Title: Low dose nitric oxide as targeted anti-biofilm adjunctive therapy to treat chronic *Pseudomonas aeruginosa* infection in cystic fibrosis

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Short Title: Low dose nitric oxide therapy in cystic fibrosis

Abstract:

Despite aggressive antibiotic therapy, bronchopulmonary colonisation by *Pseudomonas aeruginosa* causes persistent morbidity and mortality in cystic fibrosis (CF). Chronic *P. aeruginosa* infection in the CF lung is associated with structured, antibiotic-tolerant bacterial aggregates known as biofilms. We have demonstrated the effects of non-bactericidal, low-dose nitric oxide (NO), a signaling molecule that induces biofilm dispersal, as a novel adjunctive therapy for *P. aeruginosa* biofilm infection in CF in an *ex-vivo* model and a proof of concept double blind clinical trial. Submicromolar NO concentrations alone caused disruption of biofilms within *ex-vivo* CF sputum, and a statistically significant decrease in *ex-vivo* biofilm tolerance to tobramycin and tobramycin combined with ceftazidime. In the 12 patient randomized clinical trial, 10 ppm NO inhalation caused significant reduction in *P. aeruginosa* biofilm aggregates compared to placebo across the 7 days of treatment. Our results suggest a benefit of using low-dose NO as adjunctive therapy to enhance the efficacy of antibiotics used to treat acute *P. aeruginosa* exacerbations in CF. Strategies to induce the disruption of biofilms have potential to overcome biofilm-associated antibiotic resistance in CF and other biofilm-related diseases.

Introduction

Cystic fibrosis (CF) is the most common lethal, hereditary disease in Caucasian populations, with a UK and US incidence of approximately 1 in 2500 live births and an estimated worldwide prevalence of 70,000 (*1, 2*). Long-term morbidity and mortality is primarily associated with the effects of chronic *Pseudomonas aeruginosa* lung infection and the persistence of *P. aeruginosa* biofilms (*3, 4*). Bacteria in biofilms are enclosed in a self-produced biopolymeric matrix and display up to 1,000-fold higher tolerance to antibiotic challenge than their single cell, planktonic (free living) counterparts (*5*). Biofilms also exhibit resistance to phagocytosis and other components of the host's innate and adaptive immune system (*6*). Biofilm survival mechanisms include impedance of antibiotic diffusion through the biofilm matrix (*7*), altered growth or metabolic rates of bacterial subpopulations within the biofilm (*8, 9*), and physiological (*8*), biochemical (*10*) and genetic (*11, 12*) changes. In addition, sub-inhibitory levels of aminoglycoside antibiotics can enhance biofilm formation under laboratory conditions (*13*). Biofilms can be firmly attached to tissue but can also exist in the protected phenotype as aggregates in the mucus of the CF lung (14). Biofilms are extremely difficult to eradicate using conventional therapeutic regimes (15). New approaches targeting chronic biofilm infections are needed for more effective treatment of *P. aeruginosa* in CF, and other biofilm-related diseases (16).

In vivo, bacteria often transition between planktonic and biofilm lifestyles. Given the correct environmental cues, biofilm bacteria undergo coordinated dispersal and reversion to the planktonic form (17). We identified a role for the signaling molecule nitric oxide (NO) in the dispersal of *P. aeruginosa* biofilms (18, 19) (Fig. 1). At nanomolar concentrations, NO mediates dispersal by increasing bacterial phosphodiesterase activity with a consequent reduction of the intracellular second messenger and biofilm regulator cyclic-di-guanosine monophosphate (c-di-GMP) (18, 19). Here we report the effects of non-bactericidal, low-dose NO on clinical pseudomonal biofilms *ex-vivo* in the laboratory using conventional and molecular microbiological methods. We have also extended our laboratory findings to a proof of concept clinical trial in humans, demonstrating a significant direct effect on pseudomonal biofilm load in CF patients treated with NO gas plus conventional intravenous antibiotic therapy compared to intravenous antibiotics alone.

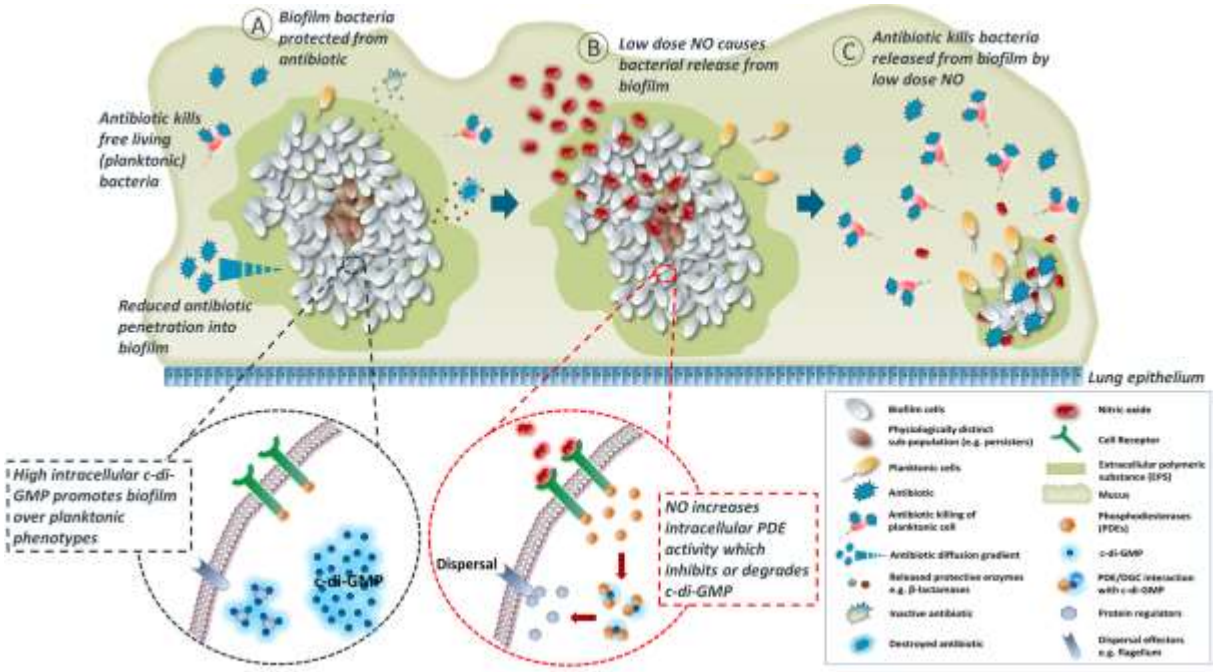

Fig. 1. Role of NO in disrupting antibiotic tolerance mechanisms associated with the biofilm structure. A) Biofilm tolerance mechanisms include reduced antibiotic diffusion, release of protective enzymes capable of destroying or inactivating antibiotics in the biofilm matrix and formation of physiologically distinct bacterial subpopulations (e.g. persister cells) resulting from nutrient and oxygen gradients. **B**) Low-dose nitric oxide diffuses into the biofilm and interacts with cell receptors that upregulate cellular phosphodiesterases (PDEs) which accelerate c-di-GMP degradation. This prevents c-di-GMP from interacting with proteins at the transcriptional, translational or post-translational level and leads to cell surface and physiological changes associated with dispersal and motility (red circle inset). **C**) Dispersal is accompanied by reversion of the bacteria to a planktonic phenotype that renders them more susceptible to antibiotic-mediated killing (18, 19).

