

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

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43  
44       **Short Title:** Low dose nitric oxide therapy in cystic fibrosis

45 **Abstract:**

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

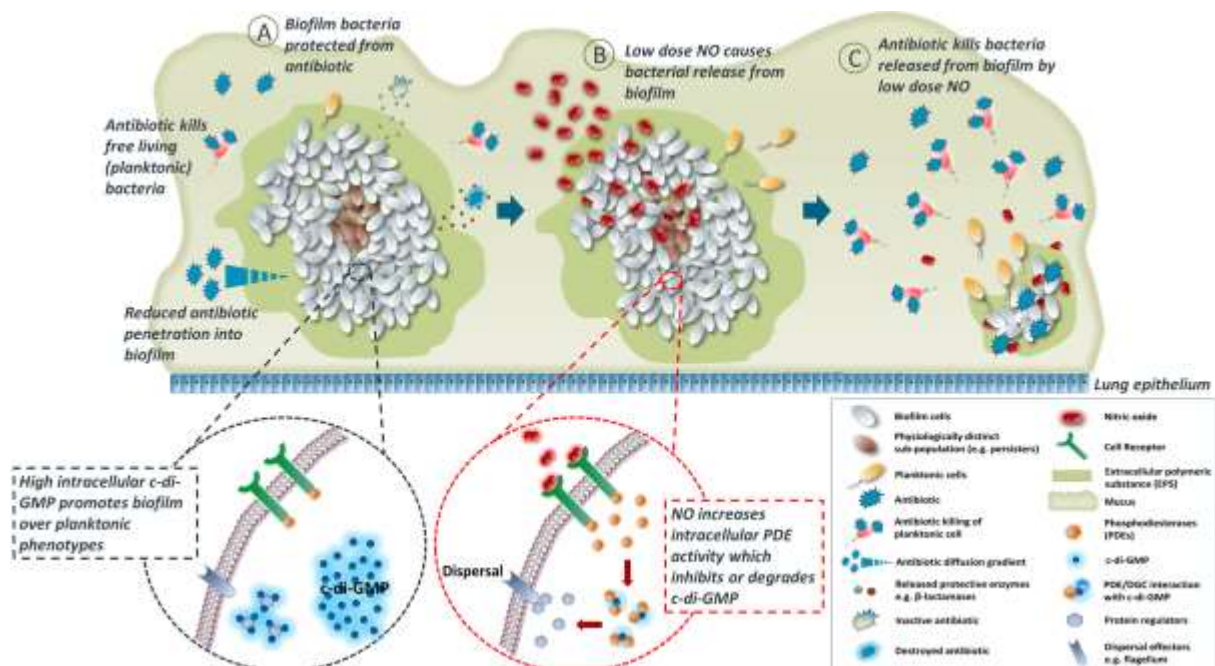
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81 **Introduction**

82 Cystic fibrosis (CF) is the most common lethal, hereditary disease in Caucasian populations,  
83 with a UK and US incidence of approximately 1 in 2500 live births and an estimated worldwide  
84 prevalence of 70,000 (1, 2). Long-term morbidity and mortality is primarily associated with the  
85 effects of chronic *Pseudomonas aeruginosa* lung infection and the persistence of *P. aeruginosa*  
86 biofilms (3, 4). Bacteria in biofilms are enclosed in a self-produced biopolymeric matrix and  
87 display up to 1,000-fold higher tolerance to antibiotic challenge than their single cell, planktonic  
88 (free living) counterparts (5). Biofilms also exhibit resistance to phagocytosis and other  
89 components of the host's innate and adaptive immune system (6). Biofilm survival mechanisms  
90 include impedance of antibiotic diffusion through the biofilm matrix (7), altered growth or  
91 metabolic rates of bacterial subpopulations within the biofilm (8, 9), and physiological (8),  
92 biochemical (10) and genetic (11, 12) changes. In addition, sub-inhibitory levels of  
93 aminoglycoside antibiotics can enhance biofilm formation under laboratory conditions (13).  
94 Biofilms can be firmly attached to tissue but can also exist in the protected phenotype as  
95 aggregates in the mucus of the CF lung (14). Biofilms are extremely difficult to eradicate using  
96 conventional therapeutic regimes (15). New approaches targeting chronic biofilm infections are  
97 needed for more effective treatment of *P. aeruginosa* in CF, and other biofilm-related diseases  
98 (16).

99  
100 *In vivo*, bacteria often transition between planktonic and biofilm lifestyles. Given the correct  
101 environmental cues, biofilm bacteria undergo coordinated dispersal and reversion to the  
102 planktonic form (17). We identified a role for the signaling molecule nitric oxide (NO) in the  
103 dispersal of *P. aeruginosa* biofilms (18, 19) (Fig. 1). At nanomolar concentrations, NO mediates  
104 dispersal by increasing bacterial phosphodiesterase activity with a consequent reduction of the  
105 intracellular second messenger and biofilm regulator cyclic-di-guanosine monophosphate (c-di-  
106 GMP) (18, 19). Here we report the effects of non-bactericidal, low-dose NO on clinical  
107 pseudomonal biofilms *ex-vivo* in the laboratory using conventional and molecular  
108 microbiological methods. We have also extended our laboratory findings to a proof of concept  
109 clinical trial in humans, demonstrating a significant direct effect on pseudomonal biofilm load in  
110 CF patients treated with NO gas plus conventional intravenous antibiotic therapy compared to  
111 intravenous antibiotics alone.

