Mann-Whitney U as appropriate. Time to reach normoxia will be compared within the two groups with a T-Test or Mann-Whitney U test, as appropriate. To exclude or confirm that the expected benefit in oxygenation by NO may be evident during the first days of treatment and then decrease we will consider the proportion of SARS-CoV-2 free patients during 28 days and compare the two groups in terms of treatment success with a log-rank test. Survival curves will be generated via the Kaplan-Meier method and compared with a log-rank test. Rates of organ dysfunction will be compared using Fisher's exact test. Ventilator-free days, ICU-free days, days free of vasopressors and time to negative SARS-CoV2 rt-PCR will be compared using the T-Test or Mann-Whitney U test, as appropriate. Fisher's Exact test will be used to estimate treatment differences in the incidence of each specified adverse event. No adjustments will be made for multiple hypothesis evaluations of safety endpoints. For all analyses, a 2-sided alpha threshold of 0.05 will be considered significant. To adjust for additional influent factors not involved in the randomization process such as ARDS severity and co-administration of other treatments, we will perform a multivariable logistic regression analysis on the primary outcome.

Power Analysis

We hypothesize that NO gas therapy leads to an improvement of oxygenation due to amelioration of ventilation perfusion matching in patients affected with SARS-CoV2. A difference of 20% in PaO_2/FiO_2 ratio between the two groups at 48 hours from enrollment is clinically relevant. Available data on oxygenation in SARS-CoV and SARS-CoV2 patients in ICU are limited [6] [7] [12]. A previous study in ARDS patients ventilated according to standard of treatment (ARDSnet table) reported a PaO_2/FiO_2 ratio at 72 hours of 190 + 71 mmHg [1]. We hypothesize that iNO gas may increase the PaO_2/FiO_2 ratio by at least 20% in the treatment group. Assuming an alpha of 0.05 and a beta (power) of 0.9, we calculated with a two-sample means test (Satterthwaite's t-test assuming unequal variances) a need for N1=91 and N2=91 patients. A 10% of dropouts after ICU discharge is foreseen and sample size is thereby increased to N=200 patients. Estimated sample sizes were calculated using Stata 14.1 software. We hypothesize that NO gas therapy leads to an improvement of oxygenation due to amelioration of ventilation perfusion matching in patients affected with SARS-CoV2. A difference of 20% in PaO_2/FiO_2 ratio between the two groups at 48 hours from enrollment is considered to be clinically relevant.

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VII. RISKS AND DISCOMFORTS

Drugs Effects and Toxicities

NO reacts with oxygen to form nitrogen dioxide (NO_2), which may cause airway inflammation and damage to lung tissues. Moreover, NO oxidizes ferrous Hb to form Met-Hb, which is unable to transport and release oxygen to tissues. [13] The binding of NO to Hb is a rapidly reversible reaction, with a half-life of 15–20 min after NO discontinuation. The side effects and adverse events related to iNO delivery are well-reported in the literature. Based on the present literature and Food and Drug Administration reports, the risks of breathing iNO at 80 ppm for 24 hours are minimal when Met-Hb levels and NO/NO_2 delivery levels are carefully monitored [14]. In the present trial, iNO is administered and continuously monitored by the respiratory care team according to MGH/BWH clinical protocols. The NO_2 will be monitored and maintained at levels below 2 ppm. Met-Hb is continuously monitored by non-invasive co-oximetry. If Met-Hb levels reaches 5%, the concentration of NO delivered will be halved and closely monitored until a reduction occurs. If Met-Hb levels persist above 5%, iNO will be progressively halved until a reduction below 5% occurs.

Performing a transthoracic echo is not associated with any additional risks to the patient. This is a safe, painless, non-invasive technology that is routinely used in hospitalized patients.

Risks of the study specimen collection procedures are primarily associated with potential discomfort. Collection of a nasal swab may cause the patients nose/throat to itch or to initiate a sneeze. Collection of the sputum and saliva samples may lead to a transient cough. Or may make the patients mouth feel dry. Risks of blood collection can include pain or bruising at the site of the blood draw, or lightheadedness. We will use several strategies to mitigate these risks. This includes collection of the specimens during routine clinical care whenever possible. Blood collection will be taken from existing indwelling lines whenever they have been placed clinically to minimize any discomfort. Further, no blood samples will be collected if a patient hemoglobin is less than 8. Additionally, we have limited the amount of blood to be collected to ensure safety of participants.

Psychosocial (Non-medical) Risks

Assignment to the treatment group only implies a different composition of inhaled gas mixture in an intubated and mechanically ventilated patients. The treatment does not cause any increase in psychosocial risk. Although remote, the possibility of a breach in data security cannot lead to the spread of sensitive interfere that would interfere with the subject quality of life, particularly referring to data that would affect the subject's social life and eventual applications in the job market.

VIII. POTENTIAL BENEFITS

Potential Benefits to Participating Individuals

Subjects with severe hypoxic respiratory failure may benefit from increased oxygenation conferred by iNO. This benefit alone may result in an increased survival and/or reduced need for extracorporeal membrane oxygenation. The putative anti-viral effect of iNO may ultimately confer a survival benefit, a reduction in multiorgan failure, and/or a reduced need for supportive interventions.

Subjects will not be prevented in any case from receiving any other potential life-saving targeted treatments.

