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**The role of nitric oxide as a
novel therapy to disrupt
bacterial biofilms in patients
with cystic fibrosis**

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ABSTRACT

FACULTY OF MEDICINE

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Doctor of MedicineTHE ROLE OF NITRIC OXIDE AS A NOVEL THERAPY TO DISRUPT BACTERIAL
BIOFILMS IN PATIENTS WITH CYSTIC FIBROSIS

By Katrina Cathie

The lungs of most patients with cystic fibrosis (CF) eventually become chronically infected with the opportunistic pathogen *Pseudomonas aeruginosa* (PA). This infection is recognised as consisting of free-living bacteria (known as “planktonic bacteria”) and bacteria within aggregates known as “biofilms”. Bacteria within biofilms are more tolerant to antibiotics than their planktonic counterparts. Current CF treatment of infective exacerbations aims to eradicate or control PA infection using aggressive antibiotic regimes. Despite repeated courses of antibiotics used early after initial positive cultures, many patients become permanently infected. This chronic infection/colonisation results in progressive airways obstruction and worsening bronchiectasis. Patients colonised with PA are more unwell and die at a younger age.

Work conducted by colleagues had established that low dose nitric oxide (NO) can disrupt pseudomonal biofilms in the laboratory and established a novel combination of assays to study biofilm obtained directly from CF sputa. This proof of concept study aimed to discover whether non-toxic levels of NO, administered to participants during an episode of acute infective exacerbation, could disrupt bacteria from biofilms and increase the effectiveness of antibiotic therapy.

A randomised, participant and outcome-assessor blind, proof of concept study was carried out to compare treatment with nitric oxide gas during acute infection with placebo. Twelve participants with CF (aged 12 or above) were randomised to receive 5-7 days of either inhaled nitric oxide gas (10ppm) or placebo (air) in addition to standard intravenous (IV) antibiotics for a pulmonary exacerbation. The primary endpoint was the microbiological effect on proportion of bacteria in biofilms in sputum between baseline and follow-up. Secondary endpoints included laboratory and clinical parameters measured at all time-points including a number of safety measures.

There was no difference between the groups in the primary outcome measure of proportion of bacteria in biofilm between baseline and follow-up. In the secondary outcomes, generalised estimating equation analysis showed a significant reduction in bacteria in biofilms in the NO group at the end of 7 days NO therapy (mean log difference between groups for measured number of >20 cell aggregates 3.49 (95%CI 0.32, 6.67; p=0.031) and for biofilm volume 4.47 (95%CI -.04, 8.98; p=0.052)). Safety measures were reassuring and other parameters (including lung function) demonstrated trends in the direction of treatment, but no other results reached significance.

All results are impacted by the small number of participants and the wide variability between and within participants. There was no effect seen on bacterial biofilms at follow-up, however, the secondary microbiological outcome data shows preliminary evidence of benefit using low dose NO as adjunctive therapy during the period of treatment. Results show inhaled NO therapy is safe when administered to CF patients during a pulmonary exacerbation alongside IV antibiotic therapy. Further studies are needed to investigate whether inhaled NO, or new drugs in development (e.g. novel targeted NO donor therapies) may have any impact on PA related morbidity and mortality in CF.

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Contribution statement, Funding and Acknowledgements

Unless otherwise stated, all work recorded in this thesis is that of the author.

Dr Rob Howlin carried out the laboratory work and analysis as detailed in section 1.6 and the microbiological data acquisition for the clinical study, except for qPCR which was carried out by Dr Geraint Rogers. Nitric oxide metabolite assays and expertise were carried out by Prof Martin Feelisch and Dr Bernadette Fernandez. Statistical analysis was directed by Dr Victoria Cornelius.

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List of Abbreviations and Definitions

AE	Adverse Event
AR	Adverse Reaction
ARDS	Acute respiratory Distress Syndrome
BALF	Broncho Alveolar Lavage Fluid
BP	Blood Pressure
c-di-GMP	Cyclic-di-guanosine monophosphate
Cet	Cetrimide Agar
CF	Cystic Fibrosis
CFQ-UK	Cystic Fibrosis Questionnaire UK
CFTR	Cystic Fibrosis Trans membrane Regulator
CFUs	Colony Forming Units
CI	Chief Investigator
CRF	Case Report Form
CRP	C Reactive Protein
CT	Computed Tomography
CTIMP	Controlled Trial of Investigational Medicinal Product
CTU	Clinical Trials Unit
DGC	diguanylate synthases
DTT	Dithiothreitol
ECMO	Extra Corporeal Membrane Oxygenation
EDRF	Endothelium Derived Relaxing Factor
EDTA	Ethylenediaminetetraacetic acid
eNO	Exhaled Nitric Oxide
EPS	Extracellular Polymeric Substance
FBC	Full Blood Count
FDA	Food and Drug Administration (US)
FEV ₁	Forced Expiratory Volume in 1 second
FiO ₂	Inspired oxygen concentration
FISH	Fluorescent In Situ Hybridisation
FVC	Forced Vital Capacity
GCP	Good Clinical Practice
GEE	Generalised Estimating Equations
GI	Gastrointestinal

GMP	Good Manufacturing Practice
HBSS	Hanks Balanced Salt Solution
HR	Heart Rate
HRQOL	Health Related Quality Of Life
IDSMC	Independent Data Safety Monitoring Committee
IL-8	Interleukin 8
IMP	Investigational Medicinal Product
iNO	Inhaled Nitric Oxide
ISF	Investigator Site File
IV	Intravenous
LFT	Liver Function Tests
MCRN	Medicines for Children Research Network
MetHb	Methaemaglobinaemia
MHRA	Medicines and Healthcare products Regulatory Agency
MSC	Microbiological Safety Cabinet
NIMP	Non-investigational Medicinal Product
NIV	Non Invasive Ventilation
NO	Nitric Oxide
NO ₂	Nitrogen Dioxide
O ₂	Oxygen
PA	<i>Pseudomonas aeruginosa</i>
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDE	Phosphodiesterase
PI	Principal Investigator
PIA	Pseudomonas isolation agar
PFA	Paraformaldehyde
PLL	Poly-L-lysine
ppb	parts per billion
ppm	parts per million
PTIO	2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide
qPCR	Quantitative Polymerase Chain Reaction
RCT	Randomised Controlled Trial
REC	Research Ethics Committee
SAE	Serious Adverse Event
SAR	Serious Adverse Reaction
Sats	Oxygen saturations

SNP	Sodium Nitroprusside
SPC	Summary of Product Characteristics
SUSAR	Suspected Unexpected Serious Adverse Reaction
TMG	Trial Management Group
TSA	Tryptic Soy Agar
TSC	Trial Steering Committee
U+E	Urea and Electrolytes
UHS	University Hospital Southampton NHS Foundation Trust

Chapter 1: Introduction

1.1 Cystic Fibrosis

1.1.1 Epidemiology

Cystic fibrosis (CF) is the UK's most common life-threatening inherited disease. It was first described in the 1940's and currently affects over 9,000 people in the UK. Around 1 in 25 of the UK population carry a gene mutation for CF of which the most common is delta F508. It is an autosomal recessive disease meaning that if 2 carriers have a child, there is a 1 in 4 chance of their offspring being affected. Each week 5 babies are born with CF in the UK and 2 young people die of CF. Although life table analyses suggest that the median life expectancy for children born with CF in 2014 might reach the mid-forties, the median age, lung function and nutritional indices have not improved for the current UK population over the last 5-6 years. It would appear that CF outcomes have now plateaued and there is a clear need for new approaches to treatment to improve on current prognoses. (Cystic Fibrosis Trust 2012).

Genetic mutations that cause CF occur within the cystic fibrosis trans membrane conductance regulator gene (CFTR). Homozygotes either fail to make, or have a defective chloride channel on epithelial surfaces, resulting in abnormalities of sodium and water re-absorption and chloride secretion. This is thought to result in dehydration of lung epithelial lining fluid. The exact cause of the CF lung's predilection to pulmonary infection remains disputed, but changes in the airway milieu clearly predispose patients to endobronchial infection with a well characterised range of pathogens. There are currently over 1900 identified CFTR mutations (CFTR mutation database). Mutations affect many other internal organs including the liver, pancreas, gallbladder, small intestine, skin, sinuses and reproductive organs. In addition to alterations in lung epithelial lining fluid there is goblet cell hyperplasia in the larger, more proximal airways. These cells produce increased amounts of mucous that remains attached to the epithelial cell surface impairing ciliary action in the large airways. Increased accumulation of airway mucus encourages opportunistic growth of *Pseudomonas aeruginosa* (PA) and other bacteria that are not cleared effectively. Inflammatory host responses to this

abnormal bacterial population within the airways importantly contribute to progressive lung damage.

All babies in the UK are screened for CF around day 5 of life via blood spot analysis. If the immunoreactive trypsinogen (IRT) screen is >99.5th centile, genetic mutation analysis is carried out looking for the four most common CFTR gene mutations in the UK. From April 2010-2011 3750 UK babies were picked up as having IRT levels >99.5th centile and went on to have mutation analysis. There were a total of 248 babies who screened positive for CF, and who were referred to their local specialist paediatric CF service for clinical evaluation. 171 babies were reported as CF carriers between April 2010 and 2011.(Morgan 2012)

The management and treatment of CF involves a multi-disciplinary team including physiotherapists, specialist nurses, pharmacists, dietitians, doctors, psychologists and social workers. Treatment is intensive and time consuming for the patient and family. Daily regimes include physiotherapy, oral and inhaled medication, exercise and a range of measures including an energy dense diet, and the ingestion of pancreatic enzyme and vitamin supplements to optimise nutrition. The aim is to reduce complications from the disease and preserve good health for as long as possible. There is a particular focus on maintaining lung function and respiratory status.

Advances in CF treatment and health care delivery have led to improved survival although in recent years these have plateaued. There is a need for further advances in care to improve survival and reduce disease burden. Most disease-related morbidity and mortality is caused by progressive lung disease as a result of bacterial infection resulting in damaging airway inflammation. Survival is directly related to lung function (FitzSimmons 1993, Koehler, Downey et al. 2004). Respiratory disease and complications thereof provide the biggest burden to patients and result in high demands on health care services due to the need for multiple hospital admissions, intensive drug therapies and their associated costs.

1.1.2 *Pseudomonas aeruginosa* (PA) infection

PA is a gram-negative rod and an opportunistic pathogen. In the environment it can be found in moist areas including soil, drains and sinks. In healthy individuals it rarely causes disease as bacteria are easily cleared by innate immunological and mechanical host defence mechanisms. In immunocompromised patients these defences fail. Situations where PA commonly causes infection are on intensive care

in ventilator assisted patients, patients suffering from severe burns where the usual skin barrier is lost, patients with impaired immunity and patients with cystic fibrosis where the normal host defence mechanisms in the respiratory tract are poorly effective.

In CF, infection with PA causes the greatest clinical concern, although in recent years it has been established that the microbiome of the CF lung is complex. Multiple other bacterial species have been identified using new molecular techniques (Rogers, Hart et al. 2003). PA is the most common cause of chronic lung infection with studies reporting it as occurring in up to 9% of pre-school children, 32% of 10-15 year olds and 59% of adults (Burns, Gibson et al. 2001, Griese, Muller et al. 2002, Langton Hewer and Smyth 2009). In one study up to 80% of adult patients were reported as infected with PA. (Hansen, Pressler et al. 2008) Chronic colonisation occurs in association with changes in the metabolic behaviour of the organism and a biofilm mode of growth (Hassett, Sutton et al. 2009). This mucoid phenotype of the organism is one in which the bacteria is immersed within large quantities of exopolysaccharides such as alginate, which protects the bacteria from phagocytosis and antibiotic exposure (Ramsey and Wozniak 2005). Once these changes have occurred within the CF airway, PA cannot be cleared by conventional antibiotic therapy and infection becomes permanent (Rosenfeld, Ramsey et al. 2003). PA, as a biofilm phenotype, is the main pathogen causing chronic infection in CF (Bjarnsholt, Jensen et al. 2009). Recently, PA biofilm has been demonstrated in explanted lungs at time of transplant and CF lung autopsy specimens (Ratjen and McColley 2012).

Patients who are colonized with PA show a more rapid decline in lung function (Emerson, Rosenfeld et al. 2002), faster decline in chest radiograph score (Kosorok, Zeng et al. 2001), poor weight gain, increased hospitalization rates and an increased need for antibiotic therapy (Ballmann, Rabsch et al. 1998). Median survival is significantly reduced and mortality increased (FitzSimmons 1993, Emerson, Rosenfeld et al. 2002).