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

125

126 **Results**

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

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

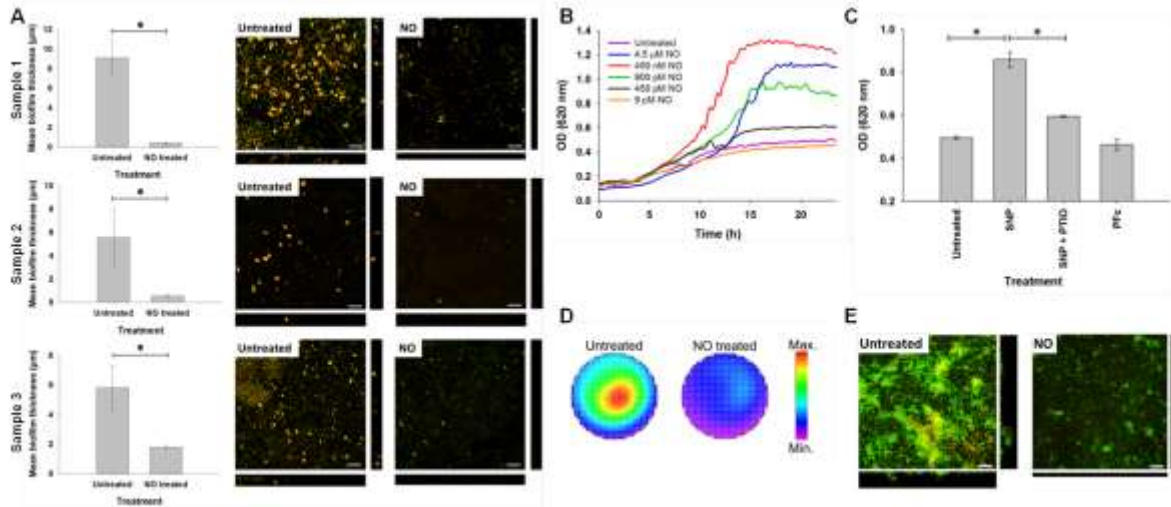
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137 **Nitric oxide mediated dispersal of CF *P. aeruginosa* isolates occurs within 5 to 10 hours**

138 Addition of NO (in the form of the NO-donor sodium nitroprusside, SNP) to 12 biofilm-forming  
139 *P. aeruginosa* clinical isolates from CF sputum samples consistently caused dispersal leading to  
140 steep increases in the optical density (turbidity) of planktonic bacterial suspensions overlying  
141 biofilms after 5 hours (Fig. 2B). The increase in OD correlated with a decrease in biofilm  
142 biomass from surfaces of plate wells as determined by fluorometric measurements and confocal  
143 microscopy, confirming the dispersal effect of NO (Figs. 2D, E). Biofilm dispersal was  
144 confirmed to be NO-specific using the NO scavenger PTIO, which reduced the dispersal of *P.*  
145 *aeruginosa* induced by SNP (p=0.002) to levels similar to the control treatment (Fig. 2C).  
146 Treatment of biofilms with potassium ferricyanide (as a control for NO-independent breakdown  
147 products of SNP) had no dispersal effect compared with untreated biofilms (p=0.394; Fig. 2C).  
148 Dispersal was observed at NO concentrations as low as 450 pM, peaking at 450 nM (Fig. 2B),  
149 with higher concentrations of NO (4.5 µM) showing reduced efficacy for biofilm dispersal (Fig.  
150 2B). NO at a concentration of 450 nM dispersed all 12 biofilm-forming CF clinical isolates  
151 tested.