Potential Benefits to Society: An Increased Understanding of the Disease Process

In countries in which this overt pandemic is reaching its peak of incidence, the local healthcare systems are struggling with providing ICU beds for severely ill subjects. Any positive effect on survival and length of stay would help tackling this unprecedented, healthcare burden. In addition, potentially demonstrating a treatment benefit may trigger the scientific community to investigate the mechanism of action. Ultimately,

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these efforts may lead to greater use, and potentially earlier use, of nitric oxide, conferring a greater impact at treating COVID-19 disease.

IX. MONITORING AND QUALITY-ASSURANCE

Independent Monitoring of Source Data

At MGH, informed consent forms, case report forms, and data will be reviewed by the principal investigator throughout the course of the study. At other sites, informed consent forms and source documents will be maintained locally and the site Principal Investigator will be responsible for reviewing and ensuring the integrity of the data. The Coordinating Center (MGH) may ask sites to verify source data that is entered into the centralized study data for accuracy.

Steering Committee (SC)

The SC will assist with the development of the study protocol and will oversee the conduct of the trial and final data analysis.

Safety Monitoring (Data Safety Monitoring Board)

An interim analysis for safety will be performed after the enrollment of 25 patients, as recommended by PHRC. A Data Safety Management Board (DSMB) comprised of three members will supervise the frequency and severity of adverse events to ensure safety of the study subjects. The safety data that will be assessed includes investigator-reported adverse events, such as adverse reactions to NO, high levels of NO_2 or metHb. Maintenance of patient confidentiality will also be assessed. At any time, the DSMB may require a safety analysis to guarantee the patients' safety.

Members of the Data Safety management Board (DSMB) consist of a microbiologist, a physician with great expertise in NO and an intensive care unit physician. Names and contacts of the DSMB members are:

Richard M Pino, MD, PhD
Professor and Head, Department of Anesthesiology
Louisiana State University Health Sciences Center
Email: rpino1@lsuhsc.edu

John G Thomas, PhD
Professor Emeritus in Microbiology
West Virginia University
Email: jgthomas@hsc.wvu.edu

Carl Nathan, MD
R.A. Rees Pritchett Professor of Microbiology
Weill Cornell Medicine
Email: cnathan@med.cornell.edu

Outcomes Monitoring

The primary outcome is the improvement of arterial oxygenation at 48 hours from enrollment. If a patient dies before the initiation of the gas, the patient will not enter the trial. If a patient dies during the first 48 hours of treatment, the last available blood gas analysis will be used. Levels of oxygenation will be calculated by the PaO_2/FiO_2 ratio. Patients will be followed for 90 days from enrollment.

Secondary outcomes include: (1) time to reach normoxia defined by a $PaO_2/FiO_2 > 300$ for at least 24 hours during the first 28 days after enrollment. If a patient dies before day 28, the patient will be considered as "never recovered"; (2) proportion of SARS-CoV-2 patients that reach normoxia during the first 28 days

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after enrollment. If a patient dies before day 28, the patient will be considered as "never recovered"; (3) Survival at 28 days and 90 days from enrollment.

The following data will be collected to assess exploratory outcomes, such as: (1) Daily oxygenation in the two groups until day 28; (2) Need for new renal replacement therapy during the first 28 days; (3) Mechanical support of circulation (i.e., ECMO, intra-aortic balloon pump, VADs) during the first 28 days; (4) Days free of vasopressors during the first 28 days; (5) Ventilator-free day at 28 days; (6) Time to SARS-CoV-2 RT-PCR negative in upper respiratory tract specimen (assessed within the first 28 days); (7) ICU-free days at 28 days; (8) overall hospital length of stay.

Adverse Event Reporting Guidelines

In accordance with PHRC policy on Adverse Event Reporting and Unanticipated Problems Involving Risks to Subjects, the Principal Investigator (Lorenzo Berra, MD) will report adverse events or other unanticipated problems to the DSMB and to the PHRC within 5 working days or 7 calendar days of the date the investigator first becomes aware of the problems. This includes adverse events that unexpected and related or possibly related to the study intervention. Additional expected or unrelated mild or moderate adverse events will be presented in progress reports at continuing reviews.

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Baseline Characteristics

Instructions to the Person Recording Data: This questionnaire should be verbally administered immediately following consent. Scoring should be recorded but not discussed with the subject.

Katz Activities of Daily Living Score

The following questions ask about the patient's health **before** they were experiencing any COVID symptoms. Please indicate whether they were able to complete each of the following tasks independently or if they required assistance:

Prior to being in the hospital, was your loved one [the patient] able to independently…	**Score** *Independence = 1 Point, Dependence = 0 Points*	
Bathe her/himself completely or need help in bathing only a single part of the body such as the back, genital area or disabled extremity?	_____	☐ No Response
Get clothes from closets and drawers and put on clothes and outer garments completely with fasteners? *This excludes tying shoes.*	_____	☐ No Response
Go to the toilet, get on and off, arrange her/his clothes, and clean her/his genital area without help?	_____	☐ No Response
Move in and out of bed or a chair unassisted? *Mechanical transfer aids are acceptable.*	_____	☐ No Response
Have complete control over urination and bowel movements?	_____	☐ No Response
Get food from her/his plate into her/his mouth without help. *Preparation of food may be done by another person.*	_____	☐ No Response
Total Katz ADL Score *The calculation should sum the points from each category.*	_____	

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28 Day Follow Up

Instructions to the Person Recording Data: This questionnaire should be verbally administered to the patient [or their LAR] during the 28 day follow up phone call. If the patient is still inpatient the questions can be asked in person. Scoring should be recorded but not discussed with the subject.