In recent years there has been a reduction in the prevalence of chronic pseudomonas infection in children in the UK to around 11%. (Smyth 2010) This is thought to be due to regular microbiological surveillance and aggressive attempts at eradication therapy when PA is first identified. Additional measures to try and

prevent cross-infection between patients may also be a contributory factor. It is still not agreed what form of eradication therapy should be used to achieve optimal results and a trial comparing oral and intravenous antibiotics is ongoing (<http://www.torpedo-cf.org.uk/> TORPEDO-CF trial).

Long term outcomes for CF patients could be improved if new methods were developed to more effectively eradicate PA when initially isolated thus reducing the occurrence of chronic infection.

1.1.3 The wider microbial community in the CF lung

It has recently been appreciated that the lungs of patients with CF and other chronic suppurative lung diseases are initially colonised with a diverse range of bacterial species. Microbiological culture media used routinely are only capable of supporting the growth of a small fraction of the total bacterial community present (Dunbar, White et al. 1997) and only a limited number of important species are routinely isolated and reported. In CF these typically include *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Burkholderia cepacia*. (HPA 2003) Conventional culture results clearly underrepresent the total population of bacterial species present within the lungs. Routine antibiotic susceptibility testing only demonstrates the effectiveness of single antibiotics against bacteria growing in aerobic conditions and usually in their planktonic phase. Patient therapy guided by such sensitivity testing is not useful, and current guidelines for antibiotic choices to treat PA infection do not support the routine use of antibiotic sensitivity testing despite rising antimicrobial resistance (Pitt, Sparrow et al. 2003, Emerson, McNamara et al. 2010). Treatment of acute exacerbations in patients with chronic *Pseudomonas* infection is more often based on 'standard' combinations of antibiotics that have been shown to produce a favourable response during previous exacerbations.

Techniques have been developed to study in detail the bacterial diversity within bacteriological specimens from CF patients. Terminal restriction fragment length polymorphism (T-RFLP) is a molecular technique for profiling microbial communities based on the position of a restriction site closest to the labelled end of an amplified gene. It has been shown to be an important means of analysing the composition and diversity of the bacterial community in samples from participants with cystic fibrosis, identifying species not previously reported in CF (Rogers, Hart et al. 2003, Rogers, Carroll et al. 2004). The importance of this diverse range of bacterial

species to lung pathology is largely unknown although it would appear that loss of bacterial diversity and the emergence of PA as the dominant infecting pathogen is typical of advanced lung disease.

Multilocus sequence typing (MLST) characterises bacterial and fungal species using the DNA sequences of internal fragments of multiple housekeeping genes to describe the genetic variation that occurs within a species. This can provide data for epidemiological surveillance and has been used to inform public health policies and develop new treatments and vaccines (Sullivan, Diggle et al. 2005).

More recently next generation sequencing has been used to look at bacterial species present in the CF lung. Gene sequencing can catalogue bacterial species present in a specimen that are unable to be detected by traditional culture methods, in particular bacteria in low numbers. Another benefit of this technique is that it can be performed rapidly, with the potential for same day results. This may be a useful clinical diagnostic strategy in the future.

(Salipante, Sengupta et al. 2013, Hauser, Bernard et al. 2014, Sharma, Gupta et al. 2014)

1.2 Nitric Oxide

Nitric oxide (NO) is an important physiological signalling molecule that is present in many body tissues and involved in a wide range of cellular processes. It was discovered by researchers investigating the regulation of vascular tone (Furchgott and Zawadzki 1980). Originally called endothelium-derived relaxing factor (EDRF), NO was initially assumed to be a protein prior to the discovery that it was nitric oxide. One of the key effects of NO in the human body is to signal the relaxation of the smooth muscle surrounding blood vessels which leads to vasodilation and allows the blood flow to increase (Ignarro, Buga et al. 1987).

NO has a half-life of a few seconds and is a highly reactive molecule. It has so many important functions (both in physiological processes and in the environment) that it was named as “molecule of the year” in 1992 (Koshland 1992).

Throughout the body NO is produced from substrate (such as L-arginine) by enzymes called nitric oxide synthases (NOS). There are 3 forms of this enzyme:(Grasemann, Knauer et al. 2000)

1. neuronal NOS (nNOS/NOS1)
2. inducible NOS (iNOS/NOS2)
3. endothelial NOS (eNOS/NOS3)

1.2.1 The role of Nitric Oxide in the airways

Nitric Oxide is important for a number of physiological processes within the respiratory tract including host defence, neurotransmission, bronchodilation, neutrophil migration into the airway, ciliary beat frequency up regulation and smooth muscle relaxation (Wooldridge, Deutsch et al. 2004, Grasemann, Kurtz et al. 2006). All 3 NOS enzymes are expressed in cells within the airways.

It is thought that iNOS in the epithelial cells within the airways is the main determinant of NO in exhaled air (eNO), although the contribution of the different NOS iso-enzymes to the levels of eNO is not known. NOS levels have been reported to increase in response to inflammation (particularly eosinophilic inflammation e.g. asthma)(Keen, Gustafsson et al. 2010) and airway NO levels have been found to be increased in a number of conditions that cause airway inflammation (Lundberg, Nordvall et al. 1996) although this appears to be the opposite to what is found in CF.

1.2.2 Nitric Oxide in Cystic Fibrosis

In CF, levels of eNO have been shown to be reduced, despite ongoing airway infection and inflammation, which would normally be expected to elevate levels within the airways. Several studies have reported reduced levels of eNO in children and adults with CF compared to healthy controls.(Balfour-Lynn, Laverty et al. 1996, Dotsch, Demirakca et al. 1996, Lundberg, Nordvall et al. 1996, Grasemann, Michler et al. 1997, Ho, Innes et al. 1998)

There are a number of reasons why eNO may be reduced in CF:

1. retention of thick, sticky mucus and subsequent metabolism of NO(Grasemann, Ioannidis et al. 1998, Linnane, Keatings et al. 1998)
2. consumption of NO by denitrifying bacteria such as PA (Everard and Donnelly 2005)
3. chronic malnutrition leading to decreased levels of the NO precursor L-arginine (Wooldridge, Deutsch et al. 2004)

4. increased arginase activity leading to reduced L-arginine availability (Grasemann, Schwartz et al. 2005)
5. decreased formation of NO due to reduced expression of NOS enzymes, in particular iNOS (NOS2) which has been shown to be reduced or absent in patients with CF. (Kelley and Drumm 1998, Meng, Springall et al. 1998, Wooldridge, Deutsch et al. 2004)

One study in patients with CF showed that there was increased arginase activity in sputum in patients treated with antibiotics for pulmonary exacerbation compared to controls. This may partly explain the reduction in eNO observed in other studies as arginase competes for the substrate L-arginine (Grasemann, Schwartz et al. 2005).

NO also contributes to the regulation of bronchial tone, and a single inhalation of nebulised L-arginine (which stimulates NO production in epithelial cells) has been shown to improve FEV₁ in CF patients (Grasemann, Kurtz et al. 2006).

eNO has been shown to increase with age in healthy children, (Buchvald, Baraldi et al. 2005) but to reduce with age in those with CF (Franklin, Hall et al. 2006). This may be due to a gradual destruction of epithelial cells leading to a reduction in iNOS, due to increased arginase, or to another as yet unestablished mechanism.

There is some evidence that lower airway NO levels may contribute to lung pathology in CF resulting in airway obstruction. (Mhanna, Ferkol et al. 2001) (Grasemann, Kurtz et al. 2006) Pulmonary function is lower in CF patients with lower eNO levels and lower levels of NO metabolites in their sputum. A study looking at genetic polymorphisms in the NOS1 gene found a slower decline in lung function, during a 5 year follow-up, of CF patients with the NOS1 genotype that was associated with higher levels of eNO (Texereau, Marullo et al. 2004). Others reported that higher numbers of repeats in the NOS1 gene polymorphism were associated with lower levels of eNO and greater colonization with PA (Grasemann, Knauer et al. 2000).

One study has shown that eNO increases in CF patients following antibiotic therapy (Jaffe, Slade et al. 2003). This could be due to the reduction in airway inflammation as a result of treatment, and consequent increase in expression of NOS2.

Alternatively, it could be secondary to the reduction in the number of denitrifying

organisms such as PA in the respiratory tract as a result of antibiotic treatment (Gaston, Ratjen et al. 2002).

The mechanism of NO reduction in CF may also be due to a reduction in NOS2. One study that looked at NOS2 expression in airway epithelial cells and macrophages in patients with CF found that there was decreased expression of NOS2 with increasing levels of Interleukin 8 (IL-8) and neutrophils in broncho-alveolar lavage fluid (BALF), both indicators of increased airway inflammation. This is contrary to the usual situation, where NOS2 levels increase with increasing inflammation in the airway (Asano, Chee et al. 1994, Warner, Paine et al. 1995, Meng, Springall et al. 1998, Saleh, Ernst et al. 1998), and suggests that NOS2 expression in response to airway inflammation in CF may be reduced or dysregulated (Wooldridge, Deutsch et al. 2004). Studies in mice and explanted CF lungs (obtained at transplant) have also demonstrated a reduction in the expression of NOS2 in CF (Meng, Springall et al. 1998, Steagall, Elmer et al. 2000). Levels of NOS2 are reduced by mutant CFTR expression and function in mice (Kelley and Drumm 1998).

1.3 Biofilms

Bacteria are now known to exist *in vivo* as single cells that float or swim independently in a liquid medium (the planktonic phase) or as structured bacterial communities known as biofilms. In biofilms, bacteria are encased in a polymeric matrix (consisting of polysaccharide, protein and DNA) (Hoiby, Ciofu et al. 2010) adherent to a biological or inert surface. Bacteria in biofilms are relatively resistant or tolerant to antibiotics. This can be explained in a number of ways (Hall-Stoodley, Costerton et al. 2004):

1. As a consequence of a protective barrier effect of the polymeric matrix.
2. Bacteria in the biofilm may be metabolically quiescent, thus reducing the activity of antibiotics that rely upon disrupting energetic processes to be maximally effective.
3. By facilitating the survival of resistant bacterial phenotypes.
4. By increasing tolerance to host defences – for example neutrophils can't penetrate the biofilm.

A bacterial population in a biofilm may have a degree of antimicrobial resistance up to 1000 times as great as an identical population that is planktonic (Brooun, Liu et al. 2000, Dales, Ferris et al. 2009). Although aggressive early antibiotic treatment can eradicate planktonic bacterial infection, the formation of biofilms, and the antibiotic resistance that they confer, are becoming increasingly recognized as risk factors for chronic infection in diseases such as CF (Singh, Schaefer et al. 2000, Costerton 2001, Bjarnsholt, Jensen et al. 2009).

1.3.1 Biofilm life cycle

Biofilms are formed as bacteria attach to a surface. The cells aggregate and proliferate and the biofilm matures. Over time, bacterial cells are released from the biofilm that revert to a planktonic phase. The dispersal that occurs as the bacteria are released from the biofilm is seen as the completion of the biofilm life-cycle and can lead to new bacterial colonization of surfaces or structures (Barraud, Schleheck et al. 2009). See Figure 1.

