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Pilot study to test inhaled nitric oxide in cystic fibrosis patients with refractory *Mycobacterium abscessus* lung infection

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ABSTRACT

Background: Airways of Cystic Fibrosis (CF) patients are Nitric Oxide (NO) deficient which may contribute to impaired lung function and infection clearance. *Mycobacterium abscessus* (*M. abscessus*) infection prevalence is increasing in CF patients and is associated with increased morbidity and mortality. Here, we assess the safety and efficacy of intermittent inhaled NO (iNO) as adjuvant therapy in CF patients with refractory *M. abscessus* lung infection.

Methods: A prospective, open-label pilot study of iNO (160 ppm) administered five times/day during hospitalization (14 days), and three times/day during ambulatory treatment (7 days) was conducted. The primary outcome was safety measured by NO-related adverse events (AEs). Secondary outcomes were six-minute walk distance (6MWD), forced expiratory volume in 1 s (FEV₁), and *M. abscessus* burden in airways.

Results: Nine subjects were recruited. iNO at 160 ppm was well-tolerated and no iNO-related SAEs were observed during the study. Mean FEV₁ and 6MWD were increased relative to baseline during NO treatment. *M. abscessus* culture conversion was not achieved, but 3/9 patients experienced at least one negative culture during the study. Mean time to positivity in *M. abscessus* culture, and qPCR analysis showed reductions in sputum bacterial load. The study was not powered to achieve statistical significance in FEV₁, 6MWD, and bacterial load.

Conclusions: Intermittent iNO at 160 ppm is well tolerated and safe and led to increases in mean 6MWD and FEV₁. iNO exhibited potential antibacterial activity against *M. abscessus*. Further evaluation of secondary endpoints in a larger cohort of CF patients is warranted to demonstrate statistical significance.

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Abbreviations: AEs, Adverse Events; ARDS, Acute Respiratory Distress Syndrome; CF, Cystic Fibrosis; CFTR, Cystic Fibrosis Transmembrane Conductance Regulator; CFU, Colony Forming Unit; CRP, C-Reactive Protein; FEV₁, Forced Expiratory Volume in 1 s; FVC, Forced Vital Capacity; SpO₂, Mean Peripheral Oxygen saturation; Methb, Methemoglobin; *M. abscessus*, *Mycobacterium Abscessus*; MABSC, *Mycobacterium Abscessus* Complex; iNO, Inhaled Nitric Oxide; NOS, Nitric Oxide Synthase; NO₂, Nitrogen Dioxide; NTM, Non-Tuberculosis Mycobacteria; qPCR, Quantitative Polymerase Chain Reaction; SAEs, Serious Adverse Events; 6MWD, Six-Minute Walk Distance; 6MWT, Six-Minute Walk Test; SNOs, S-nitrosothiols; GSNO, S-nitrosoglutathione; SD, Standard Deviation.

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1. Introduction

Lung damage induced by chronic infection and inflammation is a significant cause of morbidity and mortality in Cystic Fibrosis (CF) patients [1]. In recent years, the prevalence of Non-tuberculous Mycobacteria (NTM) lung infection has increased in these patients. NTM infections, especially *Mycobacterium Abscessus* Complex (MABSC), are complicated to treat due to biofilm generation and acquisition of multiple drug-resistance mechanisms. Prolonged and intense antibiotic treatment regimens are associated with high rates of failure, recurrence of infection, and adverse events (AEs) [2]. Therefore, currently *M. abscessus* is an incurable chronic infection in most CF patients.

Nitric Oxide (NO), formed from L-arginine by NO synthase (NOS) has been shown to play an essential role in a variety of biological processes in the lung, including host-defense against pathogens, smooth muscle

relaxation, bronchodilation, and inflammation [3]. In CF patients, the elevation of airway NO level is closely linked to improvement in lung function [4–6]. Indeed, administration of L-arginine inhalation therapy resulted in a transient improvement in pulmonary function of CF patients [7,8]. However, the treatment of CF patients with low-dose (100 ppb to 40 ppm) inhaled nitric oxide (iNO) had no immediate effect on lung function [9].