To Be Completed By the Study Team

Is the patient still in the hospital on day 28? ☐ No ☐ Yes: *Please skip to section 'Katz Activities of Daily Living'*

Phone Script

Hello, may I please speak with [*subject name, or LAR*]. My name is [*study team member*] and I am calling about a research study you [*or your loved one*] participated in when you were hospitalized. The purpose of the study was to investigate the use of inhaled nitric oxide in patients with respiratory distress and COVID19. Part of this study involves a follow up phone call to subjects 28 days after hospital discharge. That is why I am calling you.

I would like to ask you a few questions about how you [*or your loved one*] are doing. This should take about five minutes.

If speaking with patient, vital status is apparent. If speaking with a family member ask the following: Is your loved currently at home or in a health care facility?

☐ Alive, Confirmed with Patient ☐ Alive, Confirmed with LAR

☐ Died - Date of Death: _____ / _____ / _____

Were you admitted to a rehab facility for any reason? ☐ Yes ☐ No

If yes: How many days did you spend in rehab? _____ days ☐ Still in rehab

Were you readmitted to the hospital for any reason? ☐ Yes ☐ No

If yes: How many days did you spend in the hospital when you were readmitted? _____ days

☐ Still admitted

What were you readmitted for, or what was your diagnosis?

_____

During your readmission, were you mechanically ventilated or breathing on a ventilator?

☐ Yes ☐ No

Have you developed a new requirement for dialysis? ☐ Yes ☐ No

Did you experience any of the following conditions after you left the hospital? *Please indicate all that apply after your first hospital admission.*

☐ None ☐ Myocardial Infarction (*heart attack*) ☐ Stroke ☐ Pneumonia (*second episode)*

☐ New requirement for oxygen ☐ New disease/diagnosis:

_____

Did you experience any of the following alterations after you left the hospital? *Please indicate all that apply after your first hospital admission.*

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☐ None ☐ Being frequently asleep ☐ Having difficulty concentrating
☐ Altered consciousness ☐ Disorganized thinking ☐
Frank motor deficit

Katz Activities of Daily Living Score
The following questions ask about your [or your loved ones] health **today**. Please indicate whether you are able to complete each of the following tasks independently or if you require assistance.

Are you [or your loved one] able to independently…	**Score** *Independence = 1 Point, Dependence = 0 Points*	
Bathe completely or need help in bathing only a single part of the body such as the back, genital area or disabled extremity?	_____	☐ No Response
Get clothes from closets and drawers and put on clothes and outer garments completely with fasteners? *This excludes tying shoes.*	_____	☐ No Response
Go to the toilet, get on and off, arrange your clothes, and clean the genital area without help?	_____	☐ No Response
Move in and out of bed or a chair unassisted? *Mechanical transfer aids are acceptable.*	_____	☐ No Response
Have complete control over urination and bowel movements?	_____	☐ No Response
Get food from the plate into the mouth without help. *Preparation of food may be done by another person.*	_____	☐ No Response
Total Katz ADL Score *The calculation should sum the points from each category.*	_____	

Instructions to the Person Recording Data: If the LAR is completing the questionnaire, please skip to the section 'Completion'. If the patient has recovered and is completing this assessment, please complete the PROMIS questions.

PROMIS Questionnaire of Global Health
Next I am going to ask you ten questions about your overall health **today**.

	Excellent 5	**Very Good** 4	**Good** 3	**Fair** 2	**Poor** 1	**No Response**
1. In general, would you say your health is	☐	☐	☐	☐	☐	☐

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2. In general, would you say your quality of life is	☐	☐	☐	☐	☐	☐
3. In general, how would you rate your physical health	☐	☐	☐	☐	☐	☐
4. In general, how would you rate your mental health, including your mood and your ability to think	☐	☐	☐	☐	☐	☐
5. In general, how would you rate your satisfaction with your social activities and relationships	☐	☐	☐	☐	☐	☐
	Completely 5	**Mostly** 4	**Moderately** 3	**A Little** 2	**Not at All** 1	**No Response**
6. To what extent are you able to carry out your everyday physical activities such as walking, climbing stairs, carrying groceries, or moving a chair	☐	☐	☐	☐	☐	☐
	None 5	**Mild** 4	**Moderate** 3	**Severe** 2	**Very Severe** 1	**No Response**
7. In the past 7 days, how would you rate your pain on average	☐	☐	☐	☐	☐	☐
8. In the past 7 days, how would you rate your fatigue on average	☐	☐	☐	☐	☐	☐
	Excellent 5	**Very Good** 4	**Good** 3	**Fair** 2	**Poor** 1	**No Response**
9. In general, please rate how well you carry out your usual social activities at work, at home, and in your community. How well do you fulfill your responsibilities as a parent, child, spouse, employee, friend, etc	☐	☐	☐	☐	☐	☐
	Never 5	**Rarely** 4	**Sometimes** 3	**Often** 2	**Always** 1	**No Response**

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10. In the past 7 days, how often have you been bothered by emotional problems such as feeling anxious, depressed, or irritable	☐	☐	☐	☐	☐	☐
PROMIS Global Health Score	**Sum of Points:** _____		**Overall Score:** *([Sum*10] / Number of Answers)* _____			

Completion

This completes my questions today. I'll be calling you again in two months to see how you are doing. Thank you again for participating in this research study!