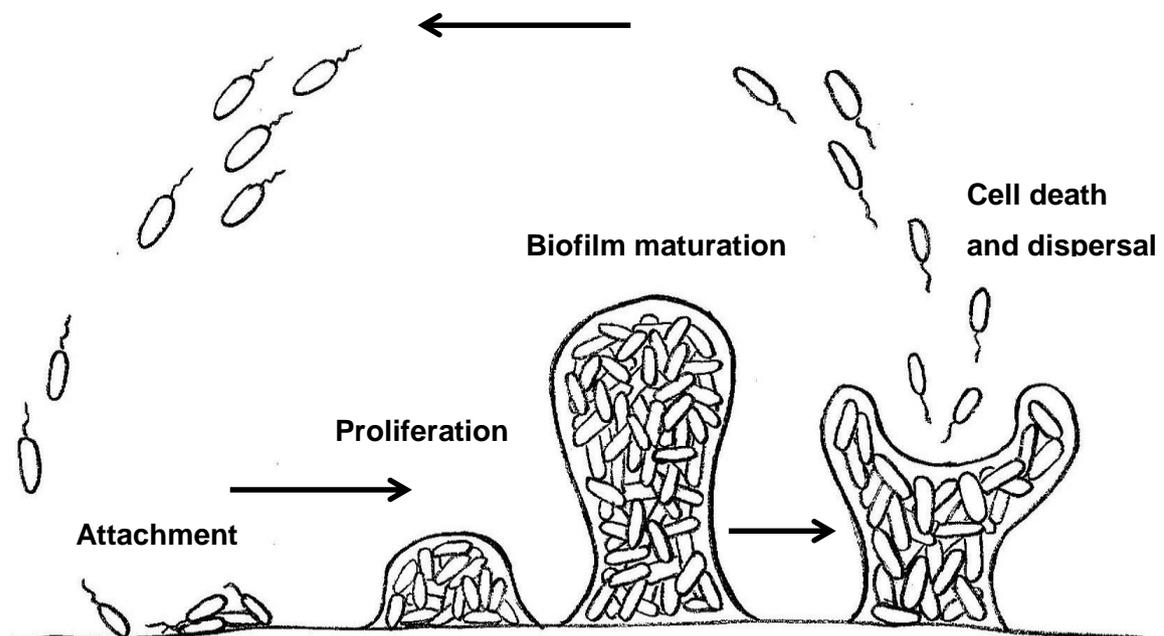


Figure 1 Biofilm Life cycle

1.3.2 Quorum sensing

Some bacteria, including PA, communicate with each other using a mechanism called quorum sensing (Hauser, Jain et al. 2011) by which bacteria sense changes in the density of the surrounding bacterial population and subsequently respond by

altering gene expression (Hasset, Cuppoletti et al. 2002). Bacteria produce permeable molecules into the environment which leads to adjacent bacteria 'sensing' those around them. The concentration of these molecules increases to a point where a 'quorum' is reached and this then causes switches in gene expression within the bacteria leading to production of the polysaccharide matrix that encourages the biofilm mode of growth.

1.4 Nitric oxide induced dispersal of biofilms

NO is known to down regulate biofilm formation *in vitro* which, together with reduced exhaled NO levels in CF (Balfour-Lynn, Lavery et al. 1996, Grasemann, Michler et al. 1997), may predispose patients to biofilm formation *in vivo* (Darling and Evans 2003). Our laboratory has shown that biofilm formation by a laboratory strain of PA was reversed *in vitro* by nanomolar, non-toxic concentrations of NO (Barraud, Hasset et al. 2006). In this model the proportion of PA in the planktonic (vulnerable) phase was increased, and a reduction in overall biofilm antibiotic tolerance observed. Exposure of biofilms to NO also greatly enhanced the ability of antibiotics, such as tobramycin (a typical antibiotic used in CF), to remove the bacteria/biofilms from a glass surface (Barraud, Hasset et al. 2006).

Further laboratory work has focused on the treatment of *ex vivo* biofilms grown from CF sputum (data submitted for publication). As part of this thesis, sputum samples were collected from participants with CF (Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease, NHS REC No: 08/H0502/126, Sponsor Code: RHM MED0837). PA was isolated from CF sputum samples and biofilms grown. This work, using PA clinical isolates, replicated the previous laboratory-strain model, demonstrating biofilm dispersal after approximately 5 hours. Further analyses assessed biofilm dispersal and bacterial removal using antibiotics combined with SNP. These studies showed that treatment of clinical isolate PA biofilms with a combination of SNP (NO donor) and antibiotic (ceftazidime and tobramycin) resulted in greater removal of the biofilm from the surface compared to antibiotic alone. The observed effect was a reduction in surface area occupied by biofilm in the order of 30% alongside an associated reduction in colony forming units (CFUs) (data submitted for publication).

From these data, there is clear evidence that nanomolar concentrations of NO causes dispersal of bacteria from biofilms in *ex vivo* CF sputum specimens. When PA biofilms are treated with antibiotic in combination with SNP, a greater degree of killing is observed than with antibiotic alone.

1.4.1 The mechanism of NO mediated biofilm dispersal

Dispersal of PA has been shown to occur through increased phosphodiesterase (PDE) activity leading to a reduction in cyclic-di-guanosine monophosphate (c-di-GMP). C-di-GMP is an important signalling molecule that is involved in the transition from a planktonic to biofilm state. It is involved in the production of the exopolysaccharide matrix (EPS) and in controlling adhesion, motility and aggregation.

Levels of c-di-GMP can be modulated by PDEs. C-di-GMP levels in planktonic and dispersed cells have been found to be 10 times lower than cells in biofilms. In contrast PDE activity was increased 3 times in dispersed cells compared to cells in the biofilm (Roy, Petrova et al. 2012). In bacteria, a biofilm lifestyle is promoted by high levels of c-di-GMP and low levels promote dispersal and motility (Hausler and Parsek 2010). In cells the levels of c-di-GMP are regulated by the synthesis of c-di-GMP by diguanylate synthases (DGC) and the degradation of c-di-GMP by PDEs (Figure 2).

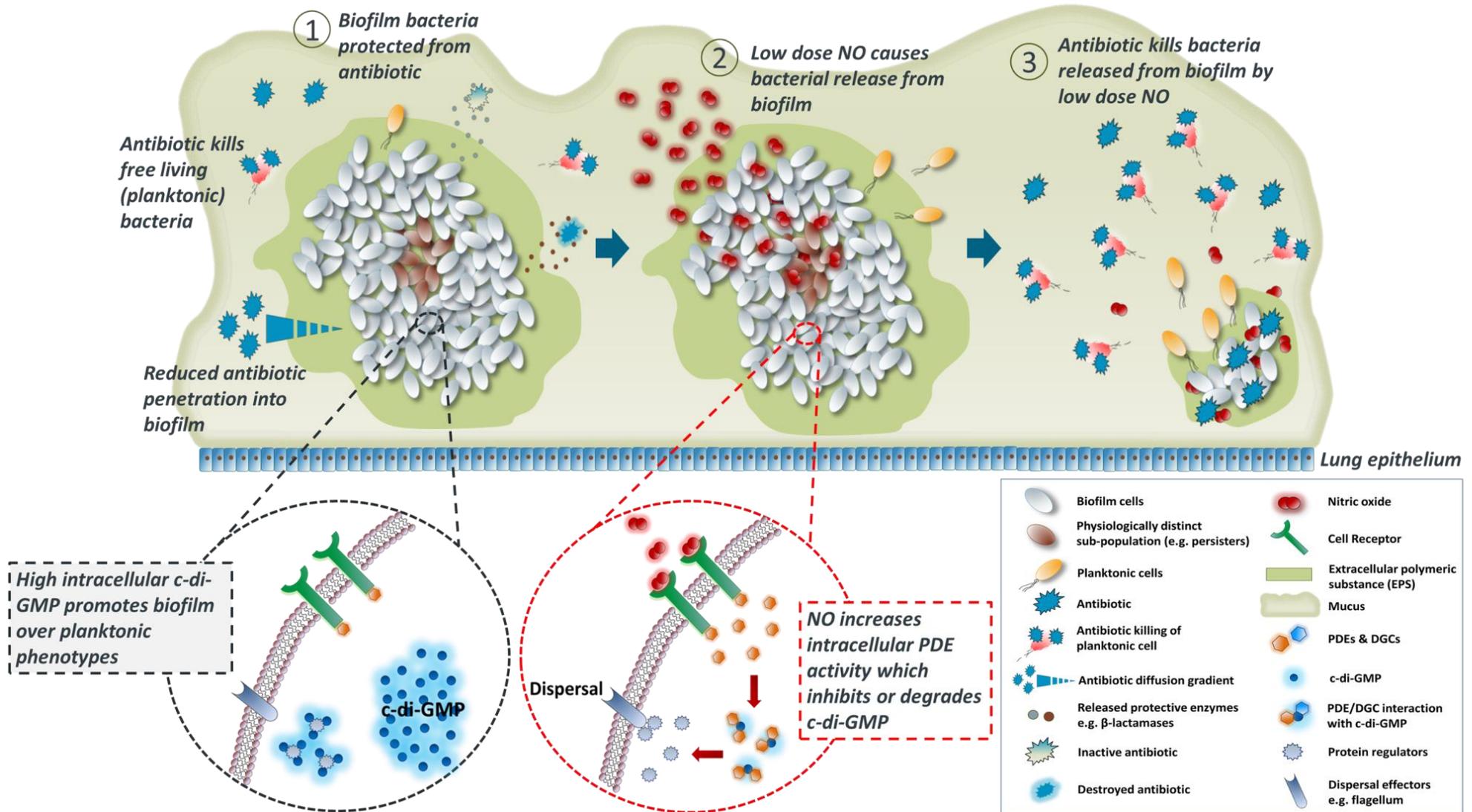


Figure 2 Nitric Oxide induced dispersal of biofilms (figure drawn by Rob Howlin and others)

Genetic studies have shown an up regulation of genes involved in motility, twitching and energy metabolism following NO treatment of PA and reduced expression of virulence factors and adhesion genes. One specific gene BdlA, has been identified as being required for biofilm dispersal by NO. This gene indirectly regulates c-di-GMP levels in the cell (Barraud, Schleheck et al. 2009).

1.5 Nitric Oxide delivery to the lungs

As preparation for a proof of concept study to assess whether nitric oxide delivered to the CF lung would have the same effect *in vivo* as in the *ex vivo* experiments, it was necessary to review the potential methods of therapeutic NO delivery to the lungs. There are a number of potential agents, but cost, delivery method, licencing and side effects were all important factors in deciding which was the most suitable for the clinical proof of concept trial.

1.5.1 Inhaled Nitric Oxide (iNO)

Nitric oxide gas has been well researched and used for a number of years in preterm neonatal, paediatric and adult patients; mainly on intensive care units. Currently it is licensed for 2 indications: infants >34 weeks gestation with hypoxic respiratory failure associated with evidence of pulmonary hypertension, and as part of the treatment of peri- and post-operative pulmonary hypertension in adults and newborn infants, older infants and toddlers. It is also used in selected children and adolescents; ages 0-17 years, undergoing heart surgery in order to selectively decrease pulmonary arterial pressure and improve right ventricular function. (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000337/WC500032784.pdf INOtheapeutics 2006). It is not compounded like other drugs and is carried within inert nitrogen. (Miller, McMullin et al. 2009). NO is used routinely in clinical care at concentrations of 20ppm, but at times increased to concentrations of 40ppm. In the neonatal ICU, iNO is often used continuously for a number of days. It acts as a vasodilator, improves oxygenation and reduces the need for extracorporeal membrane oxygenation (ECMO) (Finer and Barrington 2000).

iNO has been, and continues to be, used off-label in a number of other patient groups including adults and children with acute pulmonary hypertension, acute respiratory distress syndrome (ARDS) and children with perioperative pulmonary hypertension. (Kinsella and Abman 2005, Barr and Macrae 2010).

1.5.1.1 Inhaled Nitric Oxide in CF

There are previous studies where iNO has been given to CF patients. None of these have looked at the effect on biofilms, focussing instead on the effects of iNO on oxygenation and lung function.

In a small study iNO in concentrations of 100 parts per billion (ppb), 1 parts per million (ppm) and 40 ppm given for 5 minutes had no immediate effect on lung function or oxygenation (n=13 patients)(Ratjen, Gartig et al. 1999). In another small study, 35 CF patients were given iNO during pre-operative (lung transplant) assessment and 13 undergoing lung transplantation were also given iNO during the operation with no significant side effects or systemic effects (Della Rocca, Coccia et al. 1998).

A phase 1 study of high dose iNO (160ppm) for 30 minutes, 5 times a day for 5 consecutive days given to healthy adults, showed no adverse effects and no change in FEV₁ or other lung function parameters, serum nitrites/nitrates, prothrombin, pro-inflammatory cytokines or chemokines. Methaemoglobin levels were significantly increased after treatment, but still below 5% and normalised after therapy was discontinued (Miller, Miller et al. 2012).

A pilot study recently completed in Denver, Colorado, examined the safety and tolerability of iNO (given at doses of either 20ppm or 40ppm continuously over 44 hours) in clinically stable participants with CF. Although not looking at the effect on biofilms, this study collected induced sputum at baseline and after 44 hours, and measured quantitative bacterial counts and markers of inflammation in the sputum. iNO was safe and well tolerated in these participants with no significant antimicrobial or anti-inflammatory effects (Dr Scott Sagel, unpublished data, personal communication, outline data also available on clinical trials.gov: <http://clinicaltrials.gov/ct2/show/NCT00570349?term=nitric+oxide+and+CF&rank=3>).