Results

Nitric oxide induces *P. aeruginosa* biofilm dispersal in human CF sputum samples

NO-induced dispersal of *P. aeruginosa* biofilms was specifically measured directly in expectorated sputum samples from 5 CF patients using fluorescence *in situ* hybridization (FISH). A significant reduction in mean biofilm thickness was observed upon treatment with 450 nM NO (generated from the spontaneous NO-donor sodium nitroprusside, SNP; see Methods) and *P. aeruginosa* biofilm microcolonies (aggregates typically ~15 μm in diameter) were visibly disrupted by NO in 5/5 patient samples. Fig. 2A shows representative experiments from 3 different patients: Sample 1($p=0.003$), Sample 2 ($p=0.029$), and Sample 3 ($p=0.029$).

Nitric oxide mediated dispersal of CF *P. aeruginosa* isolates occurs within 5 to 10 hours

Addition of NO (in the form of the NO-donor sodium nitroprusside, SNP) to 12 biofilm-forming *P. aeruginosa* clinical isolates from CF sputum samples consistently caused dispersal leading to steep increases in the optical density (turbidity) of planktonic bacterial suspensions overlying biofilms after 5 hours (Fig. 2B). The increase in OD correlated with a decrease in biofilm biomass from surfaces of plate wells as determined by fluorometric measurements and confocal microscopy, confirming the dispersal effect of NO (Figs. 2D, E). Biofilm dispersal was confirmed to be NO-specific using the NO scavenger PTIO, which reduced the dispersal of *P. aeruginosa* induced by SNP ($p=0.002$) to levels similar to the control treatment (Fig. 2C). Treatment of biofilms with potassium ferricyanide (as a control for NO-independent breakdown products of SNP) had no dispersal effect compared with untreated biofilms ($p=0.394$; Fig. 2C). Dispersal was observed at NO concentrations as low as 450 pM, peaking at 450 nM (Fig. 2B), with higher concentrations of NO (4.5 μM) showing reduced efficacy for biofilm dispersal (Fig. 2B). NO at a concentration of 450 nM dispersed all 12 biofilm-forming CF clinical isolates tested.

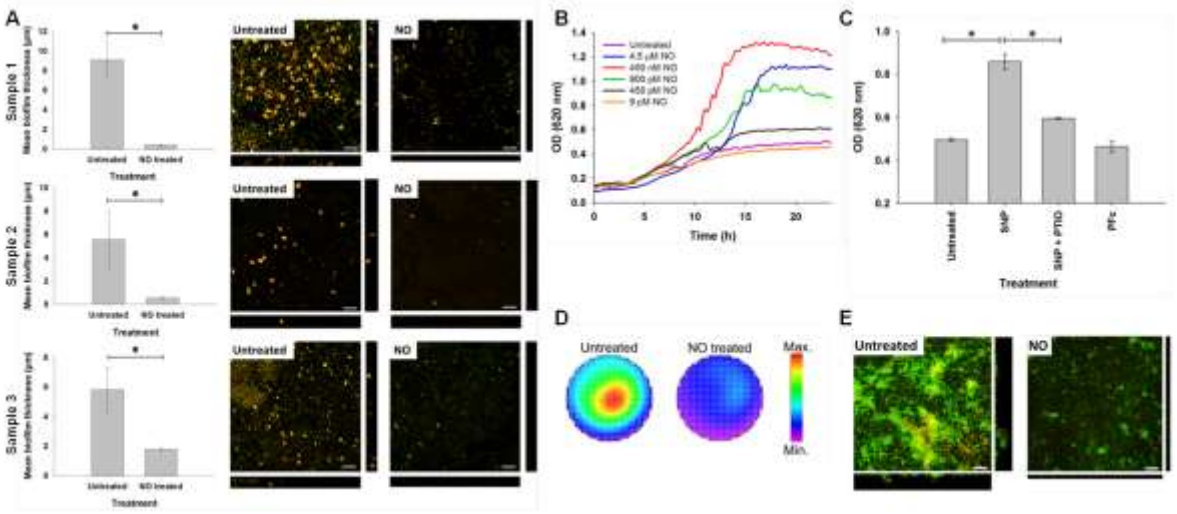

Fig. 2. A) Direct measurement of NO-induced *P. aeruginosa* biofilm dispersal in expectorated CF sputum samples. Image analysis shows a significant reduction in mean *P. aeruginosa* biofilm thickness following treatment of CF sputum samples from 3 different patients (Samples 1, 2 & 3) with 450 nM NO, compared with buffer alone (untreated) (*represents a statistically significant difference between data medians, P = 0.02). *P. aeruginosa* was identified using fluorescence *in situ* hybridisation (FISH) with both a Cy3-labeled *P. aeruginosa* specific 16S rRNA probe (green) and a Cy5-labelled eubacterial 16S probe (red). Confocal laser scanning microscopy (CLSM) images show a reduction of *P. aeruginosa* (yellow due to hybridisation with both probes) in biofilms. Images show horizontal *xy* (top-down view) sections and flanking images show vertical *z* (side view) CSLM sections of untreated (left) and NO-treated (right) CF sputum samples. Scale bar = 25 µm. **B) Nitric oxide (NO) disperses *in vitro* biofilms grown from biofilm-forming *P. aeruginosa* CF clinical isolates.** Dispersal of biofilm bacteria into the planktonic phase (measured by mean optical density (OD) of overlying planktonic suspensions) following treatment of a clinical isolate *P. aeruginosa* biofilm with low-dose NO (9 pM-4.5 µM) derived from the spontaneous NO donor, SNP. Depicted recordings are from a single isolate and representative of qualitatively identical data from 12 *P. aeruginosa* isolates studied. **C) Biofilm dispersal is NO-dependent.** Mean OD measurements of planktonic bacteria following 15 h treatment of *P. aeruginosa* biofilms with SNP alone, SNP in the presence of the NO scavenger PTIO, or with potassium ferricyanide alone (PFc); *represents a statistically significant difference between data medians, P = 0.02). Data from 3 experiments with 4 wells per experiment. **D) Dispersal causes biofilm detachment from the base of tissue-culture plate wells indicated by loss of fluorescence after NO treatment, compared with untreated controls**. Residual biofilms were fluorescently-labelled with the nucleic acid probe, Syto9. Scale indicates fluorescence intensity, with red corresponding to the highest concentration of surface-attached *P. aeruginosa* and blue-purple to the fewest remaining attached bacteria. **E) NO induces dispersal of *P. aeruginosa* biofilms *in vitro*.** Representative CSLM images indicate reduced *P. aeruginosa* in biofilms from CF isolates following NO treatment compared with untreated biofilms. Each image shows horizontal *xy* (top-down view) CLSM sections (square), and flanking images show vertical *z* (side-view) CLSM sections after staining biofilms with the BacLight Live (green)/Dead (red) kit. Scale bar = 25 µm.

Nitric oxide potentiates antibiotics to disrupt and kill clinical *P. aeruginosa* biofilms

P. aeruginosa clinical isolate biofilms treated with the antibiotic tobramycin alone or with tobramycin/ceftazidime combinations were compared to biofilms treated with NO alone, a combination of NO and tobramycin or a combination of NO, tobramycin and ceftazidime (Fig. 3). Remarkably, the biomass and thickness of the *P. aeruginosa* biofilm increased substantially following antibiotic treatments in the absence of NO. Compared with untreated biofilms, an increase in biofilm biomass and biofilm thickness was observed following tobramycin treatment alone (biofilm biomass: 243% increase compared to control, p=0.028, Fig. 3B; and mean biofilm thickness: 199% increase compared to control, p=0.065, Fig. 3C) and the tobramycin/ceftazidime combination (biofilm biomass: 155% increase compared to control, p=0.04, Fig. 3B; and mean biofilm thickness: 174% increase compared to control, p=0.04, Fig. 3C). Viability staining demonstrated that predominantly live (green) cells remained within the core of the biofilm structures (Fig. 3A). While biofilm bacteria tolerated the antibiotic treatments at the concentrations used (10 μM), free-living bacteria within the planktonic phase remained susceptible (Fig. 3D).