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90 Day Follow Up

Instructions to the Person Recording Data: This questionnaire should be verbally administered to the patient [or their LAR] during the 90 day follow up phone call. If the patient is still inpatient the questions can be asked in person. Scoring should be recorded but not discussed with the subject.

To Be Completed By the Study Team

Is the patient still in the hospital on day 90? ☐ No ☐ Yes: *Please skip to section 'Katz Activities of Daily Living'*

Phone Script

Hello, may I please speak with [*subject name*]. My name is [*study team member*] and I am calling about a research study you [*or your loved one if speaking with LAR*] participated in when you were hospitalized. The purpose of the study was to investigate the use of inhaled nitric oxide in patients with respiratory distress and COVID19. Part of this study involves a follow up phone call to subjects 90 days after hospital discharge. That is why I am calling you.

I would like to ask you a few questions about how you [*or your loved one*] are doing. This should take about five minutes.

If speaking with patient, vital status is apparent. If speaking with a family member ask the following: Is your loved currently at home or in a health care facility?

☐ Alive, Confirmed with Patient ☐ Alive, Confirmed with LAR

☐ Died - Date of Death: _____ / _____ / _____

Were you admitted to a rehab facility for any reason? ☐ Yes ☐ No

If yes: How many days did you spend in rehab? _____ days ☐ Still in rehab

Were you readmitted to the hospital for any reason? ☐ Yes ☐ No

If yes: How many days did you spend in the hospital when you were readmitted? _____ days

☐ Still admitted

What were you readmitted for, or what was your diagnosis?

_____

During your readmission, were you mechanically ventilated or breathing on a ventilator?

☐ Yes ☐ No

Have you developed a new requirement for dialysis? ☐ Yes ☐ No

Did you experience any of the following conditions after you left the hospital? *Please indicate all that apply after your first hospital admission.*

☐ None ☐ Myocardial Infarction (*heart attack*) ☐ Stroke ☐ Pneumonia (*second episode)*

☐ New requirement for oxygen ☐ New disease/diagnosis:

_____

Did you experience any of the following alterations after you left the hospital? *Please indicate all that apply after your first hospital admission.*

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☐ None ☐ Being frequently asleep ☐ Having difficulty concentrating
☐ Altered consciousness ☐ Disorganized thinking ☐
Frank motor deficit

Katz Activities of Daily Living Score
The following questions ask about your health **today**. Please indicate whether you are able to complete each of the following tasks independently or if you require assistance.

Are you [or your loved one] able to independently…	**Score** *Independence = 1 Point, Dependence = 0 Points*	
Bathe completely or need help in bathing only a single part of the body such as the back, genital area or disabled extremity?	_____	☐ No Response
Get clothes from closets and drawers and put on clothes and outer garments completely with fasteners? *This excludes tying shoes.*	_____	☐ No Response
Go to the toilet, get on and off, arrange your clothes, and clean the genital area without help?	_____	☐ No Response
Move in and out of bed or a chair unassisted? *Mechanical transfer aids are acceptable.*	_____	☐ No Response
Have complete control over urination and bowel movements?	_____	☐ No Response
Get food from the plate into the mouth without help. *Preparation of food may be done by another person.*	_____	☐ No Response
Total Katz ADL Score *The calculation should sum the points from each category.*	_____	

Instructions to the Person Recording Data: If the LAR is completing the questionnaire, please skip to the section 'Other Feedback for the Study Team'. If the patient has recovered and is completing this assessment, please complete the PROMIS questions.

PROMIS Questionnaire of Global Health
Next I am going to ask you ten questions about your overall health **today**.

	Excellent 5	**Very Good** 4	**Good** 3	**Fair** 2	**Poor** 1	**No Response**
4. In general, would you say your health is	☐	☐	☐	☐	☐	☐

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5. In general, would you say your quality of life is	☐	☐	☐	☐	☐	☐
6. In general, how would you rate your physical health	☐	☐	☐	☐	☐	☐
4. In general, how would you rate your mental health, including your mood and your ability to think	☐	☐	☐	☐	☐	☐
8. In general, how would you rate your satisfaction with your social activities and relationships	☐	☐	☐	☐	☐	☐

	Completely 5	**Mostly** 4	**Moderately** 3	**A Little** 2	**Not at All** 1	**No Response**
9. To what extent are you able to carry out your everyday physical activities such as walking, climbing stairs, carrying groceries, or moving a chair	☐	☐	☐	☐	☐	☐

	None 5	**Mild** 4	**Moderate** 3	**Severe** 2	**Very Severe** 1	**No Response**
10. In the past 7 days, how would you rate your pain on average	☐	☐	☐	☐	☐	☐
9. In the past 7 days, how would you rate your fatigue on average	☐	☐	☐	☐	☐	☐

	Excellent 5	**Very Good** 4	**Good** 3	**Fair** 2	**Poor** 1	**No Response**
9. In general, please rate how well you carry out your usual social activities at work, at home, and in your community. How well do you fulfill your responsibilities as a parent, child, spouse, employee, friend, etc	☐	☐	☐	☐	☐	☐

	Never 5	**Rarely** 4	**Sometimes** 3	**Often** 2	**Always** 1	**No Response**

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10. In the past 7 days, how often have you been bothered by emotional problems such as feeling anxious, depressed, or irritable	☐	☐	☐	☐	☐	☐
PROMIS Global Health Score	**Sum of Points:** _____		**Overall Score:** *([Sum*10] / Number of Answers)* _____			

Other Feedback for the Study Team

	Excellent 5	**Very Good** 4	**Good** 3	**Fair** 2	**Poor** 1
Overall, how was your experience with the research study?	☐	☐	☐	☐	☐

Do you have any other feedback for the study team?