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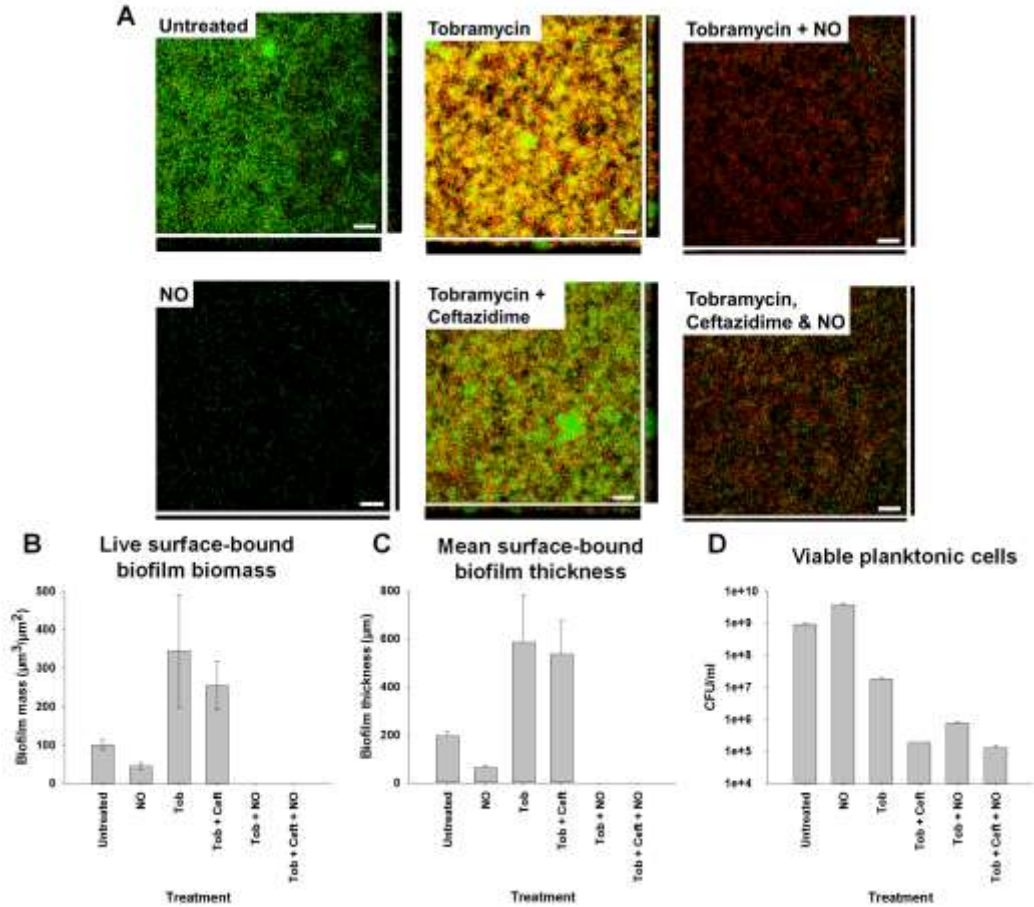
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 155 **Fig. 2. A) Direct measurement of NO-induced *P. aeruginosa* biofilm dispersal in**  
 156 **expectorated CF sputum samples.** Image analysis shows a significant reduction in mean *P.*  
 157 *aeruginosa* biofilm thickness following treatment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

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

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

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

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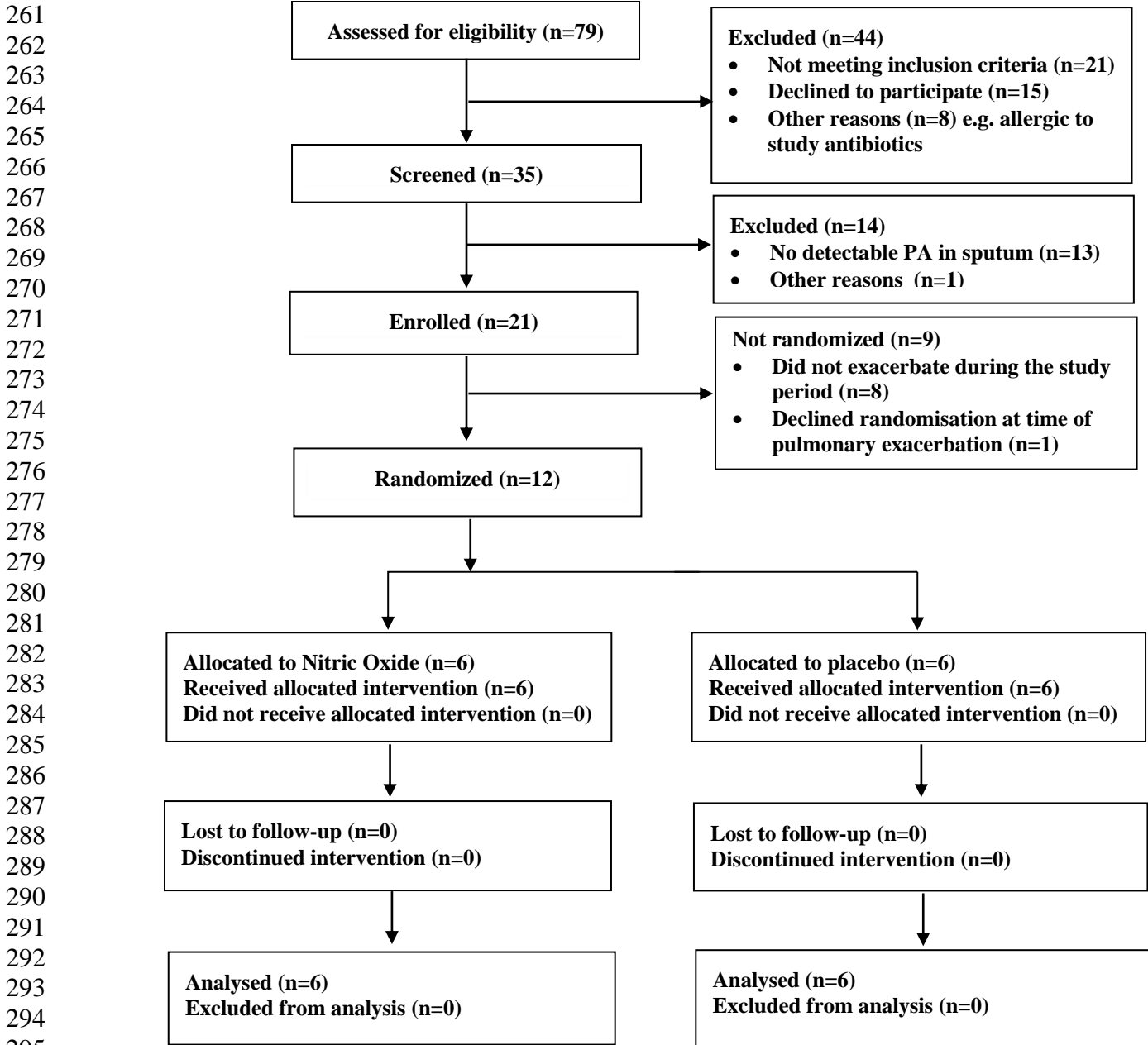
215 **Fig. 3. Antibiotic efficacy against *P. aeruginosa* clinical isolate biofilms is enhanced in the**  
 216 **presence of low-dose NO.** A) Representative confocal laser scanning microscopy (CLSM)  
 217 images showing surface-attached *P. aeruginosa* following treatment with: buffer alone  
 218 (untreated); NO alone; MBC antibiotics ( $5 \mu\text{g ml}^{-1}$  tobramycin with or without  $5 \mu\text{g ml}^{-1}$   
 219 ceftazidime); or antibiotics combined with NO. Images show horizontal xy (top-down view)  
 220 sections and flanking images show vertical z (side view) CSLM sections. Biofilms were stained  
 221 with BacLight Live (green)/Dead (red) kit to indicate viable cells. Scale bars =  $25 \mu\text{m}$ . B) **Image**  
 222 **analysis of CLSM images of residual *P. aeruginosa* biofilms with adjunctive NO** shows a  
 223 reduction in mean total biomass (**Fig. 3B**) and biofilm thickness (**Fig. 3C**) following treatment  
 224 with antibiotics (tobramycin alone and tobramycin (Tob)/ceftazidime (Ceft) combined)  
 225 indicating that NO-treatment reduces the amount of remaining biofilm bacteria (error bars  
 226 represent standard error of the mean of 5 different microscopic fields). An increase in biofilm  
 227 biomass and biofilm thickness is shown following tobramycin treatment alone (biofilm biomass:  
 228 243% increase compared to control,  $p=0.028$ , **Fig. 3B**; and mean biofilm thickness: 199%  
 229 increase compared to control,  $p=0.065$ , **Fig. 3C**) and the tobramycin/ceftazidime combination  
 230 (biofilm biomass: 155% increase compared to control,  $p=0.04$ , **Fig. 3B**; and mean biofilm  
 231 thickness: 174% increase compared to control,  $p=0.04$ , **Fig. 3C**). Viable *P. aeruginosa* in the  
 232 dispersed population (planktonic suspension), determined by colony forming unit (CFU) counts  
 233 of *P. aeruginosa* following antibiotic treatment of biofilms with or without NO, indicate that  
 234 combined NO treatment leads to killing of the bacteria released from the biofilm (**Fig. 3D**).