Over the past 10 years, iNO has been administered to numerous participants without any apparent side effects. Although iNO is considered to be safe in doses of 1-20 ppm, there are a number of precautions and safety considerations that should be taken into account. In addition, only medical grade iNO must be used (Germann, Braschi et al. 2005). Current recommendations state that scavenging is not

necessary if iNO is administered in a well-ventilated environment (Squire, Kightley et al. 1996).

Formation of nitrogen dioxide (NO₂) occurs when NO is mixed with oxygen (O₂). It is also formed from decomposition of peroxyxynitrite (a reactive nitrogen species formed from reaction of NO with reactive oxygen species such as superoxide) (Weinberger, Laskin et al. 2001). The formation of NO₂ can be reduced by limiting the concentration of O₂ in the inspired gas mixture (Griffiths and Evans 2005). Levels of NO₂ need to be monitored and kept below 2ppm (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000337/WC500032784.pdf INOtheapeutics 2006).

Systemic vasodilation does not occur with use of iNO because NO is rapidly bound and inactivated by haemoglobin (Hb) within the circulation (Ricciardolo, Sterk et al. 2004). This results in the formation of methaemoglobin (metHb). High levels of metHb cause a reduction in the O₂ carrying capacity of the blood. Clinically significant levels of metHb are unlikely to result unless concentrations of iNO higher than 20ppm are used for continuous periods over days, or if levels above 80ppm are used (Wang, El Kebir et al. 2003, Macrae, Field et al. 2004).

1.5.2 Nebulised L-arginine

L-arginine is a semi-essential amino acid with a short half-life due to rapid metabolism. Nitric oxide is produced in the airways by oxidation of L-arginine by nitric oxide synthases (NOS)(Everard and Donnelly 2005). L-arginine is therefore a precursor for endogenous NO production rather than an NO donor.

L-arginine has been used in CF to increase local airway NO levels (Everard and Donnelly 2005). In one study a single dose of IV L-arginine (500mg/kg) was given to CF patients, which resulted in increased eNO levels (Grasemann, Gartig et al. 1999). Oral L-arginine (100mg/kg or 200mg/kg) given to healthy patients has also been shown to increase eNO (Kharitonov, Lubec et al. 1995).

Others have investigated oral L-arginine effects on eNO in CF. In the first study 6 patients received oral L-arginine (150mg/kg/day in 3 divided doses) for 1 month, which resulted in an increase in plasma L-arginine but no effect on eNO or lung function (Everard and Donnelly 2005). In an unconventionally designed double-

blind, placebo-controlled, crossover trial a single dose of oral L-arginine (200mg/kg) was given to 8 patients with CF and 8 healthy controls following which the same dose was given to 10 patients with CF three times a day for 6 weeks. The single oral dose of L-arginine resulted in increased levels of L-arginine in sputum and plasma and increased eNO levels 3 hours after the dose. Supplementation of L-arginine for 6 weeks showed sustained increases in eNO (following a 14 day course of IV antibiotics) compared to placebo. There was no effect on FEV₁ (Everard and Donnelly 2005, Grasemann, Grasemann et al. 2005).

L-arginine undergoes extensive presystemic elimination due to arginase activity in the intestine (Schwedhelm, Maas et al. 2008). Therefore, higher L-arginine levels may be able to be achieved in the lungs using inhaled L-arginine compared to IV (Grasemann, Kurtz et al. 2006). Thirteen patients with CF were given nebulized L-arginine and compared to 9 healthy controls. Maximal effects on increasing FEV₁ after inhalation of L-arginine were seen after 4 hours. A rise in eNO was seen in patients with CF up to 6 hours after inhalation of L-arginine, which was statistically significant at 4 hours (statistics not reported at other time points)(Grasemann, Kurtz et al. 2006). L-arginine products in sputum were not significantly increased after inhaled L-arginine, neither were L-citrulline levels in plasma. L-ornithine levels in plasma were increased, but returned to baseline levels at 24 hours. L-arginine levels in sputum had also returned to baseline by 24 hours, suggesting that the amino acid does not accumulate in the airways. In fact, L-arginine levels in sputum were reduced close to baseline at 4 hours post inhalation (Grasemann, Kurtz et al. 2006).

More recently, a group in Canada have completed recruitment in 2009 to a trial investigating 'the short term safety and efficacy of inhaled L-arginine in patients with CF'. In personal correspondence with the PI (Felix Ratjen), he has reported that there are no safety concerns with 14 days treatment of nebulised L-arginine although data has not yet been published.

It is not known at present whether the effects on the airways seen after L-arginine administration are directly attributable to NO. There may be other active metabolites, such as the s-nitrosothiols,(Grasemann, Kurtz et al. 2006) formed in the airways that mediate some of the effects. Others have suggested NO could be 'stored' in the circulation by s-nitrosothiols, leading to a longer period of increased eNO (Griffiths and Evans 2005).

Historically, it was suggested that L-arginine had a mucolytic effect in CF, but this was not confirmed in later studies (Solomons, Cotton et al. 1971, Dietzsch, Gottschalk et al. 1975) and this hypothesis remains unlikely.

L-arginine could be a potential candidate for delivering NO to CF airways for the study. However, the main potential problem with this approach is that L-arginine may not be metabolised to NO in the airways of patients with CF due to the lack of iNOS (which is the major determinant of NO in exhaled breath) in the bronchial epithelium (Kelley and Drumm 1998, Meng, Springall et al. 1998, Keen, Gustafsson et al. 2010).

However, as described above (Jaffe, Slade et al. 2003) eNO levels increased following IV antibiotics in patients with CF. In this scenario, iNOS expression might have been expected to be down regulated following antibiotic treatment as a result of reducing the bacterial load and reduced levels of pro-inflammatory cytokines (both of which up-regulate iNOS activity), but in fact an increase in eNO was shown, suggesting L-arginine could lead to increased airway NO levels.

L-arginine is an amino acid present in food, but is currently not licensed for intravenous or nebulized use. Little data exist in the published literature (Grasemann, Kurtz et al. 2006) and so prior to any pilot experimental medicine or phase 1 trials it would be necessary to manufacture the drug to GMP standards.

1.5.3 GSNO

S-Nitrosoglutathione (GSNO) is an endogenous nitric oxide donor known to be present in pulmonary extracellular fluid (Gaston, Reilly et al. 1993). It has a number of effects in the airway including:

1. Relaxation of airway smooth muscle (Gaston, Reilly et al. 1993)
2. Improvement of airway ciliary motility (Li, Shirakami et al. 2000)
3. Promotion of neutrophil apoptosis (Fortenberry, Owens et al. 1999)
4. Antimicrobial effects (Saura, Zaragoza et al. 1999)

In CF, GSNO has been observed to increase cellular expression and maturation of $\Delta F508$ (the common mutant form of CFTR)(Zaman, McPherson et al. 2001). Low

levels of GSNO have been found in CF airways compared with controls (Grasemann, Gaston et al. 1999).

The half-life of GSNO in solution is in the order of hours, but varies depending on a number of external factors (Snyder, McPherson et al. 2002).

GSNO is not currently licensed in the European Union or United States and has only been used in a few small clinical trials. In a double-blind placebo controlled study, nebulised GSNO was given to patients with CF (n=20) (Snyder, McPherson et al. 2002). Sputum production, vital signs and spirometry were no different between the groups at 5, 10, 20 and 30 minutes. Oxygen saturations increased at all time points in the GSNO group when compared to placebo ($p < 0.05$). eNO levels increased significantly in those treated with GSNO at 5 minutes and then fell over a 30 minute period, remaining higher than the placebo group at 30 minutes ($p < 0.05$).

GSNO is metabolized to a number of compounds including ammonium (NH_4). The concentration of NH_4 in the patients treated with GSNO was log orders below that shown to be physiologically or pathologically relevant in other studies (Snyder, McPherson et al. 2002). This study excluded patients with previous gastro-intestinal or airway bleeding due to the theoretical risk of hypotension and bleeding with GSNO. This is the only study of nebulised GSNO in humans, however, it has been given intravenously (IV) to prevent platelet aggregation in a number of cardiac conditions including after carotid angioplasty (Kaposzta, Clifton et al. 2002), coronary artery bypass (Salas, Langford et al. 1998), acute myocardial infarction and unstable angina (Langford, Wainwright et al. 1996). It has also been given intravenously to 10 healthy females with no effect on BP or pulse (Ramsay, Radomski et al. 1995).

1.5.4 Proli/NO

Proli/NO is an NO donor with the full name: Disodium 1-[2-(carboxylato)pyrrolidin-1-yl]diazene-1-ium-1,2-diolate. It has a half-life of 2 seconds at physiological temperature and pH (Waterhouse, Saavedra et al. 2006). As a result, it has proved difficult to formulate for use in patients. One study found that Proli/NO could be formulated as an injectable drug that was stable and only produced physiological hydrolysis products (Waterhouse, Saavedra et al. 2006). However, there are no reports of Proli/NO being used in clinical trials. It would therefore require significant further research before being ready to be tested in a phase 2 clinical trial.

1.6 Pre-clinical study

1.6.1 Introduction

This study was designed to collect sputum samples from patients with CF to generate laboratory data concerning the effect of NO on *ex vivo* biofilms that could lead to future testing in a clinical trial. In addition, the protocol (see Appendix 1) was written to allow other projects to collect respiratory samples from patients for investigation of the methods of infection and inflammation in a wide variety of respiratory diseases that affect children and adults. The processes involved in these diseases are often similar, and knowledge that emerges about one disease can sometimes be applicable to others. The protocol for these laboratory based studies was written by the author of this thesis. Additionally, regulatory approval was obtained by the author for these studies to take place (see Appendix 1 for the protocol and regulatory approvals). The laboratory work and results described in this section were carried out by Dr Rob Howlin and used by the author to help guide design of the clinical study.

Detailed scientific analysis and investigation had been ongoing in Southampton since 2000 as part of a clinical research collaboration (LREC 306/00/w) investigating the role of inflammatory mediators, surfactant phospholipids and surfactant proteins in respiratory disease. In April 2008 the National Institute for Health Research (NIHR) awarded Southampton a Respiratory Biomedical Research Unit (BRU) contract to create infrastructure to investigate childhood and adult respiratory disease at all stages of life. Overall the BRU has a specific role to undertake translational clinical research in priority areas of disease, ill health and areas of clinical need that are currently under-represented in the NHS. This study (NHS REC No. 08/H0502/126) was designed to investigate the effect on NO on biofilms, but regulatory applications were broadened to continue the ongoing work (LREC 306/00/w) and include new areas of research into respiratory disease (see Appendix 1 for full protocol and regulatory approval documents).

This study of lung infection and inflammation describes in detail a project designed to investigate inflammatory and infective processes in the lung. Children and adults were (and continue to be) entered into the study if they are known to suffer from, or are being investigated for, a range of respiratory illness, such as (but not limited to) cystic fibrosis (CF), primary ciliary dyskinesia (PCD), bronchiectasis and asthma.

1.6.2 Aim and objectives

The overall aim of this respiratory project was to improve understanding of the interactions between inflammatory and infective processes in paediatric and adult lung disease that could lead to novel therapies, usefully impacting on the progression of CF lung damage.

Specifically the aims of the preclinical study were:

1. To quantify and describe *in vitro* the effect of nitric oxide (NO) on bacteria in biofilms from clinical isolates from CF sputum.
2. To quantify *in vitro* the ability of NO to enhance the efficacy of antibiotics conventionally used to treat infective exacerbations in CF.
3. To inform design of a proof of concept trial to investigate the effects of NO therapy on PA biofilm in CF.

1.6.3 Methods

This study was set-up to allow research use of aliquots of samples collected as part of the routine clinical management of adults and children attending the paediatric and adult respiratory service with a variety of respiratory diseases including CF. Samples were collected from patients recruited prospectively into this study.

Samples included:

1. Sputum
2. Cough swabs
3. Naso-pharyngeal aspirates
4. Broncho-alveolar lavage fluid (BAL)
5. Urine
6. Blood (if a clinically indicated blood test of cannulation was being carried out)
7. Lung tissue (removed during surgery from an ethically approved source and with appropriate consent)
8. Endobronchial and brush biopsy specimens
9. Exhaled nitric oxide levels

For the CF biofilm work only sputum samples were obtained from CF patients.