Adjunctive NO used in combination with 5 μg ml^{-1} tobramycin (with or without ceftazidime) demonstrated a pronounced and significant reduction in *P. aeruginosa* mean biofilm biomass and thickness compared with both untreated biofilms and biofilms treated with antibiotics in the absence of NO (p=0.001) (Figs. 3B & 3C). Residual surface-attached biofilms observed by CSLM appeared as only a thin monolayer indicating that the majority of the remaining surface-attached *P. aeruginosa* had been killed, shown in Fig. 3A by increased red fluorescent staining with propidium iodide. In addition, there was a marked reduction in viable planktonic cells following adjunctive NO treatments (Fig. 3D), demonstrating that bacteria released from biofilms during NO-induced dispersal are killed in the planktonic phase by the combined antibiotic treatment.

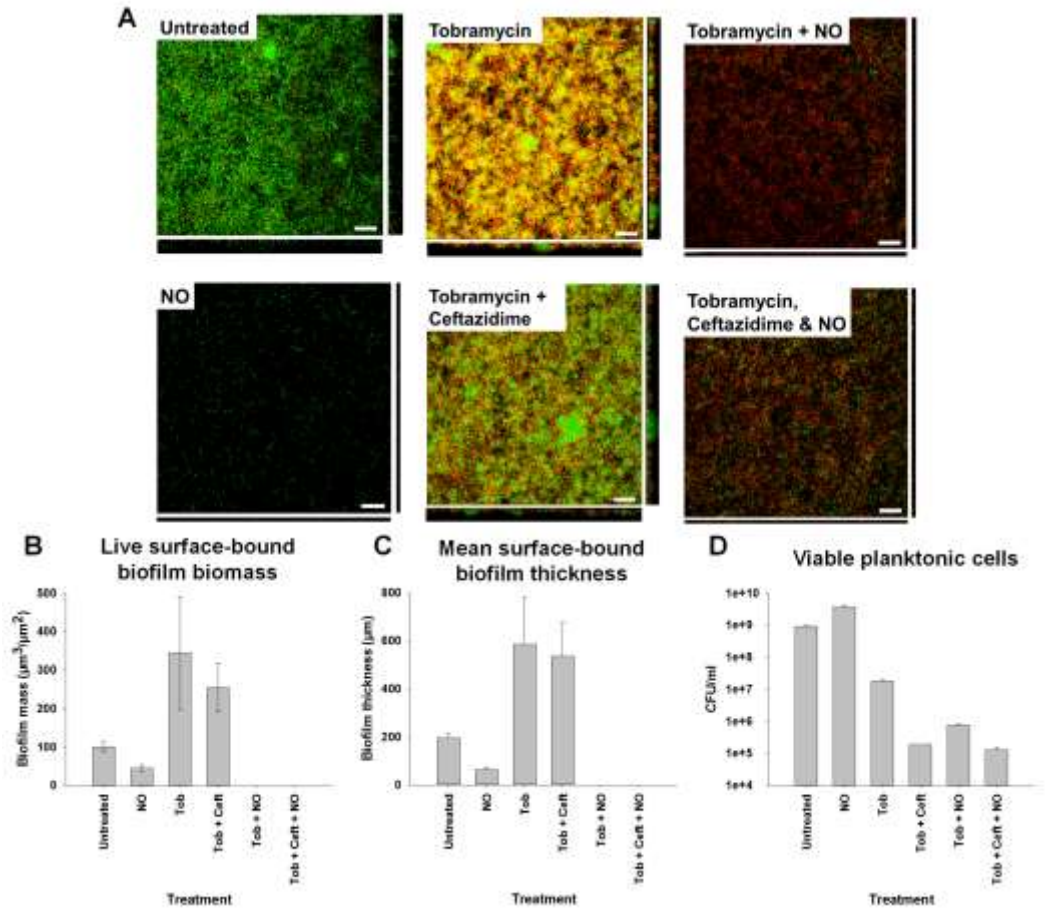

Fig. 3. Antibiotic efficacy against *P. aeruginosa* clinical isolate biofilms is enhanced in the presence of low-dose NO. A) Representative confocal laser scanning microscopy (CLSM) images showing surface-attached *P. aeruginosa* following treatment with: buffer alone (untreated); NO alone; MBC antibiotics (5 μg ml^{-1} tobramycin with or without 5 μg ml^{-1} ceftazadime); or antibiotics combined with NO. Images show horizontal *xy* (top-down view) sections and flanking images show vertical *z* (side view) CSLM sections. Biofilms were stained with BacLight Live (green)/Dead (red) kit to indicate viable cells. Scale bars = 25 μm. **B) Image analysis of CLSM images of residual *P. aeruginosa* biofilms with adjunctive NO** shows a reduction in mean total biomass (**Fig. 3B**) and biofilm thickness (**Fig. 3C**) following treatment with antibiotics (tobramycin alone and tobramycin (Tob)/ceftazidime (Ceft) combined) indicating that NO-treatment reduces the amount of remaining biofilm bacteria (error bars represent standard error of the mean of 5 different microscopic fields). An increase in biofilm biomass and biofilm thickness is shown following tobramycin treatment alone (biofilm biomass: 243% increase compared to control, p=0.028, **Fig. 3B**; and mean biofilm thickness: 199% increase compared to control, p=0.065, **Fig 3C**) and the tobramycin/ceftazidime combination (biofilm biomass: 155% increase compared to control, p=0.04, **Fig. 3B**; and mean biofilm thickness: 174% increase compared to control, p=0.04, **Fig 3C**). Viable *P. aeruginosa* in the dispersed population (planktonic suspension), determined by colony forming unit (CFU) counts of *P. aeruginosa* following antibiotic treatment of biofilms with or without NO, indicate that combined NO treatment leads to killing of the bacteria released from the biofilm (**Fig. 3D**).

A proof of concept randomized trial demonstrates low-dose nitric oxide adjunctive therapy reduces detectable *P. aeruginosa* biofilm in patients without increasing planktonic bacterial loads

12 patients were randomized to receive either low-dose NO inhalation or placebo (CONSORT diagram, figure 4). Adjunctive NO used in combination with tobramycin and ceftazidime demonstrated a significant reduction in the key primary microbiological endpoint, *P. aeruginosa* biofilm aggregates. This is shown in aggregates both over 20 cells in size and in those over 10 cells in size compared to those receiving placebo with antibiotics over the 7 days of treatment (GEE analysis, p=0.031 and p=0.029 respectively for days 5 and 7; and Fig. 5). Data suggested less *P. aeruginosa* biofilm as quantified by both the number and volume of aggregates greater than 20 or 10 cells in the NO group compared with placebo through day 7 while on NO therapy. This reduction was not fully maintained after treatment was stopped, as pseudomonal biofilm was detected in treatment group samples at timepoint 10-13 days following the cessation of NO therapy (study period days 5 through 20, Table 1 and Fig 5). See methods for rationale regarding cluster size selection. Other important secondary endpoints are shown in Table 2. From an individual participant safety perspective, there was no evidence that the biofilm dispersal increased the amount of viable *P. aeruginosa* detected in planktonic phase by CFUs. qPCR, indicative of total viable *P. aeruginosa* cells (20), did not demonstrate a difference between groups due to the small numbers and large variation between individuals. There were also no adverse clinical safety signals (FEV_1, FVC, quality of life score) in the treatment group compared to those treated with placebo. Baseline clinical data are shown in Table 3, baseline laboratory data and study adverse effects are shown in Supplementary Tables S1 and S2, and individual patient data for the primary outcome (FISH) and one clinical parameter (FEV1) are shown in Supplementary Table S3.