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

238 12 patients were randomized to receive either low-dose NO inhalation or placebo (CONSORT  
239 diagram, figure 4). Adjunctive NO used in combination with tobramycin and ceftazidime  
240 demonstrated a significant reduction in the key primary microbiological endpoint, *P. aeruginosa*  
241 biofilm aggregates. This is shown in aggregates both over 20 cells in size and in those over 10  
242 cells in size compared to those receiving placebo with antibiotics over the 7 days of treatment  
243 (GEE analysis,  $p=0.031$  and  $p=0.029$  respectively for days 5 and 7; and Fig. 5). Data suggested  
244 less *P. aeruginosa* biofilm as quantified by both the number and volume of aggregates greater  
245 than 20 or 10 cells in the NO group compared with placebo through day 7 while on NO therapy.  
246 This reduction was not fully maintained after treatment was stopped, as pseudomonal biofilm  
247 was detected in treatment group samples at timepoint 10-13 days following the cessation of NO  
248 therapy (study period days 5 through 20, Table 1 and Fig 5). See methods for rationale regarding  
249 cluster size selection. Other important secondary endpoints are shown in Table 2. From an  
250 individual participant safety perspective, there was no evidence that the biofilm dispersal  
251 increased the amount of viable *P. aeruginosa* detected in planktonic phase by CFUs. qPCR,  
252 indicative of total viable *P. aeruginosa* cells (20), did not demonstrate a difference between  
253 groups due to the small numbers and large variation between individuals. There were also no  
254 adverse clinical safety signals (FEV<sub>1</sub>, FVC, quality of life score) in the treatment group  
255 compared to those treated with placebo. Baseline clinical data are shown in Table 3, baseline  
256 laboratory data and study adverse effects are shown in Supplementary Tables S1 and S2, and  
257 individual patient data for the primary outcome (FISH) and one clinical parameter (FEV1) are  
258 shown in Supplementary Table S3.

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**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.