_____

_____

_____

This completes my questions and concludes your participation in this study. Thank you again for participating in this research study!

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STATISTICAL ANALYSIS PLAN

Nitric Oxide Gas Inhalation in Severe Acute Respiratory Syndrome in COVID-19 (NOSARSCOVID)

Statistical Analysis Plan

Principal Investigator: Lorenzo Berra, MD
Biostatistician: Timothy T. Houle, PhD
PHRC Protocol 2020P000787

Version 1.0
July 2, 2020

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This Statistical Analysis Plan (SAP) was created in accordance with the Guidelines for the Content of Statistical Analysis Plans in Clinical Trials (*JAMA December 2017*).

Section 1. Administrative Information

Title and Trial Registration
Inhaled Nitric Oxide Gas Therapy in Mechanically Ventilated Patients Affected by Severe Acute Respiratory Syndrome in COVID-19. NCT04306393 Study Acronym: NOSARSCOVID https://clinicaltrials.gov/ct2/show/NCT04306393

SAP Version
SAP 1.0, Version 07/02/2020

Protocol Version
Original Protocol Version 5/24/2020: https://www.medrxiv.org/content/10.1101/2020.03.09.20033530v2 Protocol Version at Time of SAP Development: Version 11.0, June 7, 2020

SAP Revisions – *Revision History Justification and Timing*
SAP 1.0, Version 07/02/2020 - ORIGINAL DRAFT

SAP Revision Notes
This version of the SAP document represents the most recent draft of the SAP, with all revisions to the plan noted by section, below. The original trial design was conducted using Frequentist procedures and assumptions, with these procedures reported in the protocol document. Within weeks of initiating enrollment, the SAP was adapted to incorporate Bayesian approaches for estimation of primary and secondary outcomes. These revised considerations, their rationale, and any additional changes from the initial protocol are reported in this document.

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Section 2. Introduction

Background and Rationale
Severe acute respiratory syndrome (SARS-CoV-2) due to novel coronavirus (2019-nCoV) related infection (COVID-19) is characterized by severe ventilation perfusion mismatch leading to refractory hypoxemia. To date, there is no specific treatment available for 2019-nCoV. Nitric oxide is a selective pulmonary vasodilator gas used as a rescue therapy in refractory hypoxemia due to acute respiratory distress syndrome (ARDS). In vitro and clinical evidence has shown that inhaled nitric oxide gas (iNO) has antiviral activity against other strains of coronavirus. The primary aim of this study therefore is to determine whether iNO improves oxygenation in patients with hypoxic SARS-CoV2.

Objectives
The primary objective of this study is to examine whether continuous delivery of high-dose iNO is superior to standard of care in terms of oxygenation and survival in patients with SARS-CoV-2.

Section 3. Trial Methods

Trial Design – *Description of the Trial Design*
This is a multicenter, single-blind, randomized (1:1) controlled, parallel-arm clinical trial. Secondary analyses and safety analyses will involve participants originally assigned to the control (usual care) arm that cross-over to receive nitric oxide after the assessment of the primary outcome measurement occasion at 48 hours. Interventions include either the institutional standard of care with the addition of iNO (treatment arm) or the institutional standard of care (control arm) alone. Standards of care are delivered according to each institution's protocols (such as ventilation strategies and use and dosage of antivirals and antimicrobials, steroids, inotropic-vasopressor agents and initiation of extracorporeal membrane oxygenator [ECMO]). Patients in the treatment arm receive iNO at 80 ppm for the first 48 hours after enrollment, then reduce to 40 ppm until severe hypoxemia resolves. Weaning from NO will start when patients improve the level of oxygenation to PaO_2/FiO_2 > 300 mmHg for more than 24 hours consecutively. Since abrupt discontinuation of iNO can sometimes result in rebound pulmonary hypertension, with possible oxygenation impairment and acute right heart failure, gradual discontinuation will be performed. Physicians will follow their own institution weaning protocols. In the absence of institutional protocols, iNO will be reduced every four hours in a stepwise fashion starting from 40 ppm to 20, 10, 5, 3, 2, and 1 ppm. In the case of hypoxemia (SpO2< 92%) or acute hypotension (systolic blood pressure < 90 mmHg) during weaning, iNO should be increased to the prior (higher) concentration.

Randomization
Permuted block randomization will be used to generate treatment allocation assignments. The randomization will be conducted using block sizes of 2 or 4 and stratified by site, age (≤ 60, > 60), and sex (male, female). Because of the expected uncertainty of site enrolment due to the variable nature of the pandemic, a fixed block size of two was used for the first 20 allocations at each site. The randomization procedures will be conducted suing the 'blockrand' package in the most recent version of R.