1.6.3.1 Subject selection

Potential participants for the CF biofilm work were identified in 2 ways:

1. Patients with CF admitted to a ward at University Hospital Southampton NHS Foundation Trust (UHS) for treatment.
2. Patients with CF attending an outpatient clinic at UHS.

1.6.3.2 Subject recruitment

Patients attending the CF service were sent (before their appointment) or given (on the ward or in clinic) an information sheet about the study. All participants and/or their parents and legal guardians had time to read the information sheets and discuss any concerns or questions with a doctor. They were asked to consent to aliquots of their sputum being used for research purposes. Consent was obtained from either the patient (>18 years) or from a parent/adult with legal responsibility for the child. Children over 12 years, who were able to understand, were also asked for their verbal assent (See Appendix 1 for protocol which includes information sheets and consent forms used in this pre-clinical study, these were all designed by the author).

Patients with CF who attended regularly (visits to clinic, inpatient episodes) were asked to consent to repeated collection of sputum on different occasions. Some patients were therefore able to provide a number of specimens over time. All patients were free to withdraw their consent at any time, and consent to continue in the study was confirmed verbally before collection of sputum.

1.6.3.3 Laboratory methods

Sputum samples were analysed by Dr Rob Howlin (post-doctoral scientist) who investigated:

1. The effect of NO donor sodium nitroprusside (SNP) on biofilm dispersal using clinical PA isolates from CF sputum.
2. The optimum concentration of SNP to achieve dispersal in clinical PA isolates.
3. Confirmation of the action of NO using a nitric oxide scavenger.
4. Confirmatory PCR on clinical PA isolates.
5. Combination treatment of clinical PA isolates with SNP and antibiotics.
6. FISH analysis of PA biofilm.
7. Image analysis of confocal light scanning microscope images.

1.6.4 Results

The results presented here are a summary of the output from the biofilm research group in order to show how this informed the design of the clinical trial. All data and figures have been provided by Dr Rob Howlin. 70 patients with CF were investigated over the first 2 years of this programme. They were aged between 16 and 70 years with a mean of 27.2 years and a median of 22 years. Minimal clinical

data was collected on these participants as part of this laboratory based study and only included current medications and dates of current exacerbation. Data was not collected on CF mutation or pseudomonas status or any other biological characteristics.

Sodium Nitroprusside (SNP) was used as the NO donor in these experiments because it has been used in laboratory based biofilm experiments as an NO donor in many studies (Barraud, Hassett et al. 2006, Barraud N 2009, Howlin, Cathie et al. 2011). Using Sodium Nitroprusside (SNP) to disperse biofilms has shown a concentration of 500µM to achieve optimal dispersal of PA biofilms in CF isolates. Figure 3 shows an example of a typical graph from a patient isolate. It can be seen that low dose SNP (10nM) disperses close to control and high dose SNP (5mM) gives less dispersal than 500µM which is the dose that achieves optimal dispersal.

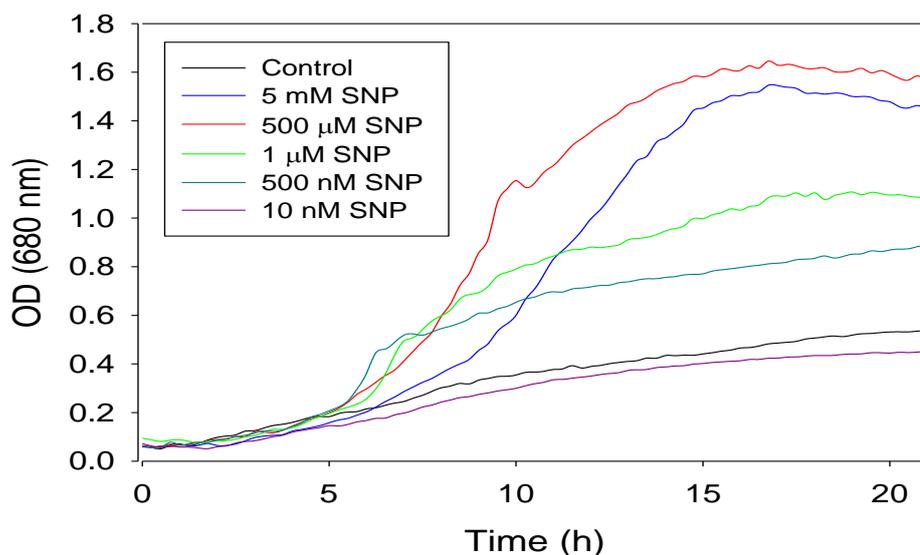


Figure 3 Dispersal curves for different concentrations of Sodium Nitroprusside showing increase in optical density in the supernatant over time

Figure 4 shows that SNP mediated dispersal of PA biofilms can be attenuated by the addition of a nitric oxide scavenger 2-Phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (PTIO). This confirms that it is nitric oxide causing the dispersal in the SNP treated biofilms.

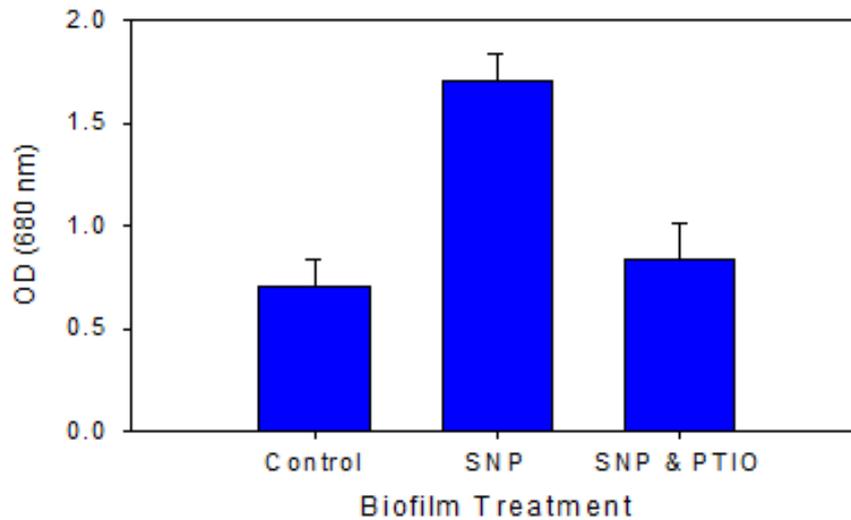


Figure 4 Optical density of supernatant after treatment with SNP +/- PTIO

Figure 5 shows scans of the bottom of the culture plate after treatment with SNP (500 μ M) and staining with SYTO 9 showing a reduction in surface bound biofilms which corresponds to the increase in optical density in the supernatant seen with dispersal (figure 3).

Before SNP treatment

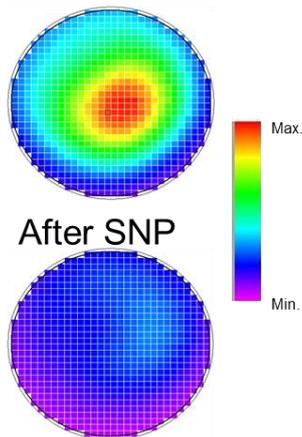


Figure 5 Scans of bottom of 96 well culture plate before and after treatment with Sodium Nitroprusside

Figure 6 shows dispersal curves over 2 graphs from 9 of the PA isolates from CF sputum using 500 μ M SNP. These show that dispersal starts to occur at around 5 hours. This was useful information to gather to inform how long the treatment is likely to be needed in patients to achieve dispersal of biofilms when given in a clinical trial.

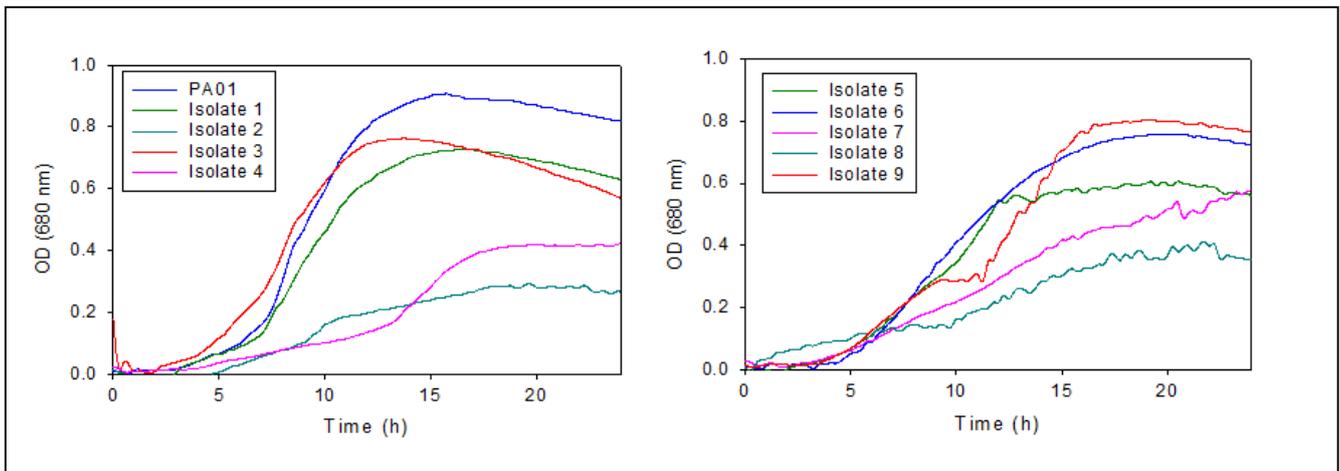
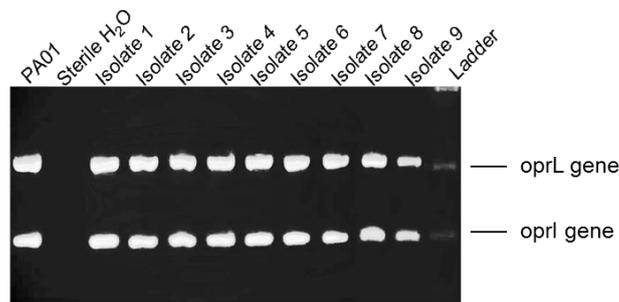


Figure 6 Dispersal curves for 9 patient isolates and the laboratory strain PA01 using SNP at a concentration of 500µM

Figure 7 shows confirmatory Polymerase Chain Reaction (PCR) performed on patient isolates confirming that the dispersed bacteria are PA.



oprL = *P. aeruginosa* genus specific gene
oprI = *Pseudomonas* species specific gene

Figure 7 Polymerase Chain Reaction (PCR) showing all 9 isolates are *P.aeruginosa*

Figure 8 shows surface plots obtained using image analysis of an epifluorescent image, stained with SYTO 9 and imaged using epi-fluorescence microscopy. There is some removal of biofilm from the surface with the antibiotics (ceftazidime and tobramycin), but a significant proportion remains. However, when combined with SNP treatment there is effective removal of the biofilm from the surface.