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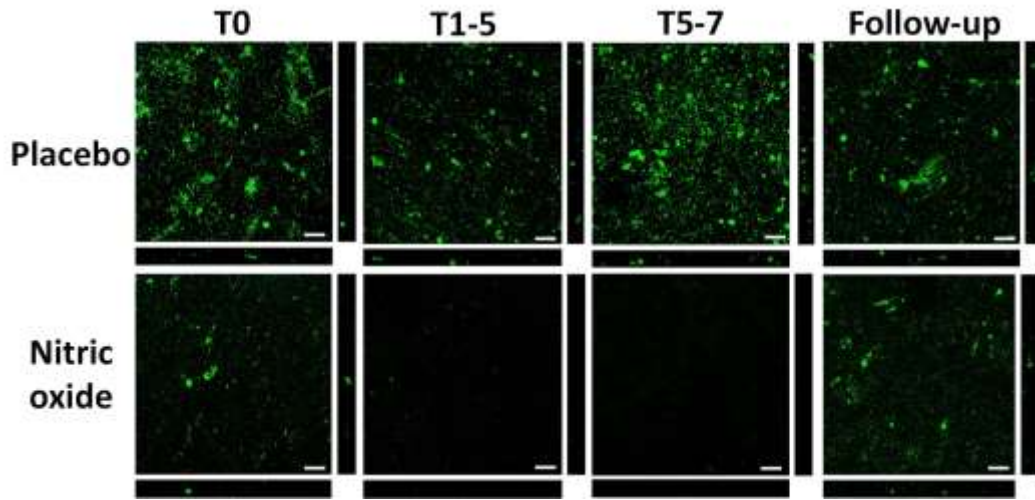
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**Fig. 5. Reduction in *P. aeruginosa* biofilm with NO adjunctive therapy.** Representative FISH confocal images from a CF patient being treated with NO adjunctive to conventional antimicrobial agents (ceftazadime and tobramycin) compared to a patient 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  $\mu$ m. 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)

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327 **Tables 1-2. Results from randomized clinical trial.**

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

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Day	Change from baseline, Mean (SD)				Treatment effect: mean difference (95%CI), p value			
	5	7	10	20	Intervention period (days 5 & 7)		Study period (days 5,7,10 & 20)	
<b>FISH: Ln <u>number</u> of aggregates &gt; 20 cells</b>								
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				
<b>FISH: Ln <u>volume</u> of aggregates &gt; 20 cells</b>								
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				
<b>FISH: Ln <u>number</u> of aggregates &gt; 10 cells</b>								
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				
<b>FISH: Ln <u>volume</u> of aggregates &gt; 10 cells</b>								
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				

334 **Table 2. Microbiological and clinical safety monitoring:** showing mean differences between  
 335 groups (NO or placebo) of change from baseline  
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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)	
<b>Ln CFU</b>								
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				
<b>Ln QPCR</b>								
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)	
<b>FEV<sub>1</sub></b>								
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)				
<b>FVC</b>								
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)				

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

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	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 <sub>1</sub> % 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

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

344 Plasma nitrate ( $\text{NO}_3^-$ ) concentrations tended to increase in response to delivery of low-dose NO,  
345 but these changes did not reach statistical significance ( $P > 0.05$ ). Plasma levels of nitrite ( $\text{NO}_2^-$ )  
346 and total nitrosation products (RXNO) paradoxically decreased during NO inhalation, although  
347 this was also not significant. With the exception of unusually high nitrite levels in erythrocytes  
348 compared to plasma values there was also no obvious effect of inhaled NO on NO metabolite  
349 status in these blood cells, which is surprising given that nitrosylhemoglobin (NO-Heme) is the  
350 most sensitive marker of NO availability *in vivo* and nitrate is the final oxidation product of NO  
351 (22) (thus, both might be expected to be elevated following prolonged NO inhalation). Direct NO  
352 measurement in sputum was impractical due to the short half-life of NO in relation to the time  
353 taken for the probe to equilibrate in individual sputum samples (data not shown). Overall,  
354 determination of a comprehensive panel of NO metabolites suggested that low-dose inhaled NO  
355 does not significantly affect circulating NO metabolites in CF (Supplementary Figure S1).

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371 **Discussion**

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

378

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

384

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

395

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

400

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



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

418  
419 The proof of concept clinical study demonstrated a significant direct effect on pseudomonal  
420 biofilm (as measured by a reduction in *P. aeruginosa* aggregate load) in CF patients treated with  
421 NO gas plus conventional IV antibiotic therapy compared to IV antibiotics alone. The effect was  
422 not sustained following the end of NO therapy in this group of adult patients with long term  
423 chronic disease. In this small study we did not detect any side effects as a result of this treatment  
424 strategy. All changes in the clinical parameters measured favored NO treatment and there was no  
425 evidence that NO treatment caused an increase in overall bacterial load or the severity of acute  
426 exacerbations. We saw no treatment effects suggestive of NO induced vasodilatation (i.e. no  
427 increase in oxygen saturations during treatment); and no adverse effects during the weaning  
428 period at the end of each day's NO therapy that might have been indicative of rebound  
429 pulmonary hypertension. Our study measured clinical parameters to ensure safety but not clinical  
430 efficacy, which will be the subject of future large clinical trials.

431  
432 Previous studies have shown that *P. aeruginosa* cells can be killed directly by high doses of NO  
433 (29). This might be the result of several possible toxic effects of NO on bacteria at high  
434 concentrations, including direct modification of membrane proteins, DNA cleavage and lipid  
435 peroxidation through mechanisms of both nitrosative and oxidative stress (30-32). The use of  
436 high dose NO in this way has potential cytotoxic and other adverse clinical effects (33) and is  
437 associated with considerable cost. Despite this, recent trials of high dose 160 ppm inhaled NO in  
438 CF did not demonstrate any adverse safety signals (34, 35). In terms of biofilm growth which  
439 has not been measured in previous clinical trials, high levels of NO might result in increased  
440 nitrate levels in CF sputum that may support growth of *P. aeruginosa* by metabolism based on  
441 anaerobic denitrification (36, 37). Our previous *in vitro* studies have shown that higher  
442 concentrations of NO can stimulate biofilm formation (18). These studies agree with another  
443 report suggesting that higher dose NO may in fact enhance aminoglycoside tolerance by  
444 blocking energy-dependent phases of drug uptake (38). The low-dose, signal-relevant  
445 concentrations of NO we used in the proof of concept clinical trial reported here are  
446 approximately three orders of magnitude lower than those shown to inhibit drug uptake and did  
447 not inhibit tobramycin efficacy against dispersed (planktonic) or biofilm *P. aeruginosa* bacteria.