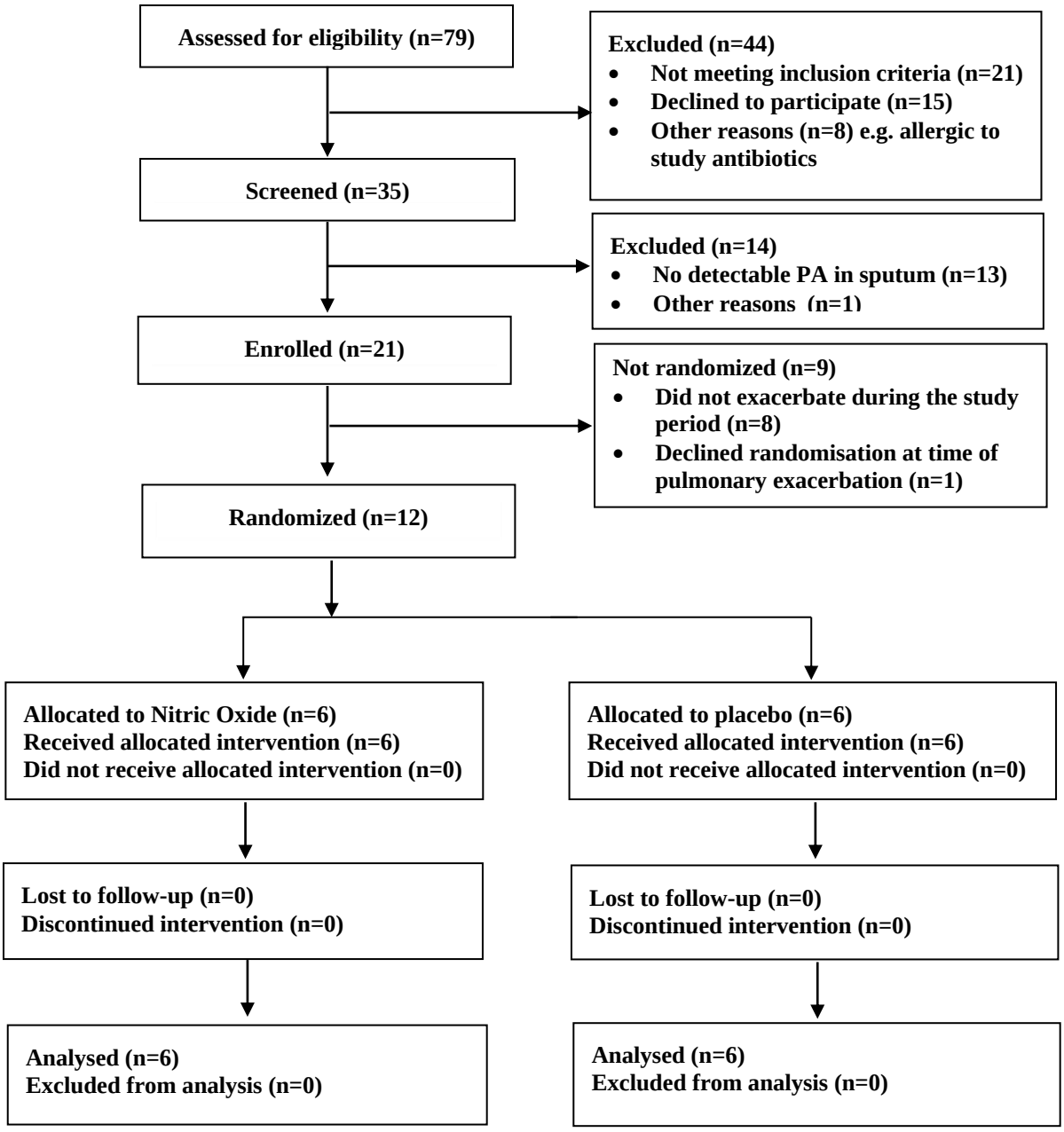

Figure 4: Clinical Study CONSORT diagram depicting the flow of patients through the study. In order for patients to be randomised they had to be admitted during pulmonary exacerbation to receive trial therapy concurrently with IV antibiotics.

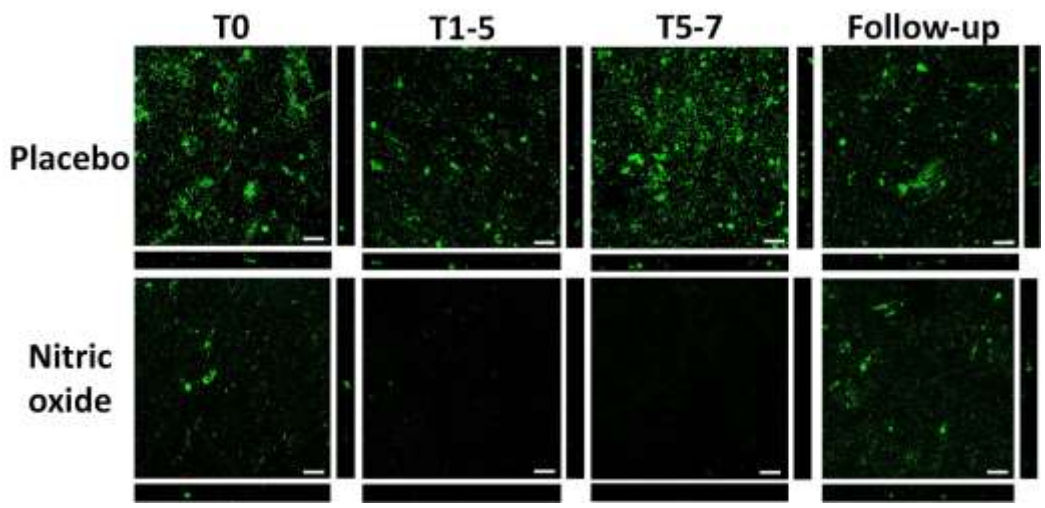

Fig. 5. Reduction in *P. aeruginosa* biofilm with NO adjunctive therapy. Representative FISH confocal images from a CF patient being treated with NO adjunctive to conventional antimicrobial agents (ceftazadime and tobramycin) compared to a patient on antibiotics alone (n=6 in both nitric oxide and placebo groups). Almost no *P. aeruginosa* biofilms were detectable in the treatment group compared to placebo. At follow up, 10-13 days after NO adjunctive treatment stopped, pseudomonal biofilm was detected in sputum having been reduced while on NO. Scale bars = 25 μm. The central panels show x-y plan views of merged image stacks (total biofilm detected in 3D imaging), the rectangular z-axis side panels show representative single side views of the biofilm (21)

Tables 1-2. Results from randomized clinical trial.