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Sample Size
We hypothesize that iNO gas therapy leads to an improvement of oxygenation due to amelioration of ventilation perfusion matching in patients affected with SARS-CoV-2. A difference of 20% in PaO_2/FiO_2 ratio between the two groups at 48 hours from enrollment is considered to be clinically relevant. Available data on expected values for oxygenation in SARS-CoV and SARS-CoV-2 patients in ICU are limited. However, a previous study in ARDS patients ventilated according to standard of treatment (ARDSnet table) reported a PaO_2/FiO_2 ratio at 72 hours of 190 ± 71 mmHg. We hypothesize that iNO gas may increase the PaO_2/FiO_2 ratio by at least 20% in the treatment group. Assuming a two-tailed alpha of 0.05, enrolling 91 patients per group provides power = 0.90 to detect an effect size of d = 0.48. This equates to approximately a 34 mmHg difference between groups. A 10% of dropouts after ICU discharge is foreseen so target sample size is thereby increased to 200 patients. The original sample size estimates for the trial were derived from a Frequentist approach to the analysis using the above assumptions. However, due to reporting requirements posed by the IRB, and the need for rapid learning in the context of the COVID-19 crisis, the plan of analysis was revised to employ Bayesian estimation with continuous (sequential) analysis. As such, the target sample size is viewed as the maximum sample size target for the trial, with earlier stopping, as described below. No additional simulations were conducted to consider posterior distribution precision based on the available sample size.

Framework
The hypotheses driving the trial will involve a superiority framework for conducting the analyses such that it is expected that the iNO group will exhibit superior oxygenation than the usual treatment control group. However, the Bayesian approach focusing on the estimated posterior probability of treatment effect allows the simultaneous evaluation of both futility and harm.

Interim Analyses and Stopping Guidance
The analyses of the trial will be conducted using a ‘rapid learning’ approach such that the treatment effect will be estimated continuously with accruing data. The use of Bayesian estimation allows the direct evaluation of the probability distribution of the treatment effect (i.e., probability of the effect conditional on the data and model). As such, multiplicity concerns are not an issue as with the concern for classical type-I errors associated with a Neyman-Pearson null hypothesis-testing framework. Therefore, there are no planned adjustments made to the inferences (i.e., posterior probabilities) as would be appropriate for statistical significance thresholds using frequentist adaptive designs. The only study stopping rule for the trial is defined as a significant increase of mortality with nitric oxide gas.

Timing of Final Analyses
The analysis is being conducted continuously with accruing data and in time for DSMB meetings. If none of the stopping rules have been reached, the final estimation will occur after the completion of final follow-up measurements for the final participant in the target sample size (N = 200).

Timing of Outcome Assessments

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Patients are assessed daily while in the hospital and at 28 and 90 days after enrollment. The primary outcome measure, oxygenation, will be defined based off of the patients PaO_2/FiO_2 at 48 hours after enrollment. Secondary outcomes and their measurement are defined in full in the study protocol.

Section 4. Statistical Principles

Confidence Intervals and P-values
The use of Bayesian estimation allows the interpretation of the posterior probability distributions for each effect. The definitions for interpreting the size of treatment effects are provided above, under the stopping rules. Interpretation of the Bayesian posterior probabilities will be based on a > 0.95 probability of effect defined as reliable evidence for superiority. Using Bayesian estimation, two-sided 95% high probability density (HPD) intervals will be constructed around treatment contrasts to communicate point estimates and their uncertainty.

Analysis Populations
Our primary analysis will utilize a modified intention to treat (mITT) principle. This is defined as all eligible participants for whom respiratory treatment was available at enrollment and for whom a secondary review of inclusion/exclusion criteria confirmed eligibility. This definition recognizes the difficulty of arranging the logistics of treatment as well as the existence of intercurrent events that precluded meaningful delivery of treatment for enrolled individuals who rapidly decompensated before treatment could be initiated.

Section 4. Trial Population

Screening Data
In accordance with requirements to collect and store only the minimum amount of data necessary to achieve the study aims, only limited information will be retained on patients who were screened but found ineligible for participation. Aggregate data will be reported on the number of patients screened (defined as accessing their medical record to assess eligibility), the total number not meeting each study inclusion criteria, the number (among those who met all inclusion criteria) of patients who were excluded and specific reasons for exclusion. This data will be presented in the trial CONSORT figure and may also be reported as frequencies and proportions.
Eligibility

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The study inclusion and exclusion criteria are as follows:

Inclusion Criteria
1. Adult patients ≥ 18 year-old
2. Patients admitted to the ICU
3. Patients who are intubated and mechanically ventilated
4. Confirmed diagnosis of SARS-CoV-2 by positive rt-PCR

Exclusion Criteria
1. Patients intubated for more than 72 hours from initiation of the treatment gas
2. Subjects enrolled in another interventional research study
3. Past medical history of lung malignancy or pneumonectomy or lung transplant
4. Tidal volume < 3 cc/Kg of IBW at the time of randomization
5. Severe burns (>40% total body surface area)
6. Cardiac arrest with CPR for longer than 30 minutes
7. Presumed severe deficit in cerebral function with fixed dilated pupil
8. Receiving renal replacement therapy at the time of enrollment
9. Impaired ability to ventilate without assistance (e.g. C5 or higher spinal cord injury, ALS, GBS, and myasthenia gravis)
10. History of malignancy or other irreversible disease/conditions with 6-month mortality >50%
11. Patients not committed to full support at the time of enrollment
12. Patients who received inhaled nitric oxide prior to enrollment
13. Admission unrelated to the COVID-19 disease
14. Physician of record opposed to enrolling the patient due to perceived safety concerns or any condition that does not allow the protocol to be followed safely

Of note, changes in study inclusion/exclusion criterion throughout the course of the study will be detailed in the Supplementary Appendix of the primary manuscript. Final study inclusion and exclusion criteria will be described in the Methods section of the primary study manuscript.