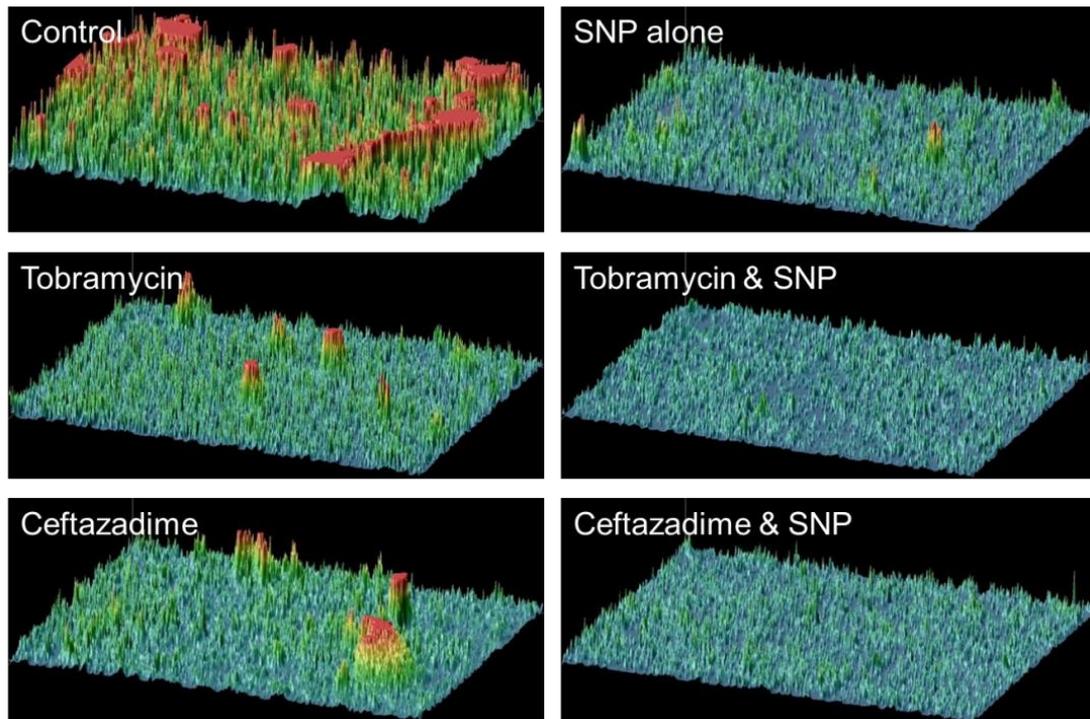


Figure 8 Surface plots obtained using image analysis of an epifluorescent image, stained with SYTO 9 and imaged using epi-fluorescence microscopy

A key study suggested that under certain conditions, tobramycin may enhance biofilm growth (Hoffman, D'Argenio et al. 2005). The mechanism was through an aminoglycoside response regulator gene (*arr*) which codes for an inner membrane PDE which uses c-di-GMP as a substrate. *Arr* gene mutants showed reduced levels of PDE activity and increased levels of c-di-GMP which promotes biofilm phenotypes over planktonic. Biofilm formation can hence be encouraged by the presence of tobramycin through increases in the level of c-di-GMP. This is opposite to the effect seen with NO induced biofilm dispersal where NO leads to an increase in PDE activity and reduction in c-di-GMP.

Tobramycin induced enhancement of biofilm growth was seen in these experiments using clinical isolates and clinically relevant concentrations of tobramycin (shown by confocal microscopy, Figure 9). Biofilms are stained with Live/Dead (green = live, red = dead). There is enhancement of biofilm growth with tobramycin and the PA in the centre of the biofilms remain alive. These images also show that with the addition of SNP this effect is reversed and the biofilm is removed and PA killed.

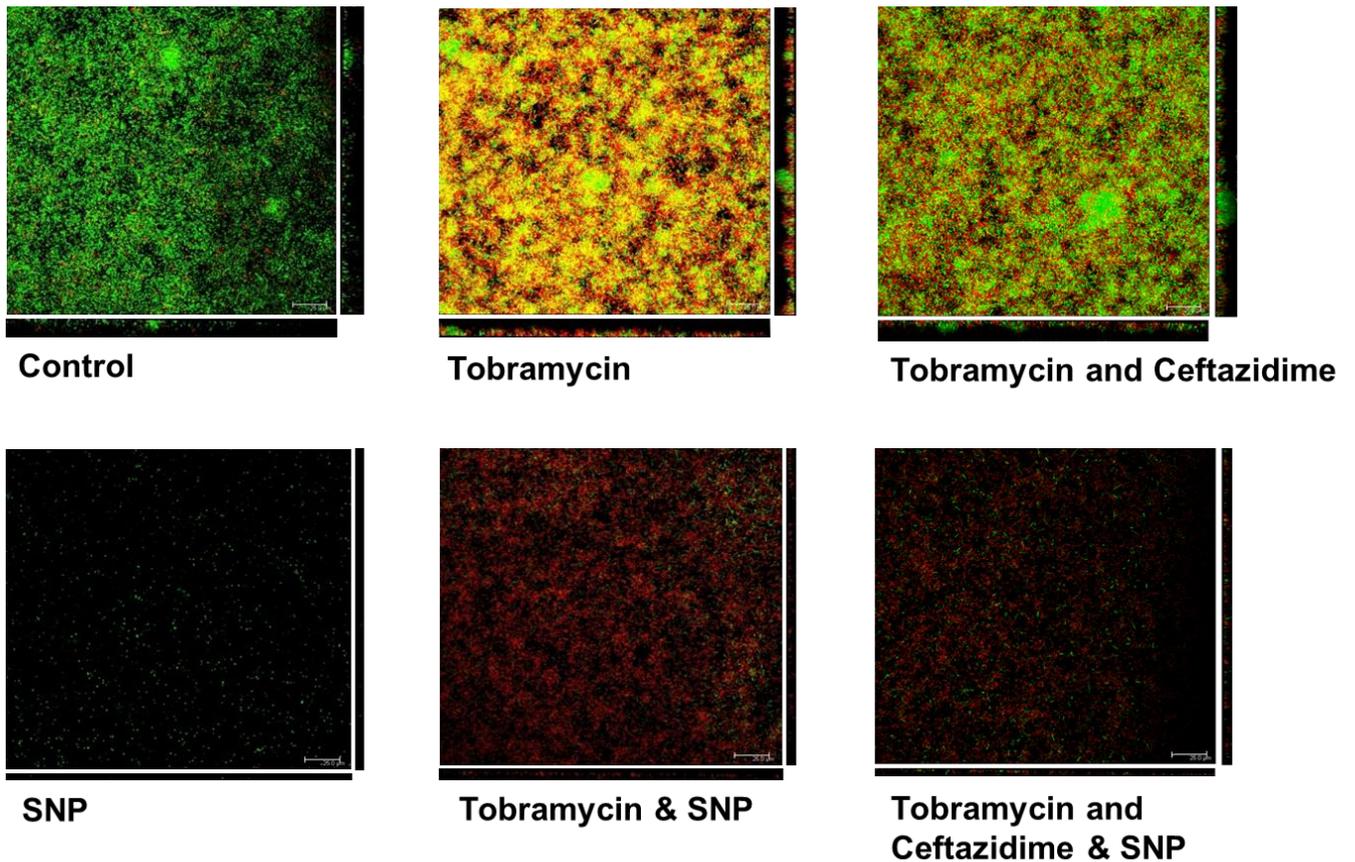


Figure 9 Confocal images showing an increase in biofilm mass with tobramycin (tobramycin 100 μ M, SNP – 500 μ M)

1.6.5 Discussion

The concentration of SNP required for optimal dispersal of PA from *ex vivo* samples was approximately 500 μ M. With higher concentrations there was reduced dispersal, which may be due to the toxic effects of NO at these concentrations. The optimum concentration was used to inform design of the clinical trial with equivalent inhaled NO dose in parts per million (ppm). The concentration of NO produced by SNP was calculated using a NO micro sensor (Unisense, Denmark) and calibrated over a range of 250 nM to 10 μ M using previously published methods (Zhang, Cardosa et al. 2000). Based on the measured linear relationship between micro molar 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 450 nM NO generated from 500 μ M SNP.

Electrochemical measurement of NO gas released in solution by approximately 500 μM SNP was measured to be around 390 nM NO (Barraud, Schleheck et al. 2009) which is equivalent to 390 nmol/L, giving 8.7 $\mu\text{L/L}$ or 8.7 ppm (not taking into account any adjustment due to the environmental temperature).

The results from the *ex vivo* sputum samples from CF patients show three things. Firstly, dispersal that has previously been demonstrated in laboratory strains of PA also occurs in PA isolates from patient sputum. Second, NO in combination with antibiotic therapy, successfully removes biofilm in *ex vivo* samples. Dispersal was greater in some samples than others, which may be explained by variation in sputum quality which can be affected by time of day, preceding nebulised therapy, co-operation of the patient and concurrent treatments. CF sputum is highly variable in consistency, between and within patients. In these laboratory studies, a large variation in the quality of the samples was observed.

PA dispersal was observed to be greater in the sputum samples of higher quality with large quantities of PA bacteria. In these specimens, changes were easier to observe than the more subtle changes seen in the more dilute (poorer quality) samples. This observation led to the development of the screening process used in the proof of concept trial which required PA to be present in the sputum in sufficient quantities before enrolment in order to allow observation of patient effect, if present, following NO therapy in the clinical study.

The third observation from the preclinical study was the effect of tobramycin on biofilms. Although tobramycin may be an effective antibiotic for treating acute infection, these results show it can lead to an increase in biofilm. Hoffman et al report these findings in CF patients (Hoffman, D'Argenio et al. 2005, Elliott, Burns et al. 2010). Tobramycin, whilst treating acute infection, may be making chronic infection worse through enhanced biofilm growth. These experiments show that this can be prevented with the addition of an NO donor.

1.7 Summary

CF is a life-limiting multi-system disease where chronic respiratory infection and inflammation play a major role in morbidity and mortality. Chronic infection with PA causes the greatest clinical concern and is thought to persist, despite antibiotic therapy, due to the formation of biofilms within the lungs. Biofilms are structured

communities of bacteria that show greater tolerance to antibiotics due to a number of properties. New ways of effectively eradicating PA biofilms from the CF lung could have a significant impact on lung function and quality of life for CF sufferers.

NO has been shown in *ex vivo* CF sputum to cause PA bacteria in biofilms to disperse and become more susceptible to antibiotic therapy.

There are a number of ways that NO can potentially be delivered to the CF lung, the simplest being inhaled NO gas. This product is already licensed in the UK for use in neonates with pulmonary hypertension. Other options including L-arginine, GSNO and Proli/NO do not currently have suitable preparations and previous studies have been experimental with small numbers.

Preliminary studies carried out within our research group confirmed the effect seen in previous laboratory work with NO and PA biofilms. They provided useful information to allow design of a proof of concept study to look at the effects of NO on biofilm when given to CF patients.

1.8 Hypothesis

Delivery of low dose NO to the CF lung will significantly reduce carriage of PA by reducing PA biofilm antibiotic tolerance.

This may:

- enhance efficacy of standard antibiotic regimes, especially ceftazidime/tobramycin
- reduce length/number of exacerbations & overall antibiotic burden
- improve respiratory function
- improve quality of life

This hypothesis will be tested through the design and completion of a pilot clinical trial. This trial will use inhaled nitric oxide, given to CF patients, at the time of pulmonary exacerbation, alongside IV antibiotic therapy and assess the microbiological effect of NO treatment on biofilms when administered directly to patients.

Chapter 2: Clinical Proof of Concept Study Design

2.1 Summary of study design

A proof of concept study, with experimental outcomes, was designed in order to investigate the effect of NO on biofilms when given to CF patients. Due to the current NO license, this was classified as phase 2 for regulatory purposes, as it was a study of a licenced drug for a new indication. The design was informed from preliminary laboratory work (section 1.6) and input from the biofilm research group, senior CF clinicians and CF patients. The study was a randomised, participant and outcome-assessor blind, placebo controlled, proof of concept study. Laboratory staff (analysing the primary outcome measures), investigators, medical staff, ward nursing staff and participants were all blinded to therapy. For practical and cost reasons, research nurses were not able to be blinded to therapy.

CF patients age 12 or above were screened for the ability to grow PA biofilms from their sputum to ensure maximum power for this pilot study. The first 12 participants who had been screened and subsequently had a pulmonary exacerbation were randomised to one of the following groups:

Group A: Nitric Oxide (using air or air/oxygen blend according to clinical need) was administered by inhalation via nasal cannula for 8 hours overnight for the first 5-7 days of IV antibiotic therapy.

Group B: Placebo (air or air/oxygen blend according to clinical need), was administered by inhalation via nasal cannula for 8 hours overnight for the first 5-7 days of IV antibiotic therapy.

Both groups also received standard IV antibiotic therapy of tobramycin and ceftazidime for 9-12 days (or greater - according to clinical need).

Primary outcome was between group differences in proportion of bacteria in biofilms (as determined by colony forming unit assessment, live/dead staining, direct visualisation of the biofilm and image analysis) between T0 and T20-25.

Secondary outcomes were:

1. Between group differences in proportion of bacteria in biofilms (as determined by colony forming unit assessment, live/dead staining, direct visualisation of the biofilm and image analysis) at all time points (T0, T5, T7, T10 and T20).
2. Between group differences in 'change in PA colony forming units' (as determined by plate counts and intensity of qPCR) between T0, T5, T7, T10 and T20-25.
3. Between group differences in whole colony CFU's at T5, T7, T10 and T20-25.
4. Between group differences in NO levels in fresh sputum at T7.
5. Between group differences in the characteristics of the wider microbial community as assessed by microbial community analysis/molecular analysis.
6. Between group differences in FEV₁ at T7, T10 and T20-25.
7. Between group differences in exhaled NO levels at T7.
8. Between group differences in HRQOL scores at T20-25.
9. Between group differences in length of course of antibiotics.