Table 1: Primary outcome results: showing mean differences between groups (NO or placebo) of change from baseline

	Change from baseline, Mean (SD)				Treatment effect: mean difference (95%CI), p value			
Day	**5**	**7**	**10**	**20**	**Intervention period (days 5 &7)**		**Study period (days 5,7,10 & 20)**	
FISH: Ln <u>number</u> of aggregates > 20 cells								
Placebo	0.11 (2.38)	0.35 (1.44)	0.38 (2.32)	NA	3.49 (0.32, 6.67)	p= 0.031	1.35 (-0.58, 3.7)	p=0.170
NO	-4.33 (5.11)	-2.19 (3.93)	0.98 (1.83)	NA				
FISH: Ln <u>volume</u> of aggregates > 20 cells								
Placebo	-0.16 (2.51)	-0.03 (1.54)	0.21 (2.20)	NA	4.47 (-0.40,8.98)	p= 0.052	2.35 (0.08, 4.63)	p=0.043
NO	-6.10 (7.50)	-3.03 (5.88)	0.97 (2.02)	NA				
FISH: Ln <u>number</u> of aggregates > 10 cells								
Placebo	0.28 (2.09)	0.26 (1.52)	0.20 (2.04)	NA	2.44 (0.25, 4.62)	p=0.029	1.09 (-0.54, 2.72)	p=0.118
NO	-1.46 (1.08)	-2.71 (4.56)	1.10 (1.19)	NA				
FISH: Ln <u>volume</u> of aggregates > 10 cells								
Placebo	0.08 (2.33)	0.05 (1.50)	-2.47 (2.12)	NA	2.68 (-.052, 5.41)	p=0.055	1.27 (-0.62, 3.16)	p=0.188
NO	-1.75 (1.14)	-3.37 (6.34)	1.07 (1.50)	NA				

Table 2. Microbiological and clinical safety monitoring: showing mean differences between groups (NO or placebo) of change from baseline

Day	Change from baseline, Mean (SD)				Treatment effect, mean (95%CI); p value			
	5	7	10	20	Intervention period (days 5 &7)		Total study period (days 5,7,10 & 20)	
Ln CFU								
Placebo	-1.62 (2.34)	-2 (3.77)	-0.89 (4.08)	NA	-0.19 (-2.95, 2.56)	p=0.891	0.03 (-2.53, 2.59)	p=0.980
NO	-1.97 (2.20)	-1.25 (2.76)	-1.30 (1.64)	NA				
Ln QPCR								
Placebo	-2.16 (1.73)	-4.33 (2.44)	-4.32 (1.92)	NA	-0.47 (-1.91, 0.97)	p=0.519	-0.37 (-1.44, 0.71)	p=0.504
NO	-1.86 (1.60)	-3.67 (1.81)	-3.09 (1.74)	NA				

Day	Change from baseline, Mean (SD)				Treatment effect, mean (95%CI); p value			
	5	7	10	20	Intervention period (day 7 only)		Study period (day 20 only)	
FEV_1								
Placebo	NA	6.67 (4.46)	9.00 (2.52)	6.17 (3.49)	-8.93 (-25.3, 7.42)	p=0.248	1.95 (-7.31, 11.20)	p=0.645
NO	NA	15.6 (17.2)	5.01 (14.2)	4.22 (9.35)				
FVC								
Placebo	NA	4.83 (6.74)	9.17 (5.46)	6.33 (4.46)	-11.6 (-30.7, 8.42)	p=0.229	8.03 (-4.10, 20.2)	p=0.168
NO	NA	16.0 (20.1)	3.75 (14.6)	-1.70 (12.3)				

Table 3: Baseline clinical characteristics of groups (A=Nitric Oxide, B=Placebo)

	Treatment Group	N	Mean	Std. Deviation
Age in years	A	6	30.0	13.99
	B	6	29.3	15.60
Height in cm	A	6	162.8	9.45
	B	6	166.0	9.27
Weight in kg	A	6	56.4	9.61
	B	6	63.0	8.32
Heart rate in bpm	A	6	89.3	18.62
	B	6	91.2	17.19
Systolic blood pressure (mmHg)	A	6	107.3	13.84
	B	6	121.0	14.97
Diastolic blood pressure (mmHg)	A	6	64.2	9.37
	B	6	75.8	13.73
Oxygen saturation (% in air)	A	6	95.2	2.23
	B	6	95.2	3.25
Respiratory rate (per minute)	A	6	20.0	1.10
	B	6	18.5	2.17
Temperature (deg C)	A	5	36.8	.31
	B	6	36.9	.48
FEV_1% of predicted (l)	A	6	40.2	20.14
	B	6	45.7	18.28
FVC % of predicted (l)	A	6	54.4	17.60
	B	6	71.5	21.11
Average exhaled NO levels (in ppb)	A	6	12.7	9.46
	B	6	9.3	8.86

Circulating NO metabolites change little during low-dose NO inhalation in CF patients

Plasma nitrate (NO_3^-) concentrations tended to increase in response to delivery of low-dose NO, but these changes did not reach statistical significance ($P > 0.05$). Plasma levels of nitrite (NO_2^-) and total nitrosation products (RXNO) paradoxically decreased during NO inhalation, although this was also not significant. With the exception of unusually high nitrite levels in erythrocytes compared to plasma values there was also no obvious effect of inhaled NO on NO metabolite status in these blood cells, which is surprising given that nitrosylhemoglobin (NO-Heme) is the most sensitive marker of NO availability *in vivo* and nitrate is the final oxidation product of NO (22) (thus, both might be expected to be elevated following prolonged NO inhalation). Direct NO measurement in sputum was impractical due to the short half-life of NO in relation to the time taken for the probe to equilibrate in individual sputum samples (data not shown). Overall, determination of a comprehensive panel of NO metabolites suggested that low-dose inhaled NO does not significantly affect circulating NO metabolites in CF (Supplementary Figure S1).

Discussion

Targeted therapy to address biofilm infection, rather than using conventional antibiotics alone, represents a potential paradigm shift in the treatment of chronic pseudomonal infection in cystic fibrosis. Our experiments show that adjunctive NO can disrupt *P. aeruginosa* biofilms and suggest a novel approach to the challenge of managing persistent *Pseudomonas* biofilm infection in CF patients.

The importance of the biofilm phenotype in promoting *P. aeruginosa* survival and persistence within the lower respiratory tract is well established (4, 23). However, there are currently no clinically recognized therapeutic approaches for eradicating established biofilm-associated *P. aeruginosa* respiratory infections. New treatment strategies for bacterial biofilms are a critical unmet need (24-26).

Our approach was to design a clinical diagnostic platform that could be used to detect changes in *Pseudomonas* biofilm from patients with CF. We used fluorescence in situ hybridization (FISH) as a primary technique to identify biofilm in clinical samples as recommended by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines for the diagnosis and treatment of biofilm infections (26) . We first used *ex vivo* samples from CF patients to establish the diagnostic platform. We tested *P. aeruginosa* clinical isolates growing in biofilms and used FISH to follow the effects of NO on aggregate size in these biofilms. We designed the proof of concept clinical study to determine whether changes in the size of *Pseudomonas* biofilm aggregates taken from patients who had been given low dose NO could be detected during and following treatments regimes.

Ex-vivo studies demonstrated that low concentrations of NO (<500 nM) significantly reduced the amount of *P. aeruginosa* biofilm aggregates in CF sputum, potentiating the effect of the aminoglycoside antibiotic tobramycin alone and in combination with the third generation cephalosporin ceftazidime.

In addition, our results suggest that adjunctive low-dose nitric oxide might prevent a previously reported potential biofilm-enhancing effect of aminoglycoside treatment (*13*). Our *ex-vivo* data show that treatment of CF *P. aeruginosa* biofilms with clinically relevant concentrations of tobramycin can lead to increased biofilm growth. Bacteria in biofilms within the CF lung are likely to be exposed to sub-inhibitory concentrations of antibiotics due to poor penetration or diffusion gradients through the biofilm (27). Such sub-inhibitory antibiotic concentrations may explain the apparently paradoxical increase in biofilm thickness we observed despite increased cell death. It is possible that initially low antibiotic concentrations within the biofilm induce bacterial growth and/or extracellular matrix production, followed by increased cell death as the antimicrobial concentration increases due to diffusion into the biofilm. An alternative explanation for the increased biofilm thickness might be enhanced cell lysis, which has been shown to contribute to *P. aeruginosa* extracellular matrix production (28). Importantly, irrespective of mechanism, the observed enhancement of *P. aeruginosa* biofilm growth in the presence of tobramycin, was completely eliminated in the presence of 450 nM adjunctive NO. Nitric oxide potentiated the effect of tobramycin alone, and ceftazidime and tobramycin in

combination, by dispersing *P. aeruginosa* biofilms and facilitating the killing of dispersed bacteria.