448  
449 The rationale for our approach using NO to treat *P. aeruginosa* infection was to exploit our  
450 discovery that low-dose NO (10 ppm, assumed to translate into submicromolar concentrations  
451 locally) mediates biofilm dispersal through increased bacterial phosphodiesterase activity and an  
452 associated decrease in c-di-GMP levels (19). We have previously shown that low-dose NO can  
453 increase the motility of *P. aeruginosa* cells *in-vitro* (18), and proposed that this increased  
454 motility promotes biofilm dispersal. In contrast and in the context of CF sputum, other studies  
455 have shown that *P. aeruginosa* isolates are frequently non-motile (39) and that sputum can  
456 repress *P. aeruginosa* flagellar activity and motility (40, 41). Cyclic-di-GMP binds to a broad  
457 range of effector components that control the physiology, development, stability, cell  
458 adhesiveness and motility of the biofilm phenotype. Factors other than motility could therefore  
459 be responsible for biofilm disruption and a reduction in tolerance to antibiotic treatment. Further  
460 studies are required to understand the specific c-di-GMP effectors responsible for NO-mediated  
461 disruption of biofilms within CF sputum.

462  
463 Chronic CF infections are often associated with multiple bacterial pathogens and complex  
464 microbial communities (42, 43). Genes that modulate c-di-GMP turnover are widely distributed  
465 in bacteria, and NO-mediated dispersal has now been observed across a number of species  
466 including many pathogenic organisms (44, 45). NO-mediated alteration of intracellular c-di-  
467 GMP levels is therefore an important new potential target to control multispecies bacterial  
468 communities in CF. NO might also be of benefit in treating younger CF patients after initial  
469 infection with *P. aeruginosa*. Used in these circumstances it might increase the effectiveness of  
470 eradication therapy and delay the onset of chronic biofilm infection with this organism.

471  
472 Our clinical trial data appear to differ from the reported effects of inhaled NO on circulating NO  
473 metabolite levels in infants with pulmonary hypertension (46), where a clear increase in NO  
474 metabolite levels was reported to occur with twice the concentration of inhaled NO used in our  
475 study. There is a paucity of information on circulating levels of NO metabolites in CF.  
476 Nevertheless, our observations are in general agreement with the notion that NO concentrations  
477 are lower in the exhaled breath of CF patients while systemic NO production does not appear to  
478 be compromised (47). Possible mechanisms for this include accelerated degradation as a result of  
479 increased oxidative stress in epithelial cells, increased NO consumption by bacterial biofilms, or  
480 impaired gas exchange as a result of mucus obstruction. All of these factors would be expected  
481 to prevent exogenous inhaled NO to reach the systemic circulation, limiting its effects to the site  
482 of administration.

483  
484 The main limitation of the clinical component of our study is the small sample number and  
485 between-patient variation in clinical and microbiological parameters. This has made formal  
486 statistical analyses difficult but we were able to incorporate repeated measurements over time to  
487 improve power. Variability in the qPCR results between NO and placebo groups was probably  
488 due to sample heterogeneity in chronically infected patients. Despite these limitations, FISH  
489 image analysis data demonstrate a treatment effect and provide proof of concept for our low-dose  
490 NO approach. Similarly, our analysis of the changes in systemic NO status following low-dose  
491 NO inhalation is likely compounded by inter-individual differences in NO processing. However,  
492 the lack of an observed rise in blood nitrate and NO-heme levels are consistent with well-  
493 documented perturbations in NO production and handling in CF patients (48, 49).

494  
495 Our study has demonstrated the potential for the use of low dose NO to enhance the antibiotic  
496 treatment of biofilm infections. Although the practical challenges in delivering inhaled NO gas to  
497 CF patients were considerable, future novel NO donor antibiotics might prove to be a more  
498 feasible approach to targeting biofilms (50). Biofilm-related chronic infections are responsible  
499 for at least half a million deaths per year at an estimated cost of over \$94 billion in the United  
500 States alone (16). More effective anti-biofilm therapies are needed to address this significant  
501 unmet need.