Outcomes were measured using a variety of methods including sputum samples, quality of life questionnaires and clinical parameters such as lung function. Samples were collected from the participants over the course of the 10 day period of IV antibiotics. Additionally, participants were asked to attend a follow-up visit 10-15 days after completing their IV antibiotics for further samples and clinical evaluation.

Ethical approval was obtained from Southampton Research Ethics Committee A on 11th January 2011 (11/H0502/7). University Hospital Southampton NHS Foundation Trust was the sponsor (RHM CHI 0548). The Medicines and Healthcare products Regulatory Agency (MHRA) approved the study (Clinical trial authorisation 11709/0236/001-0001), Eudra CT number 2010-023529-39, The study was publically registered on ClinicalTrials.gov NCT02295566.

2.1.1 Patient and public involvement in design

This study was designed through collaboration and repeated discussions with the co-investigators, adult and paediatric CF teams (including CF nurses, physiotherapists, psychologists and doctors), research nurses and doctors and study statisticians. CF patients, both adult and adolescent, were consulted at a number of stages of the design process, to contribute their thoughts and ideas, and

to ensure they felt the study would be acceptable. This was carried out through interviews with CF patients who were admitted to hospital or attending clinic. Discussions occurred between the research team and patients as well as the clinical CF teams with their patients. The study concept was explained and the likely procedures and treatment required was discussed regarding acceptability to patients. Outcomes that were relevant to patients were also discussed. The patients felt that blinding and delivery of NO via nasal cannula were acceptable, and they were happy with the requirement for additional tests (blood/sputum/lung function) to assess outcome measures. Generally, they were not keen on anything that would prolong their hospital stay. The patients expressed a preference for receiving the inhaled therapy overnight for 8 hours, as they felt it would be less disruptive to their lives and other treatments in hospital. They preferred the research nurse to sit outside the room to monitor them through the window, coming into the room when measurements were required, rather than the research nurse observing them from within the room for the whole night. Patients reported that their quality of life, level of symptoms and level of functioning was important to them. All of these factors were taken into account when designing the study.

2.2 Hypothesis/Objective

The primary objective of this pilot study was to test the hypothesis that adjunctive low dose NO given concurrently with standard IV antibiotic therapy would lead to enhanced antibiotic efficacy due to the disruption and killing of PA biofilms.

2.3 Outcomes

Selection of the correct outcome measures for a clinical trial is vital in order to be able to assess the effect of treatment on the participants. For clinical trials, different endpoints will be appropriate according to which phase the trial is (I, II, III or IV) as the purpose of the study at each phase requires a different outcome assessment.

Appropriate outcome measures need to be clinically and biologically relevant, sensitive and specific, reproducible and feasible. In earlier phase studies, less feasible measurements (in terms of patient burden and cost) may be easier to implement than in larger later phase studies.

2.3.1 Primary endpoint selection

For this proof of concept study the primary endpoint was microbiological.

A number of factors were key in the discussion and decision making which involved clinical and non-clinical members of the research team, senior biofilm scientists and CF patients.

From a microbiological perspective, the preliminary studies (introduction section 1.6) had established methods for examining biofilm in *ex vivo* sputum samples and assessing effect of exogenous NO (using SNP as an NO donor) on those biofilms. There had been unsuccessful attempts by our research group to look at the biofilms in real time, so this was not an option for the study. Direct visualization of biofilms in sputum and image analysis was shown to be the most effective way to demonstrate changes over time in NO and antibiotic treated biofilm. However, full microbiological assessment of biofilm volume in expectorated sputum samples at a number of time points was known to be time consuming and laborious (from work carried out by scientists in the group), in addition to the time required to carry out image analysis using a confocal light microscope (CLSM). More established measures of bacterial density, such as CFUs and qPCR, had been used in the preliminary work, but are not direct measures of biofilm volume/size.

There is a known clinical difficulty in assessing biofilm deep in the lung. Initially, consideration was made for repeated bronchoscopy to obtain broncho-alveolar lavage samples from different areas of the lung before, during and after treatment. This would also have enabled direct visualization of the airways. However, bronchoscopy was ruled out due to the increased risk to participants, tolerability of the procedure making the study unappealing to potential participants leading to difficulties in recruitment, as well as the feasibility and cost of staffing, equipment and the availability of a bronchoscopy suite at short notice. It would not have been possible to arrange these factors in advance, as the participants were randomized at the time of pulmonary exacerbation when admitted to hospital as an unplanned emergency. Additionally, a small study looked at sputum, broncho-alveolar lavage and bronchoscopy brushings from 12 adult patients found no increase in yield of planktonic or biofilm grown bacteria from bronchoscopy samples compared with sputum (Aaron, Kottachchi et al. 2004).

The primary endpoint selected was the quantitative microbiological assessment of biofilm in sputum and comprised of fluorescent in situ hybridization (FISH) and image analysis, quantitative PCR (qPCR) and colony forming units (CFUs). This was felt to be the most effective way to demonstrate the effect of NO on the biofilms with a small number of patients in this pilot study. The use of FISH and image analysis for assessing biofilm in response to clinical treatment have not previously been described in the literature, there was potential for a new biomarker to be developed for use in future clinical trials investigating the effects of treatment on biofilm. CFUs and qPCR were selected in order to assess the safety of NO therapy directed at dispersal of bacteria from biofilms.

2.3.2 Secondary endpoint selection

There are many clinical endpoints that have been used in trials with CF patients including number of pulmonary exacerbations, time to pulmonary exacerbation, treatment with IV antibiotics, hospitalization, growth and quality of life (QOL)(Ramsey, Pepe et al. 1999, Geller, Flume et al. 2011, Ramsey, Davies et al. 2011, Treggiari, Retsch-Bogart et al. 2011). QOL measures reflect how the patient is feeling or functioning and may have an impact on how long they survive.

True impact on clinical status will only be seen after several years of follow-up, particularly in the young patients where therapies are aimed at preventing progression of lung disease. Therefore, in many clinical trials surrogate endpoints are used as substitute measures in order to keep trials of a reasonable cost, size and duration. These clinical or laboratory measures act as a substitute for clinically meaningful endpoints, or are short term measures that can reflect long term outcomes for example when the follow-up period is short (De Gruttola, Clax et al. 2001). Surrogate endpoints that are well recognized and have been used in many CF trials include FEV₁, Computed Tomography (CT) and laboratory biomarkers such as inflammatory markers, bacterial density (CFUs) and CFTR functional measures (Geller, Flume et al. 2011, Ramsey, Davies et al. 2011, Sanders, Li et al. 2011).

In this small proof of concept study we were not expecting to see any statistically significant differences in the clinical outcomes, however, due to the potential for bacterial release with iNO therapy, they were considered important to include for safety monitoring and to inform design of any future larger studies.

2.3.3 Selected secondary endpoints

The following endpoints were selected for use in the study.

Lung Function

For this study FEV₁ was selected as an outcome measure. It is a well established predictor of survival in CF and is the only surrogate outcome measure currently accepted by the Food and Drug Administration (FDA US) for CF lung disease. It is used in almost all clinical trials of CF therapy. The two main ways to evaluate FEV₁ are at a given time point or to assess the rate of decline, which requires a larger sample size and longer duration of follow-up. The short follow-up period of this study meant that rate of decline of lung function would not be a useful measure, but evaluation of FEV₁ (and FVC) at various time points was selected for its' importance as an outcome measure and to inform design and power calculations of future larger studies.

Quality of life

QOL assessment is crucially important when evaluating the effect of treatment on patients. CF has a significant negative impact on quality of life, so any treatment that might lead to an improvement in this area would be beneficial to patients. To be able to assess significant differences between groups, a large sample size would be required, but due to the importance of this to patients, QOL was included as an endpoint in order to assess its' usefulness in this setting to inform design of future larger studies.

IV antibiotics

Length of IV antibiotic therapy was selected as an endpoint to ensure that patients receiving NO therapy did not require a longer course of antibiotics due to bacteria release from biofilms. All participants in the study received IV antibiotics due to the nature of the study therapy being delivered during a pulmonary exacerbation and IV antibiotics being required in order to treat the infection and target any dispersed bacteria as a result of the NO therapy. Length of antibiotic therapy was initially planned to be 10 days total (with ethical approval to vary this between 9-12 days), but the CF clinicians felt strongly that they wanted to be able to alter the length of course of participants antibiotics depending on their clinical status, as would usually occur during admission to hospital for pulmonary exacerbation. Hence the inclusion of length of course of antibiotics as a secondary outcome measure. The clinical

teams involved in making decisions about antibiotic courses were blinded to study therapy. In some studies, episodes of treatment with IV antibiotics are used as an outcome measure. However, use of IV antibiotics for treatment of pulmonary exacerbations is variable between clinicians and is affected by patient compliance and choice and hence is difficult to standardize for a clinical trial. A longer follow-up period is also required in order to assess further courses of IV antibiotics required by participants over a period of time. For these reasons episodes of IV antibiotic use was not suitable in this study as an outcome measure, but length of course of IV antibiotics was included.

2.3.4 Unsuitable secondary endpoints for this study

Pulmonary exacerbations

Pulmonary exacerbations are defined as “a CF related pulmonary condition requiring admission to hospital or use of home IV antibiotics” and are important clinical events. However, rate of exacerbations limits its use as an endpoint. The lower the general rate of exacerbations in the population studied, the larger the sample size needed in order to detect a small yet significant difference between the intervention and control group. Rate of exacerbation is lower in younger patients and those with better FEV₁ (Goss and Burns 2007). Long follow-up periods and large numbers of participants are required for this outcome measure to be helpful. In this pilot study the follow-up period was short (2 weeks after completing IV antibiotics) so it was highly unlikely that any participants would have another exacerbation during that time. In addition the sample size was small; therefore number of exacerbations or time to exacerbation was not a feasible clinical outcome measure to use. However, the rate of pulmonary exacerbations is related to respiratory decline in CF and therefore linking an outcome measure, such as FEV₁, to the rate of exacerbation strengthens the validity of the lung function measurement.

Hospitalisation

Hospitalization has similar drawbacks to use of IV antibiotics and is complicated by the fact that some CF patients receive their IV antibiotics at home rather than in hospital, despite having an episode which may result in hospitalisation in another patient. There are also a number of other reasons that CF patients may be hospitalised and this would affect the validity of this outcome measure aimed at

assessing respiratory status. In addition, the follow-up period was not long enough in this study for this to be a useful outcome measure.

Computed Tomography (CT)

CT is able to demonstrate lung injury before clinical signs and symptoms become apparent (Brody, Sucharew et al. 2005). It is quite widely used now as an outcome measure and radiation doses can be quite low (Tidden and Stick). However, this approach was not felt to be relevant to this study as the participants were patients with chronic PA infection and likely to demonstrate clinical signs and symptoms and hence exposing them to unnecessary radiation was not required.

Lung Clearance Index (LCI)

Lung clearance index is measured by multiple breath inert gas washout. It can demonstrate abnormal ventilation distribution in the airways. It is thought to more sensitively reflect abnormalities in the smaller airways, whereas FEV₁ reflects mainly disease in the proximal airways. As such it can detect earlier changes as a result of lung damage in CF (Davies, Cunningham et al. 2008, Davies and Bilton 2009). When this study was designed, LCI was not selected as an outcome measure as the trial site was not experienced in using this measure. In addition there have been concerns about the heterogeneity of results, especially in relation to changes seen following treatment for pulmonary exacerbations (Sonneveld, Stanojevic et al. 2015). However, recent studies have shown that LCI can predict pulmonary exacerbation in young patients who have a normal FEV₁. (Vermeulen, Proesmans et al. 2014) It is therefore a promising future surrogate outcome measure for use in clinical trials.

2.3.5 Outcome measures used in the study

Table 1 shows the final outcome measures selected for use in this proof of concept study.

Table 1 Outcome measures used in the study

Primary outcome	Between group differences in proportion of bacteria in biofilms (as determined by colony forming units, live/dead staining, direct visualisation of the biofilm and image analysis) between T0 and T20-25.
Secondary microbiological outcomes	Between group differences in proportion of bacteria in biofilms (as determined by colony forming units, live/dead staining, direct visualisation of the biofilm and image analysis) at all time points (T0, T5, T7, T10 and T20).
	Between group differences in 'change in PA colony forming units' (as determined by plate counts and intensity of qPCR) between T0, T5, T7, T10 and T20-25.
	Between group differences in whole colony CFU's at T5, T7, T10 and T20-25.
	Between group differences in NO levels in fresh sputum at T7.
	Between group differences in the characteristics of the wider microbial community as assessed by microbial community analysis/molecular analysis.
	Between individual and between group differences in the level of nitric oxide metabolites in the blood.
Secondary clinical outcomes	Between group differences in FEV ₁ at T7, T10 and T20-25.
	Between group differences in exhaled NO levels at T7.
	Between group differences in HRQOL scores at T20-25.
	Between group differences in length of course of antibiotics.