Recruitment

In addition to the limited screening data collected on participants, we will also collect minimal data on the recruitment process. This information will be reported in the CONSORT flow diagram and includes the number of eligible participants, aggregate numbers of patients who were not approached for participation and reasons why (ex. study staff availability, unable to contact the legally authorized representative, etc.), as well as the number of eligible participants who are approached for recruitment but decline to participate.

Extensive data will be collected and reported on patients who are eligible and agree to participate in the trial, as detailed below in Section 5, Analysis.

Withdrawal/Follow-up

Patients (or their legally authorized representative) may elect to withdraw from the study at any time. Further, patients can be withdrawn from the study at the discretion of the treating clinician or the PI if it is deemed unsafe for the patient to continue participating. The type and timing of withdrawal will be noted and classified as (1) after enrollment, but prior to randomization, (2) after randomization but prior to drug administration, (3) while in-hospital, or at (4) 28 day or (5) 90 day follow up. Frequency counts for periods of withdrawal will be tabulated and reported.

Baseline Patient Characteristics

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Descriptive statistics of the data will be collected and reported. Continuous data will be reported as means ± standard deviations or medians (interquartile range). Categorical data at baseline will be reported as frequencies and relative percentages. Absolute standardized differences among baseline characteristics will be estimated for which values > 0.10 will be utilized to signify potential imbalance between groups.

Baseline patient characteristics include but may not be limited to the following:
Age, *continuous value in years*
Sex, *male or female*
Race, *categorized as White, Black or African American, Asian, American Indian or Alaska Native, Native Hawaiian or Other Pacific Islander, Multiracial, Other or Unknown/Not Specified*
Ethnicity, *categorized as Hispanic or Latino, not Hispanic or Latino and Unknown*
Height*, continuous value in centimeters*
Weight*, continuous value in kilograms*
Body mass index*, continuous value in kg/m^2*
Presence of comorbidities, *including non-distinct categories of hypertension, coronary heart disease, diabetes, malignancy, cerebrovascular disease, chronic pulmonary disease, chronic kidney disease, immune deficiency, or none of the above*
Smoking history*, distinct categories for current smoker, former smoker, never smoked or unknown*
APACHE II score on enrollment
Time between confirmed diagnosis and the enrollment*, days*
Physiologic and respiratory data at baseline (enrollment) including:
Respiratory Rate*, Breath/min*
Oxygen Saturation, %
Fraction of inspired oxygen
Positive end expiratory pressure
Heart rate, *beats/min*
Mean arterial blood pressure, *mmHg*
Temperature on enrollment, *◦C*
Laboratory values on enrollment including:
Serum Creatinine*, mg/dL*
ALT*, g/dL*
AST*, g/dL*
White Blood Cell Count*, x10^9 L*
Hemoglobin*, g/dL*
Platelet Count*, K/uL*
Total Lymphocytes*, %*

Section 5. Analysis

Outcome Definitions

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The primary outcome is the change in arterial oxygenation at 48 hours from enrollment. Levels of oxygenation will be calculated by the PaO_2/FiO_2 ratio. If a patient dies during the first 48 hours of treatment, the last available blood gas analysis will be imputed for the 48-hour timepoint.

Secondary outcomes include:
Time to reach normoxia defined by a $PaO_2/FiO_2 > 300$ for at least 24 hours during the first 28 days after enrollment. If a patient dies before day 28, the patient will be considered as "never recovered"
Proportion of SARS-nCoV-2 free patients (i.e., normoxiemic patients) during the first 28 days after enrollment. If a patient dies before day 28, the patient will be considered as "never recovered"
Survival at 28 days from enrollment
Survival at 90 days from enrollment

The following data will be collected to assess exploratory outcomes, including:
Daily oxygenation in the two groups until day 28
Need for new renal replacement therapy during the first 28 days
Mechanical support of circulation (i.e., ECMO, intra-aortic balloon pump, VADs) during the first 28 days
Days free of vasopressors during the first 28 days
Ventilator-free day at 28 days
Time to SARS-CoV-2 RT-PCR negative in upper respiratory tract specimen (assessed within the first 28 days)
ICU-free days at 28 days

Analysis Methods

The primary and secondary outcome analyses will be conducted using Bayesian estimation of generalized linear models. For the primary outcome, the PaO_2/FiO_2 ratio will be modeled using a normal distribution and identify link function. The PaO_2/FiO_2 ratio at 48 hours will be regressed on the baseline (i.e., enrollment) PaO_2/FiO_2 ratio, several additional predictors (*see 'Adjustment for Covariates' below*), and randomized group assignment.

Secondary outcomes and endpoints will be conducted using the same prognostic covariates and treatment group indicator with outcome distributions and link functions appropriate to the outcome. For the 24-hour timepoint, the primary model will be applied to the 24-hour outcome. For mortality outcomes and other binary outcomes (e.g., 28-day, 90-day), a Bernoulli outcome with a logit link will be used. It is anticipated that the time to normoxia will be normally distributed in this setting, so a normal distribution with an identity or log link (if skewed) will be applied.

For the exploratory outcomes, a generalized linear model will be conducted as above with an appropriate distribution and link function (e.g., normal, gamma, negative binomial) using the same predictors and baseline status (where appropriate).