502 **Materials and Methods**

503

504 **CF sputum collection and *P. aeruginosa* isolation**

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

515

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

517 We first evaluated the ability of NO at different doses to disperse clinical isolates of *P.*  
518 *aeruginosa* biofilms *in-vitro* and within sputum from CF patients. Biofilm forming *P. aeruginosa*  
519 clinical isolates (n=12) were inoculated using overnight cultures grown in M9 minimal medium  
520 (20 ml per litre of 20 % glucose, 2 ml per litre of 1 M MgSO<sub>4</sub> and 100 µl per litre of 1 M CaCl<sub>2</sub>).  
521 Overnight cultures were diluted to give optical density readings corresponding to 10<sup>6</sup> cells per  
522 millilitre and 200 µl aliquots were inoculated into a 96 well plate and incubated at 37 °C for 24  
523 hours. The medium was aspirated and replaced with fresh M9 medium with/without increasing  
524 concentrations of the NO-donor sodium nitroprusside (SNP), concentration range 9 pM - 4.5 µM  
525 (Sigma Aldrich). The concentration of NO produced by SNP was calculated using a NO  
526 microsensor (Unisense, Denmark) and calibrated over a range of 250 nM to 10 µM using  
527 previously published methods (55). Based on the measured linear relationship between  
528 micromolar concentration of SNP producing nanomolar concentrations of NO (where  $y =$   
529  $0.9022x$ , ( $R^2 = 0.9617$ ,  $n=6$  data points)), NO concentrations were calculated to be nearly 1000  
530 fold less than the starting concentration of SNP, resulting in approximately 450 nM NO  
531 generated from 500 µM SNP. To confirm that effects were specific to NO, assays were also  
532 carried out with SNP (500 µM) in the presence of 5 mM NO scavenger carboxy-2-phenyl-  
533 4,4,5,5-tetramethyl-imidazole-1-oxyl-3-oxide (carboxy-PTIO; Sigma-Aldrich). M9 medium  
534 containing 500 µM potassium ferricyanide (Sigma-Aldrich), used to generate breakdown  
535 products of SNP (56, 57), was also used as a control. Optical density measurements of the  
536 supernatant containing planktonic cells were made using a BMG Labtech Omega plate reader  
537 (620 nm and chamber temperature of 37 °C) over 24 hours with measurements taken every 15  
538 minutes. Experiments were repeated 3 times with 4 replicates for each experiment.

539

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

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

547  $\mu\text{M}$  SNP). Samples were fixed in freshly prepared 4 % paraformaldehyde in phosphate-buffered  
548 saline (PBS) at 4 °C and washed with PBS and PBS-ethanol (1:1 v/v) and 20  $\mu\text{l}$  drops of sputum  
549 were spotted onto poly-L-lysine (PLL) coated slides and left to dry overnight. *P. aeruginosa*  
550 detection was performed using FISH with the 16S ribosomal probe sequences: PseaerA, 5'-  
551 GGTAACCGTCCCCCTTGC-3', specific for *P. aeruginosa* (58), labelled with Cy3 and  
552 EUB338 5'- GCTGCCTCCCGTAG GAGT-3' (Domain bacteria) (59), labelled with Cy5  
553 (Integrated DNA Technologies Inc, Leuven, Belgium). Hybridization conditions for FISH were  
554 optimised and stringently evaluated *in vitro* to ensure specificity of the PseaerA probe. We  
555 independently confirmed the previously reported optimal hybridization conditions for the  
556 specificity of the Pseaer probe for *P. aeruginosa* (21, 58). Hybridisation with the sample was  
557 carried out using 20% formamide, and a 2 hour incubation at 46 °C was followed by washing for  
558 15 minutes at 48 °C in pre-warmed wash buffer as previously described (58, 60). Cover slips  
559 were placed on samples and imaged using an inverted Leica DMI600 SP5 confocal laser  
560 scanning microscope (CLSM; Leica Microsystems, Cambridge, U.K.). Control experiments with  
561 both positive and negative controls demonstrated that low concentrations of NO in the  
562 concentration range used for our studies did not interfere with the eubacterial or species-specific  
563 FISH signal for *P. aeruginosa* including no fluorescence quenching in the presence of NO  
564 (Supplementary Figure S2).

565  
566 *P. aeruginosa* biofilms were examined for antibiotic sensitivity using adjunctive treatment of  
567 450 nM NO with or without the aminoglycoside tobramycin. The antibiotic was added alone or  
568 in combination with the cephalosporin ceftazidime (both antibiotics at the minimum bactericidal  
569 concentrations (MBC) to induce killing of planktonic cells, determined to be 5  $\mu\text{g ml}^{-1}$ ). Biofilms  
570 were grown from colony sweeps as described above in culture plates (MatTek Corporation,  
571 Ashland, MA, USA) and treatment carried out for 15 hours at 37 °C. Ceftazidime is not used  
572 alone to treat CF exacerbations due to the emergence of resistant bacterial strains and so was  
573 used only in combination with tobramycin in this study. Viable bacterial cell counts were  
574 determined on cetrimide agar and residual surface bound biofilms were examined using CLSM  
575 and the BacLight Live/Dead viability stain (Invitrogen).

### 576 577 **Proof of concept randomized clinical trial**

578 We subsequently conducted a randomized, participant and outcome-assessor blind, placebo  
579 controlled, proof of concept study of inhaled NO gas in hospitalized participants aged 12 and  
580 above with CF and chronic pseudomonas colonisation between August 2011 and September  
581 2012 (UK NHS REC 11/H0502/7, EudraCT 2010-023529-39, ClinicalTrials.gov NCT02295566)  
582 (CONSORT diagram Fig. 4).

### 583 **Study design and placebo**

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

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### **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.