The proof of concept clinical study demonstrated a significant direct effect on pseudomonal biofilm (as measured by a reduction in *P. aeruginosa* aggregate load) in CF patients treated with NO gas plus conventional IV antibiotic therapy compared to IV antibiotics alone. The effect was not sustained following the end of NO therapy in this group of adult patients with long term chronic disease. In this small study we did not detect any side effects as a result of this treatment strategy. All changes in the clinical parameters measured favored NO treatment and there was no evidence that NO treatment caused an increase in overall bacterial load or the severity of acute exacerbations. We saw no treatment effects suggestive of NO induced vasodilatation (i.e. no increase in oxygen saturations during treatment); and no adverse effects during the weaning period at the end of each day's NO therapy that might have been indicative of rebound pulmonary hypertension. Our study measured clinical parameters to ensure safety but not clinical efficacy, which will be the subject of future large clinical trials.

Previous studies have shown that *P. aeruginosa* cells can be killed directly by high doses of NO (29). This might be the result of several possible toxic effects of NO on bacteria at high concentrations, including direct modification of membrane proteins, DNA cleavage and lipid peroxidation through mechanisms of both nitrosative and oxidative stress (30-32). The use of high dose NO in this way has potential cytotoxic and other adverse clinical effects (33) and is associated with considerable cost. Despite this, recent trials of high dose 160 ppm inhaled NO in CF did not demonstrate any adverse safety signals (34, 35). In terms of biofilm growth which has not been measured in previous clinical trials, high levels of NO might result in increased nitrate levels in CF sputum that may support growth of *P. aeruginosa* by metabolism based on anaerobic denitrification (36, 37). Our previous *in vitro* studies have shown that higher concentrations of NO can stimulate biofilm formation (18). These studies agree with another report suggesting that higher dose NO may in fact enhance aminoglycoside tolerance by blocking energy-dependent phases of drug uptake (38). The low-dose, signal-relevant concentrations of NO we used in the proof of concept clinical trial reported here are approximately three orders of magnitude lower than those shown to inhibit drug uptake and did not inhibit tobramycin efficacy against dispersed (planktonic) or biofilm *P. aeruginosa* bacteria.

The rationale for our approach using NO to treat *P. aeruginosa* infection was to exploit our discovery that low-dose NO (10 ppm, assumed to translate into submicromolar concentrations locally) mediates biofilm dispersal through increased bacterial phosphodiesterase activity and an associated decrease in c-di-GMP levels (19). We have previously shown that low-dose NO can increase the motility of *P. aeruginosa* cells *in-vitro* (18), and proposed that this increased motility promotes biofilm dispersal. In contrast and in the context of CF sputum, other studies have shown that *P. aeruginosa* isolates are frequently non-motile (39) and that sputum can repress *P. aeruginosa* flagellar activity and motility (40, 41). Cyclic-di-GMP binds to a broad range of effector components that control the physiology, development, stability, cell adhesiveness and motility of the biofilm phenotype. Factors other than motility could therefore be responsible for biofilm disruption and a reduction in tolerance to antibiotic treatment. Further studies are required to understand the specific c-di-GMP effectors responsible for NO-mediated disruption of biofilms within CF sputum.

Chronic CF infections are often associated with multiple bacterial pathogens and complex microbial communities (42, 43). Genes that modulate c-di-GMP turnover are widely distributed in bacteria, and NO-mediated dispersal has now been observed across a number of species including many pathogenic organisms (44, 45). NO-mediated alteration of intracellular c-di-GMP levels is therefore an important new potential target to control multispecies bacterial communities in CF. NO might also be of benefit in treating younger CF patients after initial infection with *P. aeruginosa*. Used in these circumstances it might increase the effectiveness of eradication therapy and delay the onset of chronic biofilm infection with this organism.

Our clinical trial data appear to differ from the reported effects of inhaled NO on circulating NO metabolite levels in infants with pulmonary hypertension (46), where a clear increase in NO metabolite levels was reported to occur with twice the concentration of inhaled NO used in our study. There is a paucity of information on circulating levels of NO metabolites in CF. Nevertheless, our observations are in general agreement with the notion that NO concentrations are lower in the exhaled breath of CF patients while systemic NO production does not appear to be compromised (47). Possible mechanisms for this include accelerated degradation as a result of increased oxidative stress in epithelial cells, increased NO consumption by bacterial biofilms, or impaired gas exchange as a result of mucus obstruction. All of these factors would be expected to prevent exogenous inhaled NO to reach the systemic circulation, limiting its effects to the site of administration.

The main limitation of the clinical component of our study is the small sample number and between-patient variation in clinical and microbiological parameters. This has made formal statistical analyses difficult but we were able to incorporate repeated measurements over time to improve power. Variability in the qPCR results between NO and placebo groups was probably due to sample heterogeneity in chronically infected patients. Despite these limitations, FISH image analysis data demonstrate a treatment effect and provide proof of concept for our low-dose NO approach. Similarly, our analysis of the changes in systemic NO status following low-dose NO inhalation is likely compounded by inter-individual differences in NO processing. However, the lack of an observed rise in blood nitrate and NO-heme levels are consistent with well-documented perturbations in NO production and handling in CF patients (48, 49).

Our study has demonstrated the potential for the use of low dose NO to enhance the antibiotic treatment of biofilm infections. Although the practical challenges in delivering inhaled NO gas to CF patients were considerable, future novel NO donor antibiotics might prove to be a more feasible approach to targeting biofilms (50). Biofilm-related chronic infections are responsible for at least half a million deaths per year at an estimated cost of over $94 billion in the United States alone (16). More effective anti-biofilm therapies are needed to address this significant unmet need.

Materials and Methods

CF sputum collection and *P. aeruginosa* isolation

Sputum samples (51, 52) from 72 patients with CF (median age at informed consent 21 years, range 17-62; UK NHS Research Ethics Reference 08/H0502/126) were obtained by CF physiotherapist-assisted sample expectoration. For isolation of *P. aeruginosa* from sputa, samples were digested using Mucolyse (Pro-Lab Diagnostics) containing dithiothreitol and phosphate buffer for 15 minutes at 37 °C, followed by culture on *P. aeruginosa*-specific cetrimide agar (Sigma-Aldrich). Multiplex PCR was used to confirm *P. aeruginosa* as previously described (53). Because *P. aeruginosa* colonisation of the CF lung often consists of multiple clonal lineages (54), colony sweeps (sterile loops drawn across a confluent streak of bacterial growth on cetrimide agar) were used in preference to single colony isolates for routine subculture and biofilm growth of *P. aeruginosa*.