At higher doses (160–200 ppm), exogenous NO has been shown to exert potent antimicrobial activity in preclinical models against a broad range of bacteria including *Mycobacterium smegmatis* and drug-resistant pathogens [10–15]. An intermittent delivery protocol (30 min of 160 ppm iNO every 4 h) was developed in order to overcome potential AEs associated with continuous exposure to high-dose iNO such as methemoglobinemia. Administration of intermittent high-dose NO at 30-min cycles of 160 ppm for five days was shown to be safe and well-tolerated in healthy adults and CF patients [16,17]. Our group previously reported a prospective compassionate treatment in two CF patients with adjunctive iNO therapy at 160 ppm for 30 min, five times/day for up to 26 days. Reductions in sputum *M. abscessus* load and improvement in lung function and 6MWD were found [18]. In the present study, we evaluated the safety and tolerability of intermittent high-dose iNO (160 ppm) in CF patients with refractory *M. abscessus* lung infection.

2. Materials and methods

2.1. Study design

This prospective, open-labeled, multi-center trial included nine CF patients with refractory *M. abscessus* lung infection admitted to three medical centers in Israel. This study was conducted in accordance with the amended declaration of Helsinki. Local institutional review boards approved the protocol, and written informed consent was obtained from all patients (IRB's: RMB – 0161-17, SMC – 4126-17, SOR – 0240-17). Participants were aged >6 and < 65 years, with a history of at least six months of chronic *M. abscessus* lung infection prior to screening, FEV₁ > 30%, able to perform 6MWD test (<700 m). Key exclusion criteria included pregnancy, diagnosis of methemoglobinemia (>2%), cardiac disease, pulmonary hypertension (documented by previous echography) and/or high blood pressure, significant prior hemoptysis (>30 ml blood in 24 h during last 30 days before enrollment), history of lung transplant, continuous oxygen supplementation and pulmonary tuberculosis. Detailed inclusion and exclusion criteria are summarized in Table S1.

2.2. Safety

The primary outcome of this study was safety and tolerability to intermittent exposure to 160 ppm iNO by monitoring blood MetHb and inspired NO₂ levels as well as any NO-related AEs (see Table 2). Blood MetHb (safety threshold <7%) and mean peripheral oxygen saturation (SpO₂, safety threshold <89%) levels were continuously monitored during treatments using a co-oximeter (RAD57 or RAD87, Masimo, Ivrine, CA, USA). Vital signs, physical examination, hematological and coagulation markers were also assessed throughout the study.

2.3. Efficacy measurements

Spirometry tests (FEV₁ and FVC) and 6MWD test were performed at screening, baseline (day 1), during treatment (weeks 1, 2 and 3) prior to first daily NO treatment, and during follow-up (weeks 7 and 11). Spirometry was performed using a KoKo spirometer (n-Spire Healthcare, Inc., Longmont CO, USA) in accordance with the American Thoracic Society (ATS) and European Respiratory Society Task Force [20]. Results are presented as absolute values and predicted percent derived from Polgar and Quanjer [21]. 6MWD test was performed according to ATS

guidelines for 6MWD at screening, baseline (day 1) prior to daily NO treatments, during treatment (weeks 1, 2, and 3) and each follow-up visit [22].

Sputum specimens were assessed at screening, baseline (day 1), during treatment (week 1, 2 and 3), and during follow-up visits (week 7 and 11). During screening, *M. abscessus* was identified via solid and liquid culture (BD BACTEC MGIT 960, Becton Dickinson, USA; Lowenstein Jensen/Middle brook 7H10 broths, Hy-Laboratories, Israel) and via Polymerase Chain Reaction (PCR) based on rpoB gene sequence. *M. abscessus* load (CFU/ml) in sputum was quantitatively assessed by Time to Positivity in liquid culture (BD BACTEC MGIT 960; from sample collection to positive culture) and by quantitative PCR (qPCR), as previously described [19]. Microbial loads (CFU/ml) of *Pseudomonas aeruginosa* (*P. aeruginosa*) in sputum were evaluated by plating on solid agar (Chocolate and MacConkey agar) following serial dilutions using sterile saline solution. Plates were incubated at 37 degrees (5% CO₂) for 48 h before CFU count.

2.4. Nitric oxide treatment

All patients received intermittent inhalations (30-min each, every 4 h) of 160 ppm NO combined with O₂/Air blend in addition to standard of care for CF-NTM pulmonary disease including antibiotic treatment for *M. abscessus* infection. NO treatments were administered for 21 days – five times/day during hospitalization (14 days), and three times/day during the ambulatory period (Day 15 to 21). Two monthly follow-up visits were conducted (once a month). Inhaled NO was delivered in a controlled manner via designated NO tubing and flow meters into the subject's inhalation facemask as described previously [18]. NO (ppm), NO₂ (ppm) and Fraction of inhaled O₂ (%) concentrations delivered were continuously monitored using a dedicated AeroNox monitoring system (International Biomedical, USA). Inspired NO₂ was measured at a point closest to the patient mask and separated for exhaled gas by a one-way valve to avoid measuring exhaled NO₂.