629

### **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

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

#### 646 647 **Clinical study outcomes**

648 The primary outcome was the between group difference in proportion of bacteria in biofilms (as  
649 determined by direct visualization of the biofilm by FISH (21, 58-60) and image analysis).  
650 Secondary outcomes were between group differences in CFUs and quantitative PCR (q PCR)  
651 (20), measures to assess safety including lung function ( $\text{FEV}_1$  and FVC) and health related  
652 quality of life assessment (CFQ-UK) (62).  
653

#### 654 **Determination of nitric oxide in sputum**

655 We attempted to determine the free NO concentrations in expectorated sputum samples  
656 following inhaled NO therapy by using a Unisense nitric oxide electrochemical probe (Unisense  
657 Nitric Oxide Microsensor, glass sensor NO-10). However, due to difficulties in equilibrating and  
658 calibrating the probe within CF sputum and insufficient volumes of sputum produced by patients  
659 to carry out NO measurement alongside FISH and molecular analyses, these data are not  
660 presented.  
661

#### 662 **Image analysis**

663 For the *ex-vivo* experiments, quantification of *P. aeruginosa* biofilm thickness and biomass was  
664 made from three-dimensional (3D) CSLM stacks using the freely available COMSTAT (63)  
665 software. In order to avoid subjectivity in the selection of sample regions, treatment groups were  
666 blinded to the researchers carrying out the sample analysis. In order to specifically avoid  
667 subjective bias, sample areas selected for study were chosen in a predetermined pattern. Means  
668 and standard deviations were calculated from 5 random fields of view per treatment group. For  
669 clinical trial samples, FIJI ([//fiji.sc/Fiji](http://fiji.sc/Fiji)) 3D object counter software was used to analyze and  
670 quantify *P. aeruginosa* “biovolume” analysis of confocal stacks. The range of volumes of a  
671 single *P. aeruginosa* cell from the literature (64) ( $0.16\text{-}3.67\ \mu\text{m}^3$ ) was used to filter fluorescently-  
672 labeled objects in the stacks into the following groups: a) noise (all objects below single cell size,  
673 estimated as less than  $0.16\ \mu\text{m}^3$ ); b) single cells; c) clusters (aggregates) over 10 cells in volume;  
674 and d) clusters over 20 cells in volume. After thresholding, the volume of a *P. aeruginosa* cell  
675 was assessed using the 3D object counter and compared to literature values for concordance. The  
676 3D object counter was then used to record all objects in each sample and results for each of the  
677 10 image stacks per sample collated into databases and grouped for analysis. For the primary  
678 analysis, aggregated cell cell clusters containing both over 20 cells and over 10 cells in size were  
679 selected as all patients had microcolonies over this size at baseline, so changes could be seen  
680 over the timecourse of the study. There were not enough clusters greater than 40 cell size to  
681 analyse, however as the 20 cell size microcolonies were estimated using the upper limit of a PA

682 cell size based on literature values (3.67  $\mu\text{m}^3$ ), aggregates of >20 cells by our definition were  
683 likely to contain greater than 20 cells.

#### 684 **Measurement of nitric oxide metabolites in blood**

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

699

#### 700 **Statistical analysis**

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

706

707 The mean difference of the treatment effect between arms during the intervention period (days  
708 5,7) and total study period (days 5,7,10, 20) was estimated by conducting linear regression using  
709 the method of generalized estimating equations (GEE) (68) to take account for longitudinal  
710 dependence (where study time points were available). Residuals were examined to assess model  
711 assumptions. Analyses were performed in Stata software, version 11.

712

713

#### 714 **Author Contributions**

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

723

724

#### 725 **Funding, Conflicts of Interest and Acknowledgements**

726 Funding was provided via the UK National Institute of Health Research (NIHR) Southampton  
727 Respiratory Biomedical Research Unit (RBRU) and supported by staff and resources at the  
728 Southampton NIHR Wellcome Trust Clinical Research Facility (NIHR WTCRF). Additional  
729 resources were made available by the University of Southampton Institute for Life Sciences. As  
730 the study was conducted via NIHR infrastructure funding, nitric oxide and the delivery system  
731 were provided in completely unrestricted fashion by Peter Rothery, INO Therapeutics (UK, now  
732 part of Linde group) who took no part in the study design or analysis, had no contractual  
733 oversight and has not seen the data or paper prior to submission for publication. JSW was funded  
734 in part by a Biotechnology and Biological Sciences Research Council Sir David Phillips  
735 Fellowship award. KC was funded in part by a NIHR Academic Clinical Training Fellowship.  
736

737 TD, GC, MC, JJ, LHS, PS, SCC and SNF have participated as clinical trial or study investigators  
738 on behalf of their employing University or Hospital for trials Sponsored or funded by  
739 pharmaceutical, vaccine or device manufacturers entirely unconnected with this work but have  
740 not received personal payments. No author has any pecuniary or personal interest in any  
741 company manufacturing or supplying nitric oxide. JSW, SK, SR, NB are named on the original  
742 patent (US 8425945 B2) identifying low dose nitric oxide as a therapeutic possibility to break  
743 pseudomonal biofilms. MK, SK, NB and SR are named inventors of a novel antimicrobial  
744 compound designed to disrupt pseudomonal biofilms via NO release (Australia App No  
745 2011901872). MF is a member of the Scientific Advisory Board of AOBiome. No other authors  
746 have declared any conflicts of interest.  
747

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

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