To estimate the model parameters, prior probability distributions are specified for each of the parameters. Weak or non-informative priors with default regularization will be used for each parameter. Briefly, this formulation assumes that the likely treatment estimate is centered around zero (for logit and Gaussian models) with likely estimates of the parameter residing within 2.5-fold of the variables' scale (i.e., 2.5 times the expected standard deviation for PF). These priors will regularize estimation with sparse data, and act as modestly pessimistic priors for the treatment effect.

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The models will be conducted using the 'brms' package with implementation using 'rstan'. Prior to conducting the models, prior predictive checks will be conducted to examine the suitability of the prior specification and sampling methods.

Statistical Methods – *Adjustment for Covariates*

Each primary and secondary model will be conducted using a Bayesian framework that estimates the treatment effect conditional on several additional prognostic variables included in the model (defined *a priori*):
Randomized treatment group
Baseline PaO_2/FiO_2 ratio
Age + Age^2
APACHE-II baseline score
Sex
Body mass index

Statistical Methods – *Sensitivity Analyses*

Several sensitivity analyses are planned.

The primary model will be conducted using the specified predictors as well as any variables that are imbalanced between groups (i.e., standardized difference > 0.20).

The primary and secondary models will be conducted using the study site as an additional a fixed effect.

The unexpected nature of the pandemic may produce variability in patient populations, routine care, and outcome expectations. The influence of any temporal effects on outcomes will be considered by adjusting relevant models for calendar time.

Statistical Methods – *Subgroup Analyses*

Treatment heterogeneity may be considered as a function of site and using an approach outlined in the PATH guidelines (https://www.acpjournals.org/doi/10.7326/M18-3667).

Missing Data

If warranted (i.e., > 10%), missing data in the primary model will be further considered using fully conditioned multiple imputation.

Harms

NO reacts with oxygen to form NO_2, which may cause airway inflammation and damage to lung tissues. Moreover, NO oxidizes ferrous Hb to form Met-Hb, which is unable to transport and release oxygen to tissues. The binding of NO to Hb is a rapidly reversible reaction, with a half-life of 15–20 min after NO discontinuation. The side effects and adverse events related to iNO delivery are well reported in the literature. Based on the present literature and Food and Drug Administration reports, the risks of breathing iNO at 80 ppm for 24 hours are minimal when Met-Hb levels and NO/NO2 delivery levels are carefully monitored.

To improve safety, in the present trial, iNO is administered and monitored by trained clinicians. The NO2 will be monitored and maintained at levels of below 2 ppm. Met-Hb is continuously monitored by non-invasive co-oximetry. If Met-Hb levels exceed 5% of circulating Hb, the concentration of NO

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delivered is halved and closely monitored until a reduction occurs. If Met-Hb levels persist above 5%, iNO is progressively halved until a reduction below 5% occurs.

Patients will thus be monitored throughout the study for adverse events. Adverse events that are unexpected and related or possibly related to the research and that indicate there are new or increased risks to subjects will be reported to the IRB within 5 working days (7 calendar days) of the date the investigator first became aware of the event. Expected adverse events will be documented in the study case report forms and reviewed at regularly scheduled DSMB meetings to evaluate if the frequency of adverse events exceeds what is expected.

Frequency counts for measurements exceeding these thresholds, as well as commonly experienced adverse events will be tabulated and reported. Expected adverse events are defined as:
$NO_2 > 2$ppm,
Met-Hb > 5%
$SpO_2 < 92\%$, or
Hypotension, including either SBP < 90mmHg for > five minutes or hypotension that requires the start of a vasopressor or increase in the existing dose, or rebound pulmonary hypertension.

Software
All analyses will be conducted using the most recent version of R and R-Studio. The Bayesian estimation will be conducted using the MCMCPack. Research Electronic Data Capture (REDCap v9.5.23 or later) will be used for data collection and management. The study REDCap will be housed on a Mass General Brigham research server.

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CONSORT CHECKLIST

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	Title Page
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	1
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	3-4
	2b	Specific objectives or hypotheses	4
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	4
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	6
Participants	4a	Eligibility criteria for participants	4
	4b	Settings and locations where the data were collected	4
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	5
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	5
	6b	Any changes to trial outcomes after the trial commenced, with reasons	not applicable
Sample size	7a	How sample size was determined	6
	7b	When applicable, explanation of any interim analyses and stopping guidelines	7
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	4
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	4
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	4
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	4
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	4

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		assessing outcomes) and how	4
	11b	If relevant, description of the similarity of interventions	not applicable
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	6
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	6
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	6-7
	13b	For each group, losses and exclusions after randomisation, together with reasons	6-7
Recruitment	14a	Dates defining the periods of recruitment and follow-up	6
	14b	Why the trial ended or was stopped	7
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	18-19-20
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	6
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	7
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	not applicable
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	8-9
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	8
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	14
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	14
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	14
Other information			
Registration	23	Registration number and name of trial registry	4
Protocol	24	Where the full trial protocol can be accessed, if available	4
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	Title Page

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

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Online Data Supplement References

1. World Health Organization. Modes of transmission of virus causing COVID-19: implications for IPC precaution recommendations. 2020;at <https://www.who.int/news-room/commentaries/detail/modes-of-transmission-of-virus-causing-covid-19-implications-for-ipc-precaution-recommendations>.

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AJRCCM Articles in Press. Published September 29, 2023 as 10.1164/rccm.202304-0637OC