2.5. Statistical analysis

No formal sample size calculation was performed, as the study endpoints were not expected to show statistical significance. Results were expressed as descriptive parameters, mean ± SD or 95% confidence intervals (CI). Binary comparisons were made with Wilcoxon test for paired and Mann-Whitney for non-paired tests, where appropriate. All statistical tests were conducted using SAS® version 9.3 or higher (SAS Institute, Cary North Carolina).

3. Results

3.1. Patients and treatment

Nine out of fourteen recruited patients with a confirmed diagnosis of CF and pulmonary *M. abscessus* infection were enrolled in the study (Table S1). The baseline demographics are summarized in Table 1. All nine patients completed the iNO (160 ppm) treatment.

3.2. Inhaled NO safety

The primary endpoint of this pilot study was safety and tolerability of intermittent high-dose iNO in NTM-CF patients. Study treatment was well-tolerated and there were no iNO-related SAEs reported. A total of twenty-five AEs were reported, with three patients experiencing five possible/probable treatment-related AEs (Table 2).

All iNO-related AEs were minor, transient, and patients recovered to continue the subsequent scheduled treatment. There was one iNO-unrelated SAE: papilledema, which was diagnosed due to blurred vision 30 days post-treatment (during follow up period). Patient's CT was normal, no retinal bleeding observed and the condition was resolved.

Table 1
Baseline demographics of the study population.

N (female:male)	9 (5:4)
Age (Years)	22.7 ± 6.2
Weight (kg)	46.5 ± 11.5
BP _{systolic} (mm Hg)	117 ± 10
BP _{diastolic} (mm Hg)	75 ± 9.5
HR (beats/min)	96 ± 13
Pulmonary function	
FVC (%)	69.2 ± 20.8
FEV ₁ (%)	54.6 ± 22.4
CFTR genotype	
F508del/F508del	2
F508del/H1054D	2
F508del/R1066C/R1070Q	1
W1282X/W1282X	1
W1282X/G542X	1
N1303K/N1303K	1
N1303K/1717 T	1
Pulmonary colonization (no. pts) ^a	
<i>M. abscessus</i>	9
<i>P. aeruginosa</i>	4
<i>S. aureus</i>	1
<i>A. flavus</i>	1
<i>C. albican</i>	6

Data for age, vital signs, pulmonary function (%) are given as mean ± SD. Data for bacterial colonization are given as number of patients. BP: blood pressure; HR: heart rate; FVC: forced vital capacity; FEV₁: forced expiratory volume in one second.

^a Number of patients with pulmonary colonization of specific microorganism is based on culture evaluation at the screening.

In this study, out of 817 total inhalations, only one transient episode of elevated MetHb (7.1%) was reported, which recovered to baseline levels before next scheduled treatment. Mean MetHb levels post iNO treatment ($3.27 \pm 1.0\%$) remained well below the 7% threshold across all inhalations (Fig. 1, panel A). Furthermore, MetHb peak levels in all patients returned to baseline before subsequent daily NO inhalation (Fig. S1). Moreover, the mean MetHb level between all subjects at the end of the study was measured to be $1.6 \pm 0.44\%$.

SpO₂ levels remained within acceptable range after NO treatment in all patients with no reported incidences of hypoxemia (SpO₂ < 85%, Fig. S2, panel D). NO₂ levels in the delivery line did not exceed the 5 ppm

Table 2
Incidence of adverse events.

Event	n (pts) / n (event)	Comments
Common cold	1/1	
Pulmonary exacerbation	5/6	
Dizziness	1/2	Possibly NO-related, resolved
Dry mouth	1/1	Probably NO-related ^a , resolved
Hemoptysis	3/4	Possibly NO-related (n = 1), minor, resolved
Musculoskeletal pain	1/1	
Pain in extremity	1/2	
Headache	1/1	
Vomiting	2/2	
Fever	2/3	
MetHb elevation	1/1	Probably NO-related (7.1%)
Papilledema (blurred vision)	1/1	transient, resolved, SAE
Patients with probable/possible NO-related AEs	3	minor, resolved
Patients with NO-related SAEs	0	

Event: AE description; n (pts): number of patients who experienced each AE; n (events): total number of AE's per event recorded during the study. Relation to study treatment is described where appropriate

^a Dry mouth is a common occurrence during inhalation therapy without supplementary humidification and not necessarily caused by iNO.