Nitric-oxide mediated dispersal of clinical *P. aeruginosa* isolates

We first evaluated the ability of NO at different doses to disperse clinical isolates of *P. aeruginosa* biofilms *in-vitro* and within sputum from CF patients. Biofilm forming *P. aeruginosa* clinical isolates (n=12) were inoculated using overnight cultures grown in M9 minimal medium (20 ml per litre of 20 % glucose, 2 ml per litre of 1 M $MgSO_4$ and 100 µl per litre of 1 M $CaCl_2$). Overnight cultures were diluted to give optical density readings corresponding to 10^6 cells per millilitre and 200 µl aliquots were inoculated into a 96 well plate and incubated at 37 °C for 24 hours. The medium was aspirated and replaced with fresh M9 medium with/without increasing concentrations of the NO-donor sodium nitroprusside (SNP), concentration range 9 pM - 4.5 µM (Sigma Aldrich). The concentration of NO produced by SNP was calculated using a NO microsensor (Unisense, Denmark) and calibrated over a range of 250 nM to 10 µM using previously published methods (55). Based on the measured linear relationship between micromolar concentration of SNP producing nanomolar concentrations of NO (where $y = 0.9022x$, ($R^2 = 0.9617$, n=6 data points)), NO concentrations were calculated to be nearly 1000 fold less than the starting concentration of SNP, resulting in approximately 450 nM NO generated from 500 µM SNP. To confirm that effects were specific to NO, assays were also carried out with SNP (500 µM) in the presence of 5 mM NO scavenger carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (carboxy-PTIO; Sigma-Aldrich). M9 medium containing 500 µM potassium ferricyanide (Sigma-Aldrich), used to generate breakdown products of SNP (56, 57), was also used as a control. Optical density measurements of the supernatant containing planktonic cells were made using a BMG Labtech Omega plate reader (620 nm and chamber temperature of 37 °C) over 24 hours with measurements taken every 15 minutes. Experiments were repeated 3 times with 4 replicates for each experiment.

Nitric-oxide mediated dispersal of *P. aeruginosa* biofilms in CF sputum and antibiotic sensitivity testing

The use of fluorescence *in situ* hybridisation (FISH) to identify microbial biofilms in situ is recommended within the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines for the diagnosis and treatment of biofilm infections (26). Expectorated sputum samples (n=5) were divided in half (v/v) and treated for 15 hours with either Hanks Buffered Salt Solution (HBSS; Sigma-Aldrich) alone or HBSS containing 450 nM NO (i.e. 500

μM SNP). Samples were fixed in freshly prepared 4 % paraformaldehyde in phosphate-buffered saline (PBS) at 4 ºC and washed with PBS and PBS-ethanol (1:1 v/v) and 20 μl drops of sputum were spotted onto poly-L-lysine (PLL) coated slides and left to dry overnight. *P. aeruginosa* detection was performed using FISH with the 16S ribosomal probe sequences: PseaerA, 5'-GGTAACCGTCCCCCTTGC-3', specific for *P. aeruginosa* (58), labelled with Cy3 and EUB338 5'- GCTGCCTCCCGTAG GAGT-3' (Domain bacteria) (59), labelled with Cy5 (Integrated DNA Technologies Inc, Leuven, Belgium). Hybridization conditions for FISH were optimised and stringently evaluated *in vitro* to ensure specificity of the PseaerA probe. We independently confirmed the previously reported optimal hybridization conditions for the specificity of the Pseaer probe for *P. aeruginosa* (21, 58). Hybridisation with the sample was carried out using 20% formamide, and a 2 hour incubation at 46 ºC was followed by washing for 15 minutes at 48 °C in pre-warmed wash buffer as previously described (58, 60). Cover slips were placed on samples and imaged using an inverted Leica DMI600 SP5 confocal laser scanning microscope (CLSM; Leica Microsystems, Cambridge, U.K.). Control experiments with both positive and negative controls demonstrated that low concentrations of NO in the concentration range used for our studies did not interfere with the eubacterial or species-specific FISH signal for *P. aeruginosa* including no fluorescence quenching in the presence of NO (Supplementary Figure S2).

P. aeruginosa biofilms were examined for antibiotic sensitivity using adjunctive treatment of 450 nM NO with or without the aminoglycoside tobramycin. The antibiotic was added alone or in combination with the cephalosporin ceftazidime (both antibiotics at the minimum bactericidal concentrations (MBC) to induce killing of planktonic cells, determined to be 5 μg ml^{-1}). Biofilms were grown from colony sweeps as described above in culture plates (MatTek Corporation, Ashland, MA, USA) and treatment carried out for 15 hours at 37 °C. Ceftazidime is not used alone to treat CF exacerbations due to the emergence of resistant bacterial strains and so was used only in combination with tobramycin in this study. Viable bacterial cell counts were determined on cetrimide agar and residual surface bound biofilms were examined using CLSM and the Baclight Live/Dead viability stain (Invitrogen).

Proof of concept randomized clinical trial

We subsequently conducted a randomized, participant and outcome-assessor blind, placebo controlled, proof of concept study of inhaled NO gas in hospitalized participants aged 12 and above with CF and chronic pseudomonas colonisation between August 2011 and September 2012 (UK NHS REC 11/H0502/7, EudraCT 2010-023529-39, ClinicalTrials.gov NCT02295566) (CONSORT diagram Fig. 4).

Study design and placebo

The design for proof of concept was randomized and placebo controlled, where participants and primary outcome assessors were blind to the treatment group. Participants randomized to the placebo arm of the trial received medical air (BOC, UK) or medical air/oxygen blend according to clinical need (determined by oxygen saturation monitoring as per standard clinical practice). This was administered through nasal cannula in the same manner as the nitric oxide so that participants did not know whether they received the trial treatment or placebo, including pre-defined sham weaning procedures.

Sample size and end of study

The primary aim of this study was to gain evidence that NO could reduce the proportion of aggregated bacteria in biofilms (with regards to reduction in surface area and reduction in average colony size) in the sputum of participants treated with NO. In order to demonstrate that the treatment with NO is better than the control we calculated the sample size required to achieve a 90% probability of observing the correct ordering (consistent with a treatment effect) of the proportion of bacteria in biofilms for each group (estimated taking into account the results observed from the laboratory experiments) (61). It was estimated that the proportion of bacteria in biofilms with regards to surface area (as a measure of aggregate size) in the patients treated with would be 0.7 for placebo and 0.4 for patients treated with NO. A sample size of 10 participants in each treatment group would have been sufficient to determine that the NO treatment arm is superior to the control group (by reducing the proportion of biofilm bacteria) with 90% probability assuming a change from 0.7 to 0.4. It was recognized that this study would have limited ability to detect important but rare treatment-related adverse events which would need to be identified in a future larger RCT.The study was ended at the end of the funding period when 6 participants had been recruited to each group. The data was analyzed according to the statistical plan despite the lower than expected recruitment.

Inclusion and exclusion criteria

Adolescents and young adults with cystic fibrosis were eligible for inclusion if aged 12 or above colonized with *P. aeruginosa* confirmed on sputum sample. Patients were excluded for colonization with *Burkholderia cepacia;* known hypersensitivity to the antibiotics used in the study; other known contraindications to the antibiotics to be used in the study including known aminoglycoside related hearing/renal damage; patients requiring non-invasive ventilation; patients who had a pneumothorax; patients who were admitted for specific treatment of nontuberculous mycobacteria; patients who could not tolerate nasal cannula e.g. those who could not breathe through their nose; patients who had nasal polyposis causing significant blockage of the nasal passages; adolescents not Gillick competent (and therefore not able to give their own assent in addition to parental consent); patients not likely to survive the time period of the study washout period (4 months from enrolment); treatment with an investigational drug or device within the last 3 months prior to enrolment; patients who were pregnant (a pregnancy test was carried out for females of 11 years and above); and immediate families of investigators or site personnel directly affiliated with the study. Immediate family is defined as child or sibling, whether biological or legally adopted.