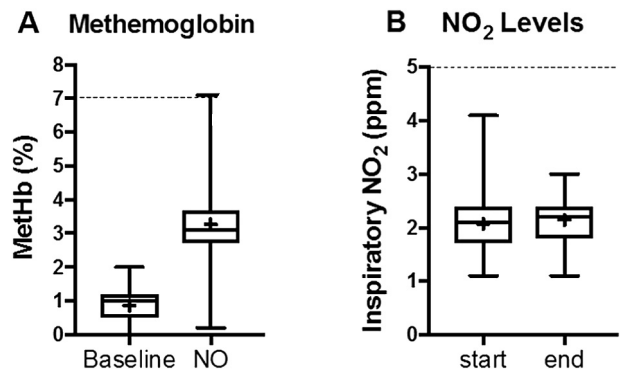


Fig. 1. Methemoglobin and NO₂ remained within accepted range during intermittent iNO treatment. *Panel A:* Pooled MetHb levels from before (baseline) and after NO inhalation (NO) are shown in a Box-Whisker plot. The dotted line marks the 7% MetHb safety threshold set in this study. *Panel B:* Inspired nitrogen dioxide (NO₂) levels in delivery line were continuously monitored by sampling from the mouthpiece sample port with an AeroNOx electrochemical analyzer. Pooled inspired NO₂ levels from start to end of NO inhalation are shown. The dotted line marks the 5% NO₂ safety threshold set in the study. Box and Whisker plot description: boxes extend from the 25th to 75th percentile, middle line represents the median, and + symbol shows the mean value. Whiskers extend to minimum and maximum values.

safety threshold during iNO treatments with an overall mean peak level of 2.1 ± 0.4 ppm (Fig. 1, panel B).

Vital signs and blood pressure readings remained within normal limits for age and with respect to baseline values (Fig. S2). Hematological evaluation including inflammatory marker C-reactive protein (CRP) did not reveal a significant difference between baseline and post iNO treatment (Fig. S3). Coagulation markers Prothrombin Time, International Normalized Ratio, and activated Partial Thromboplastin Time remained stable during and after iNO treatment (Fig. S3).

3.3. Lung function and 6MWD

Relative to baseline, mean FEV₁ ($2.0 \pm 6.0\%$) and FVC ($1.56 \pm 8.9\%$) values increased at the end of iNO treatment (week 3). The mean gains in FEV₁ and FVC were not sustained at the second follow-up, 11 weeks after the last iNO treatment (Fig. 2A, B) period. The gains observed in the mean 6MWD, relative to baseline, at the end of iNO treatment ($+43.2 \pm 96.2$ m) were partially sustained at the last follow-up ($+27.7 \pm 117.2$ m). Paired analysis of absolute FEV₁ and 6MWD data revealed that only a subset of patients responded positively to iNO therapy (5/9 in FEV₁; 6/9 in 6MWD) and the rest showed a reduction in FEV₁ and 6MWD or remained unchanged (Fig. S4). The study was not powered to achieve statistical significance and matched non-parametric tests did not reveal a significant difference in changes described above.

3.4. Sputum microbiology

Positive *M. abscessus* culture were confirmed in all patients at screening. Three patients experienced a negative culture of *M. abscessus* (in both solid and liquid media) throughout the study, with one patient remaining negative at 7- and 11-week follow-up visits (Fig. S5). *M. abscessus* culture conversion (three consecutive negative cultures) was not achieved in this study. Time to positivity assay (BACTEC MGIT 960) was used to quantify *M. abscessus* burden in sputum samples. The median time to positivity in *M. abscessus* liquid culture increased relative to baseline during iNO treatment and at follow-up periods, suggesting a reduction in *M. abscessus* bacterial burden (not statistically significant) (Fig. 3A). This observation correlated with qPCR estimate of sputum *M. abscessus* DNA load showing a downward trend after the first week of NO treatment and at follow-up visits (Fig. 3B).

In addition to *M. abscessus*, two out of five patients co-infected with multi-drug resistant *Pseudomonas aeruginosa* achieved negative culture

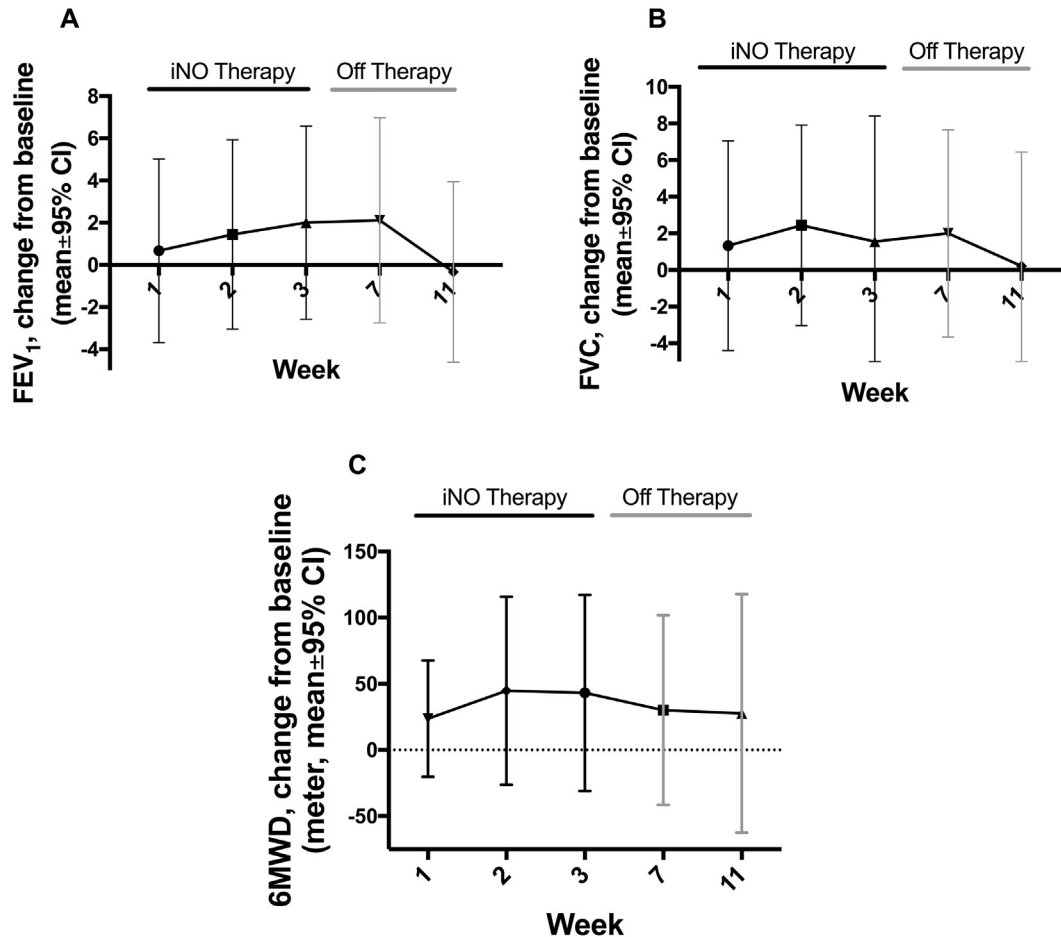


Fig. 2. The effect of iNO treatment on lung function and 6MWD. Mean changes from baseline (day 1) in FEV₁ (panel A) and FVC (panel B) measured by spirometry in all patients ($n = 9$) during iNO treatment (iNO Therapy: week 1–3) and at follow up (Off Therapy: weeks 7 and 11). CI indicates 95% confidence intervals. Matched non-parametric ANOVA (Friedman test) did not reveal a significant difference (FEV₁ $p = .220$; FVC $p = .41$). 6MWD in meters (panel C) was assessed each morning before NO inhalation (iNO Therapy) and at follow up (Off Therapy). Matched non-parametric ANOVA (Friedman test) did not reveal a significant difference ($p = .71$). Values above each bar represent means change of all patients.

at 11-week follow-up with a third patient showing a $> 2\text{-log}_{10}$ reduction (data not shown).