Study intervention and randomization

Nitric oxide gas (10 ppm, INOmax 400 ppm mol/mol inhalation gas, INO Therapeutics UK), delivered via INOvent, Ikaria Inc., Hampton, NJ, USA, supplied by INO Therapeutics UK), or identically delivered placebo (air or air/oxygen mix) was administered via nasal cannulae to 12 participants admitted for intravenous (IV) antibiotics to treat pulmonary exacerbations. The study intervention was administered by inhalation via nasal cannula for 8 hours overnight for the first 5-7 days of IV antibiotic therapy. This dose was based on extrapolation from *in vitro* work, also informed by the low dose used in hypoxic respiratory failure associated with evidence of

pulmonary hypertension in preterm infants. Electrochemical measurement of NO gas released in solution by approximately 500 μM SNP was measured to be around 390 nM NO (19) which is equivalent to 390 nmol/L, giving 8.7 uL/L or 8.7 ppm (not taking into account any adjustment due to the environmental temperature). Participants, medical and laboratory staff were blinded to treatment allocation. Block randomization with block length 2 and 4 was undertaken via an online randomization service in a 1:1 ratio to ensure concealment of treatment allocation. Participants were monitored closely by a research nurse during the overnight study intervention period and monitoring and safety data were collected.

Clinical study outcomes

The primary outcome was the between group difference in proportion of bacteria in biofilms (as determined by direct visualization of the biofilm by FISH (21, 58-60) and image analysis). Secondary outcomes were between group differences in CFUs and quantitative PCR (q PCR) (20), measures to assess safety including lung function (FEV_1 and FVC) and health related quality of life assessment (CFQ-UK) (62).

Determination of nitric oxide in sputum

We attempted to determine the free NO concentrations in expectorated sputum samples following inhaled NO therapy by using a Unisense nitric oxide electrochemical probe (Unisense Nitric Oxide Microsensor, glass sensor NO-10). However, due to difficulties in equilibrating and calibrating the probe within CF sputum and insufficient volumes of sputum produced by patients to carry out NO measurement alongside FISH and molecular analyses, these data are not presented.

Image analysis

For the *ex-vivo* experiments, quantification of *P. aeruginosa* biofilm thickness and biomass was made from three-dimensional (3D) CSLM stacks using the freely available COMSTAT (63) software. In order to avoid subjectivity in the selection of sample regions, treatment groups were blinded to the researchers carrying out the sample analysis. In order to specifically avoid subjective bias, sample areas selected for study were chosen in a predetermined pattern. Means and standard deviations were calculated from 5 random fields of view per treatment group. For clinical trial samples, FIJI (//fiji.sc/Fiji) 3D object counter software was used to analyze and quantify *P. aeruginosa* "biovolume" analysis of confocal stacks. The range of volumes of a single *P. aeruginosa* cell from the literature (64) (0.16-3.67 μm^3) was used to filter fluorescently-labeled objects in the stacks into the following groups: a) noise (all objects below single cell size, estimated as less than 0.16 μm^3); b) single cells; c) clusters (aggregates) over 10 cells in volume; and d) clusters over 20 cells in volume. After thresholding, the volume of a *P. aeruginosa* cell was assessed using the 3D object counter and compared to literature values for concordance. The 3D object counter was then used to record all objects in each sample and results for each of the 10 image stacks per sample collated into databases and grouped for analysis. For the primary analysis, aggregated cell cell clusters containing both over 20 cells and over 10 cells in size were selected as all patients had microcolonies over this size at baseline, so changes could be seen over the timecourse of the study. There were not enough clusters greater than 40 cell size to analyse, however as the 20 cell size microcolonies were estimated using the upper limit of a PA

cell size based on literature values (3.67 um3), aggregates of >20 cells by our definition were likely to contain greater than 20 cells.

Measurement of nitric oxide metabolites in blood

Venous blood was collected in EDTA tubes 1 and 7 hours after starting inhaled NO/placebo therapy on day 1, and immediately separated into plasma and blood cells by centrifugation for 10 min at 800 x g; aliquots of plasma and red blood cell (RBC) pellet were snap frozen in liquid nitrogen and stored at -80°C until analysis. NO metabolite concentrations in plasma and RBC lysate were quantified immediately after thawing of frozen samples in the presence of excess N-ethylmaleimide (in PBS, 10 mM final concentration) as described previously (65-67). Briefly, nitrite and nitrate were quantified simultaneously via high pressure liquid ion chromatography (ENO-20, Eicom) with post-column Griess diazotization following on-line reduction of nitrate to nitrite. Total nitrosation products (including low-molecular weight S-nitrosothiols, N-nitrosamines and nitrosated proteins) were measured using group-specific de-nitrosation/reduction and subsequent liberation of NO, detected using gas phase chemiluminescence (CLD77am sp, Ecophysics). NO-heme concentrations were quantified by injection of RBC lysate into an oxidizing reaction solution (ferricyanide in PBS) (67), and generated NO was quantified by gas phase chemiluminescence as above.

Statistical analysis

Data for the laboratory study was compared using a Mann-Whitney Rank Sum test for non-normally distributed data. For the clinical study an intention-to-treat analysis was undertaken. For all outcomes the change from baseline to endpoint was calculated. The primary outcome (FISH, the number and volume of aggregates >20 cells) and microbiological and clinical safety outcomes (CFU and q-PCR) were analysed on the natural log scale.

The mean difference of the treatment effect between arms during the intervention period (days 5,7) and total study period (days 5,7,10, 20) was estimated by conducting linear regression using the method of generalized estimating equations (GEE) (68) to take account for longitudinal dependence (where study time points were available). Residuals were examined to assess model assumptions. Analyses were performed in Stata software, version 11.

Author Contributions

The project was conceived by SCC, SNF and JSW. KC wrote the protocol first draft and led regulatory applications. RPH, LH-S and PS led laboratory method development, RPH carried out microbiological data acquisition. For the clinical study, SNF acted as chief investigator, GC and TD paediatric and adult clinical principal investigators respectively, VC was study statistician and JSW laboratory lead investigator. Additional biofilm and microbiology laboratory expertise and analysis was by CD, RA, NB, KB, JJ, MK, SK, SR, GR and SCC. Clinical trial staff and investigators included SP, CS, PS, RS, JL, MC and TD. Nitric oxide metabolite assays and expertise was by MF and BOF.

Funding, Conflicts of Interest and Acknowledgements

Funding was provided via the UK National Institute of Health Research (NIHR) Southampton Respiratory Biomedical Research Unit (RBRU) and supported by staff and resources at the Southampton NIHR Wellcome Trust Clinical Research Facility (NIHR WTCRF). Additional resources were made available by the University of Southampton Institute for Life Sciences. As the study was conducted via NIHR infrastructure funding, nitric oxide and the delivery system were provided in completely unrestricted fashion by Peter Rothery, INO Therapeutics (UK, now part of Linde group) who took no part in the study design or analysis, had no contractual oversight and has not seen the data or paper prior to submission for publication. JSW was funded in part by a Biotechnology and Biological Sciences Research Council Sir David Phillips Fellowship award. KC was funded in part by a NIHR Academic Clinical Training Fellowship.

TD, GC, MC, JJ, LHS, PS, SCC and SNF have participated as clinical trial or study investigators on behalf of their employing University or Hospital for trials Sponsored or funded by pharmaceutical, vaccine or device manufacturers entirely unconnected with this work but have not received personal payments. No author has any pecuniary or personal interest in any company manufacturing or supplying nitric oxide. JSW, SK, SR, NB are named on the original patent (US 8425945 B2) identifying low dose nitric oxide as a therapeutic possibility to break pseudomonal biofilms. MK, SK, NB and SR are named inventors of a novel antimicrobial compound designed to disrupt pseudomonal biofilms via NO release (Australia App No 2011901872). MF is a member of the Scientific Advisory Board of AOBiome. No other authors have declared any conflicts of interest.

SNF and JSW have had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. The collaboration would like to thank Prof Ratko Djukanovic (NIHR RBRU Director), Ms Christine McGrath, Ms Kathy Holding, Ms Lesley-Ann Castle, Mr Malcolm North, and all nursing and laboratory staff at the Southampton NIHR WTCRF and NIHR RBRU, and Dr Julian Wharton at nCATS for use of the BMG plate reader.

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