4. Discussion

In this prospective open-labeled pilot study, we assessed the safety and tolerability of intermittent iNO at 160 ppm administered as an adjunct to antibiotic therapy in CF patients with chronic *M. abscessus* infection. Inhaled NO therapy at 160 ppm was found to be safe and well-

tolerated, with no reports of iNO-related SAEs. While the study was not powered to achieve statistical significance in secondary outcomes, increases in the overall mean values of FEV₁, FVC, and 6MWD were observed during iNO treatment.

Low-dose of iNO (below 80 ppm) is FDA-approved for treating neonates with persistent pulmonary hypertension with minimal pulmonary and systemic toxicity [23]. However, there is limited evidence on the safety of high-dose iNO in CF patients. Two previous pilot studies evaluating the safety of high-dose iNO (160 ppm) did not report any

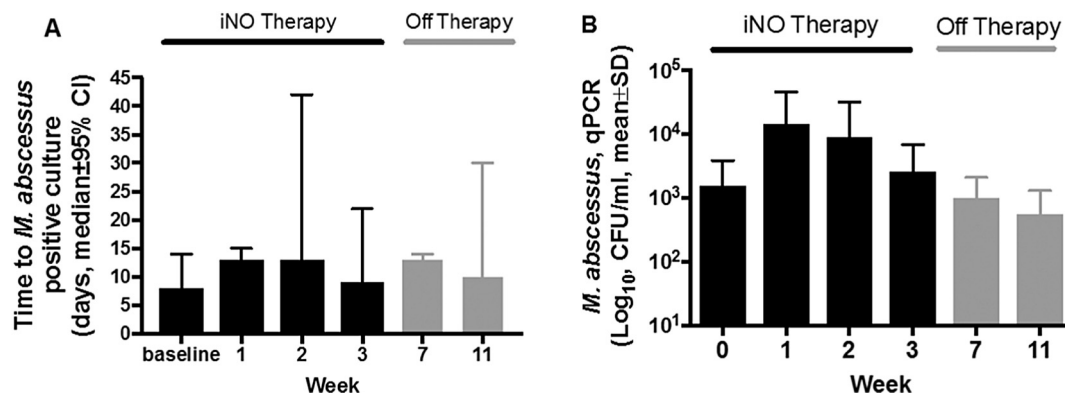


Fig. 3. iNO treatment increases time to positivity in *mycobacterium* culture. **Panel A:** time to positivity of *mycobacterium* culture was assessed in sputum samples of all patients at screening, during NO treatment (NO Therapy), and at follow up (Off Therapy). Median values plus 95% confidence intervals (CI) are presented here. No significant difference was found from baseline (Wilcoxon matched-pairs signed rank test). **Panel B:** assessment of uncultured sputum samples for *M. abscessus* DNA by qPCR using *M. abscessus*-specific *rpoB* gene. Data generated in qPCR was converted to CFU/ml by generating a CFU/qPCR standard curve. Mean values (plus standard deviation – SD) from all 9 patients are represented here. No significant difference was observed between baseline and time points (Wilcoxon matched-pairs signed rank test).

significant NO-related AEs [16,17]. As prolonged treatment with iNO may be required to achieve a clinically meaningful reduction in lung bacterial load, especially in advanced CF patients with persistent NTM infection, we explored the safety of iNO treatment for up to 21 days. The absence of NO-related SAE in our study is consistent with previous reports. Furthermore, all possible NO-related AEs (5 out of total 25 AEs) were minor, resolved within 3 h, and patients continued to receive subsequent NO inhalation in all cases.

A large portion of iNO combines with oxyhemoglobin to form methemoglobin (MetHb) and nitrates. Elevated MetHb or methemoglobinemia (defined as >7%) results in impaired oxygen delivery to tissues and is monitored as the key marker for iNO systemic toxicity [24,25]. Deppisch et al. reported a mean MetHb level of $2.7 \pm 0.4\%$ during 5-day treatment with intermittent iNO at 160 ppm for 30 min, three times daily [17]. In this study, the mean MetHb levels for individual patients were well below the safety threshold of 7% and in line with previous findings. More importantly, all patients exhibited a steady and consistent recovery to baseline MetHb levels after each 30-min intermittent iNO treatment.

A potential side-effect of iNO treatment could be its effect on bleeding diathesis, even though the prior studies have yielded conflicting results. Several studies treating healthy volunteers with 30–80 ppm iNO for up to 30 min demonstrated limited effects on bleeding time and hemostasis and of little clinical significance [26–29]. While other investigators have reported significant inhibition of platelet aggregation or prolonged bleeding time in healthy volunteers or patients receiving iNO [30–35]. Therefore, one of the key exclusion criteria in our study was significant hemoptysis, defined as >30 ml blood in 24 h, in the previous month from enrolment. The hemoptysis events experienced during this study were minor and self-limited, most likely due to the intermittent NO inhalation protocol. Nevertheless, we recommend that platelets function and bleeding time should be assessed in future iNO studies and patients monitored closely for coagulation and bleeding problems.

Although NO is a naturally produced signaling molecule with a short half-life (seconds), the indirect systemic effects of exogenous iNO therapy should be considered. Inhaled NO is used as rescue therapy for severe hypoxemia in patients with Acute Respiratory Distress Syndrome (ARDS) [23]. Systemic reviews of ARDS patients receiving iNO rescue therapy suggest an association between iNO therapy and increased risk of renal dysfunction, especially at high cumulative-doses of iNO and in older aged patients (>65 yr) [36,37]. Renal dysfunction in these trials was usually defined as an excess of creatinine level (2–3.5 mg/dl) or the need for renal replacement therapy. It is important to note that in a recent safety study of intermittent high-dose iNO (160 ppm) in CF patients, authors did not observe a significant increase in the serum creatinine level during iNO treatment or follow-up period and did not report any adverse event related to renal function [17]. It is plausible that intermittent iNO regimen minimizes the systemic side effects of high-dose iNO compared to continuous iNO studies in ARDS patients. Nonetheless, as many concomitant medications in the treatment of CF patients have potential nephrotoxicity, we recommend monitoring renal function by measuring creatinine levels and creatinine clearance in future iNO studies.

Another systemic effect that should be discussed is the potential mutagenic activity of iNO following sustained exposures to high concentrations. NO has complex and conflicting effects in cancer. Chronic exposure to NO can mediate damage to DNA or hinder DNA repair mechanisms to promote tumorigenesis. However, NO production also plays a critical role in the tumoricidal activity of the immune system [38,39]. Dixon and colleagues have recently suggested an association between iNO treatment in the neonatal intensive care unit (NICU) and increased risk of childhood cancer later in life compared to other NICU therapies [40]. Although concerning, this association has not been reported in adult patients. It is imperative to note that relatively low percentage of iNO-treated children who developed cancer later in life (1% vs 0.22–0.56% in other NICU therapies) may have died from the severe

respiratory disease without receiving iNO treatment. A similar argument can be for adult CF patients with refractory NTM infection. The majority of these patients have minimal treatment options and will succumb to their lung infection if research and development of novel therapeutics are not advanced. Taken together, a family history of cancer diathesis, especially lung cancer and leukemias or lymphomas, should be considered as an exclusion criterion in future studies.

Pulmonary function in CF patients has been closely associated with airway NO production, as patients with improved function show higher exhaled NO and sputum NO₂/NO₃ levels (NO by-products) [4,5,41]. Previous attempts to supplement NO-deficient airways in CF patients by iNO [17] or NO pre-cursor L-arginine [8] exhibited improvements in lung function parameters FEV₁ and FVC. This is in line with our observation of an increase in the mean value of FEV₁, FVC, and 6MWD during NO treatment. Although the study was not statistically powered, gains in the overall mean 6MWD values were partially maintained during 7- and 11-week follow up visit, long after the last iNO treatment. As life expectancy is steadily rising in CF patients, quality of life and endurance tests such as 6MWD could be relevant endpoints in assessing iNO efficacy in future studies. In fact, previous studies point to the prognostic potential of 6MWD in adult CF patients and its positive correlation with lung function parameters, especially in subjects with FEV₁ below 60% [42].

The NO-related mechanism for improvement in functional lung capacity is complex and could be related to a decrease in inflammation and edema, increase in oxygenation, or a reduction in bacterial load [23,43]. Inhaled NO can also act indirectly by promoting the formation of S-nitrosothiols (SNOs) such as S-nitrosoglutathione (GSNO) in the lungs [44–46]. GSNO has been shown to increase cellular expression, maturation, and function of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), more specifically F508Del CFTR, in human airway epithelial cells [46,47]. Airway GSNO levels are very low in CF patients and aerosolized replacement GSNO therapy revealed modest and sustained improvement in oxygen saturation in CF patients [48]. In line with this proposed mechanism, the small gains in the overall mean FEV₁ can theoretically be attributed to improved CFTR maturation. However, the small number of patients precluded analysis of CFTR mutation-specific response to iNO in this study.

A partial reversal from earlier gains was observed in patients' lung function (FEV₁, FVC) at 11-week follow-up (8 weeks after the last iNO treatment), which could be due to the progressive lung injury caused by chronic NTM infection. Alternatively, NO reaction with O₂-enriched air produces NO₂, which at high levels can result in nitrosative stress induced injury in lung airways with symptoms including coughing, fatigue, nausea, headache and difficulty in breathing [49–51]. Intermittent iNO regimen described in this study was designed to minimize the buildup of airway NO₂ and maintain low blood MetHb levels. The mean inspired NO₂ level during 30 min intermittent iNO treatment was 2.1 ppm with 95% CI of [1.4, 2.8], well below the 5 ppm short-term exposure limit recommended by World Health Organization [52]. Nevertheless, to monitor any potential airway injury caused by nitrosative stress during iNO therapy or follow-up period, we recommend monitoring patient sputum and serum 3-nitrotyrosine levels as a marker for nitrosative stress in future studies.

Three patients experienced at least one negative *M. abscessus* culture during the study. Besides, increases in median time to positivity in culture and a gradual reduction in mean *M. abscessus* DNA in sputum by qPCR provide evidence that iNO treatment may have caused a reduction in airway bacterial load. However, treatment did not result in culture conversion (defined as 3 consecutive monthly negative culture of sputum samples). Deppisch et al. also reported a reduction, but not eradication, of selected bacteria and fungi species, including *M. abscessus*, in CF patients treated with 160 ppm iNO [17]. Interestingly, three out of five patients with chronic *P. aeruginosa* co-infection responded to iNO treatment in our study by exhibiting negative cultures or >2-log reduction in bacterial load at follow-up. The difference in bacterial species susceptibility to NO is unknown, however, it may be attributed to the presence

of intrinsic NO-resistant mechanisms in *Mycobacterium*, such as elevated mycothiol, which can neutralize the effect of NO [12]. This could suggest that a longer duration of iNO treatment may be necessary to achieve a significant bactericidal activity in CF patients with chronic NTM lung infection. It is noteworthy to examine the transient burst in sputum *M. abscessus* DNA (rpoB gene detected by qPCR) during the first week of iNO treatment. While rpoB-qPCR based quantification of viable *M. abscessus* in non-cultured sputum has lower sensitivity compared to CFU count (solid media) or time to positivity (liquid media) of cultured sputum, it can provide valuable information about *M. abscessus* DNA load in patient airways [19]. The sharp increase in *M. abscessus* DNA content in sputum could be attributed to the biofilm dispersal activity of NO [53–55], which results in the release of planktonic bacteria into sputum. It is tempting to speculate that prolonged iNO treatment, beyond 3 weeks tested here, could increase the susceptibility of NTM biofilm to antibiotics and achieve an improved bactericidal activity in CF airways.

The main limitation of our study is the small number of patients and the results should be interpreted with caution. Additional exclusion criteria should be considered in future studies. Careful monitoring of additional parameters such as creatinine and platelet function as a marker of bleeding time should be included. The small number of AEs observed in our study may be due to the low number of participants and short duration of treatment.

Taken together, in this pilot study, the delivery of high-dose iNO was found to be safe and well-tolerated; thus, it may provide additional benefit to CF patients infected with *M. abscessus*. The potential value of iNO as an additional novel anti-NTM agent is encouraging, considering the complexity of the current antibiotic treatment, the significant side effects, and the lack of long-term efficacy. Based on our findings, additional clinical studies in larger cohorts and increases in the duration of iNO therapy are recommended to elucidate whether NO can serve as a potential adjunctive therapy for *M. abscessus* lung infection in CF.

Conflict of interests

The following authors; Michal Gur, Moshe Ashkenazi, Yuval Geffen, Micha Aviram, and Ori Efrati have no conflicts of interests. Lea Bentur, the principal investigator of this study received from the sponsor, AIT Therapeutics Inc. funding for travel and accommodation in order to present preliminary data at international conference (the American Thoracic society 2018 international conference, May 18–23, San-Diego, California, USA). The co-authors, Mark Mizrahi, Asher Tal and Abdi Ghaffari are all employed by AIT Therapeutics Inc. and hold stocks and/or options in the company.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jcf.2019.05.002>.

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