2.3.6 Defining an exacerbation

A clear definition of a pulmonary exacerbation was important for this study as this would determine when enrolled patients were eligible for randomisation.

There is no gold standard for defining a pulmonary exacerbation and there is a large diversity in the characteristics used by different clinicians in diagnosing a pulmonary exacerbation. Additionally, much of the information used to inform the decision is based on reported symptoms by the patient, which can be highly variable (Rosenfeld, Emerson et al. 2001). It is generally accepted that due to changes in treatment modalities and variation in practice amongst physicians, the definition of an exacerbation should not include the use of IV antibiotics or hospitalisation. This is because in some areas it is standard practice to give regular IV antibiotics regardless of clinical condition and in others a large proportion of patients may be managed at home when on IV antibiotics rather than admitted to hospital.

Criteria for defining a pulmonary exacerbation were published in the 1994 cystic fibrosis foundation microbiology and infectious disease consensus conference (Ramsey and Boat 1994). These have been used in a number of large trials (Aaron, Vandemheen et al. 2005) and were used in our study: The criteria include the presence of 3 of the following 11 findings in clinical status when compared to the most recent baseline visit:

- Increased cough
- Increased sputum production, change in appearance of expectorated sputum production, or both
- Fever (greater than or equal to 38 C for at least 4 hrs in a 24 hrs period) on more than one occasion in the previous week
- Weight loss greater than or equal to 1kg or 5% of bodyweight associated with anorexia and decreased dietary intake
- School or work absenteeism (due to illness) in the previous week
- Increased respiratory rate, increased work of breathing, or both
- New findings on chest examination (e.g. rales, wheezing, crackles)
- Decreased exercise tolerance
- Decrease in FEV₁ of greater than or equal to 10% from previous baseline study within the past 3 months
- Decrease in haemoglobin saturation (as measured by oximetry) from baseline value within past 3 months of greater than or equal to 10%
- New finding on chest radiograph

2.4 Inclusion and exclusion criteria

In order to detect an effect of NO on biofilms in this proof of concept study, patients needed to have sufficient PA in their sputum to be able to observe changes occurring as a result of treatment. Screening was offered to CF patients with chronic PA infection (50% or more of all cultures in the last 12 months positive for PA) (Lee, Brownlee et al. 2003). CF patients who did not have chronic PA infection were not included in this study. Teenagers producing PA able to consent for themselves were eligible for inclusion.

There were a number of factors that would make a patient unsuitable for the study, some of which are obvious, e.g. allergy to the study antibiotics. As a result of the small sample size, additional exclusion criteria were used in order to try and ensure the two groups were comparable for analysis and factors that may have an 'unknown' influence on the results were excluded e.g. colonisation with *Burkholderia cepacia*. This reduced variability in this early phase proof of concept study, with a plan to include a wider range of participants in any future larger scale studies. Some exclusion criteria were for practical reasons, such as those receiving non-invasive ventilation and those with nasal polyps who couldn't breathe through their nose, both which would have made delivery of NO via nasal cannula impossible.

2.4.1 Detailed Inclusion Criteria

- Adolescents and young adults with cystic fibrosis aged 12 or above
- Colonised with PA (confirmed on sputum sample)

2.4.2 Detailed Exclusion Criteria

- Colonisation with *Burkholderia cepacia*
- Known hypersensitivity to the antibiotics used in the study (ceftazidime and tobramycin)
- Other known contraindications to the antibiotics to be used in the study including known aminoglycoside related hearing/renal damage
- Patients requiring non-invasive ventilation (NIV)
- Patients who have a pneumothorax
- Patients who are admitted for specific treatment of non-tuberculous mycobacteria (NTM)

- Patients who cannot tolerate nasal cannula e.g. those who cannot breathe through their nose
- Patients who have nasal polyposis that is causing significant blockage of the nasal passages
- Adolescents who are 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 are pregnant (a pregnancy test was carried out for females of 11 years and above as is standard practice for clinical trials)
- 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.

2.5 Identification of potential participants

Patients aged 12 or above with CF (under the care of the CF clinicians at UHS) who were known to have chronic PA infection (50% or more of all cultures in the last 12 months positive for PA)(Lee, Brownlee et al. 2003), and regularly produced sputum, were approached about the study. They were identified using the Port CF registry database of patients within the adult and paediatric CF services and suitability for inclusion was discussed with the lead clinician before approach to ensure the research team were aware of other factors that might affect the appropriateness of inclusion in the study such as mental illness, work commitments precluding an admission to hospital (required for randomisation), needle phobia and lack of engagement by the patient with clinical services. In some cases this did not prevent the patient being given the information sheet to consider the study, as it was felt they should have the opportunity to discuss it, but it meant the research team were then aware of the likelihood that such participants (e.g. those with needle phobia) would decline to take part.

The initial contact was by a member of the clinical team either in person (at clinic or on the ward), by letter, e-mail or phone call (see Appendix 2 for letter to potential participants). They were given information sheets (see Appendix 3 for participant, parent and young person information sheets) and the study was explained to them.

The letter was written to explain the study in brief and prepare the patient for a further discussion when they attended clinic. The information sheets were much more detailed, including all the relevant information the patient would need to be able to make an informed decision about taking part in the study. Three separate information sheets were designed for adult participants, parents (who would legally need to consent to the study for their child) and young people (12-17 year olds). The young person information sheet was written in collaboration with the paediatric CF team and paediatric research nurses to ensure the suitability of the information for the age of adolescent who would be reading it. CF patients including adolescents were consulted about the information provided to ensure they felt there was enough given in order for them to be able to make an informed decision about taking part in the study.

Patients approached were given time to read the information sheets and consider the study before making a decision about whether to participate or not. If they were interested, they were given the opportunity to discuss the study in more detail with a member of the research team and ask any questions before deciding whether to take part. If the patient and/or parent agreed, they were asked to sign a consent form. 12-17 year olds were asked to give written assent in addition to parental consent.

2.5.1 Co-enrolment

Participants were not permitted to be recruited into other trials at the same time as this study. Potential participants who had participated in a trial testing a medicinal product likely to influence PA isolation (such as oral quinolones or intravenous or nebulised beta-lactams or aminoglycosides) within the 3 months prior to enrolment were ineligible for this study. Where recruitment into another trial was considered to be appropriate and not to have a detrimental effect on this study it was discussed and agreed with the Chief Investigator. This applied to one patient who had ongoing involvement in a psychological observational study that was felt to have no impact on their involvement in this study.

2.6 Informed consent process

Once a participant and/or parent had decided to take part in the study they were asked to sign a consent form to document their decision. For those under 18, their parent/legal guardian signed the consent form and they signed to document their

assent (See Appendix 4 for consent forms). All participants and parents were fully aware that they were able to withdraw their consent at any time during the study. Consent was verbally confirmed on presentation with a pulmonary exacerbation before randomisation. During the study, there were participants who had consented and screened positive, who declined to be randomised.

2.7 Screening

Once informed consent had been given, a sputum sample was collected for confirmation of PA biofilm growth. If PA could not be isolated from the sample, the participant was informed that they could not be enrolled in the study. If sufficient PA biofilm was grown from the sputum, the participant was enrolled. The participants received a phone call from a member of the research team to inform them whether they had been enrolled or not (as detailed in the participant information sheet (see appendix 3)).

A log of all potential participants was kept (screening and enrolment log). Anyone considered eligible for the study, including those who decided not to participate, or who were found unsuitable for the study, were documented.

The following assessments were performed during screening:

1. Confirmation of CF diagnosis (sweat test +/- genotype confirmation of disease causing mutations).
2. Confirmation of chronic PA infection (50% or more of all cultures in the last 12 months positive for PA).
3. Confirmation of fulfilment of inclusion/exclusion criteria.
4. Obtaining written informed consent.

Once enrolled, the participant and the participant's CF physician were asked to contact the research team if they presented with an acute exacerbation requiring IV antibiotic therapy. Participants were given a contact card with a 24 hour telephone number and a member of the research team attended daily handover in the CF department to identify any enrolled participants who might be admitted when exacerbating. Once a participant was experiencing a pulmonary exacerbation, consent was confirmed, and if the participant agreed, they were randomised to receive either inhaled nitric oxide therapy or placebo in addition to standard antibiotic therapy.

2.8 Randomisation

Eligible participants were randomised once they were admitted to hospital with a pulmonary exacerbation requiring IV antibiotic therapy.

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 and that the two groups were well balanced. The randomisation list was run by an online randomisation service (Tenlea) using a 24 hour web-based randomisation service. The strategy for randomisation was only known to the study statistician who set up the online randomisation service at the time of the study. The research trials team were unaware of the randomisation strategy until after the trial had ended.

An emergency telephone randomisation service was available for use should a temporary problem have been experienced with the web based randomisation system, however, this was not required during the study.

2.9 Blinding

Laboratory staff carrying out microbiological endpoint evaluation (including primary endpoint) were blinded to which therapy the participant had received. Medical staff were also blind to treatment allocation, but this was not specified in the regulatory approvals, so that in the event of it being necessary to reveal treatment allocation for clinical reasons, it did not result in a breach of protocol. However, to reduce bias in results and reporting the intention was for medical staff to remain blinded and this was achieved. Blinding of nursing staff was uneconomical for this pilot study without additional scientific benefit, but would be considered for future larger randomised controlled trials.

Participants were blinded to therapy by the use of an NO delivery system (INOvent) with a blinding screen so that they did not know whether they were breathing NO or placebo. As well as to avoid bias, blinding was important to avoid the risk of participants deciding to discontinue in the study if they were randomised to placebo rather than the treatment group.

2.10 Treatment

2.10.1 Introduction

12 enrolled participants who had a pulmonary exacerbation were randomised to one of the following groups:

Group A received inhaled nitric oxide therapy, 8 hours continuous therapy through nasal cannula overnight in every 24 hour period for 5-7 nights.

Group B received inhaled placebo (air or air/oxygen blend), 8 hours continuous placebo through nasal cannula in every 24 hour period for 5-7 nights.

Both groups also received standard IV antibiotic therapy of tobramycin and ceftazidime for 9-12 days.

The protocol plan had been to randomise 20 participants, 10 into each group, however, due to factors that limited recruitment to the study, a total of 12 randomised participants was achieved during the pre-defined study period and with the funding available. This change to the planned recruitment and the difficulties encountered is discussed in further detail in chapter 6.

The decision to interrupt or discontinue trial treatment was at the discretion of the treating clinician. Therapy could be interrupted or discontinued at any time during the trial period for reasons such as unacceptable adverse events, intercurrent illness, development of a serious disease or any change in the participants' condition that the clinician believed warranted a change in medication, however, this was not necessary for any participant.

2.10.2 Nitric Oxide Dosage and administration

Nitric Oxide for the study was manufactured by INO Therapeutics AB in Sweden and supplied in the UK by INO Therapeutics UK (Kent Science Park, Sittingbourne, Kent, ME9 8PX). The finished product name was Inomax 400 ppm mol/mol inhalation gas and the active ingredient Nitric Oxide (NO) 400 ppm mol/mol. It was supplied in a 10 litre aluminium gas cylinder (identification with aquamarine shoulder and white body) filled under a pressure of 155 bar, equipped with a stainless steel positive pressure (residual) valve with a specific outlet connection and has a shelf life of 2 years.

Nitric oxide was given at a dose of 10 ppm and delivered to the participant via nasal cannula after dilution with an oxygen/air mixture using an approved (CE marked) Nitric Oxide delivery system (INOvent). The inspired INOmax concentration was measured continuously in the inspiratory limb of the circuit near the participant. The nitrogen dioxide (NO₂) concentration and inspired oxygen (FiO₂) concentration were also measured at the same site using calibrated and approved (CE marked) monitoring equipment. For participant safety, appropriate alarms were set for INOmax (± 2 ppm of the prescribed dose), NO₂ (1 ppm) and FiO₂ (± 0.05). The INOmax gas cylinder pressure was displayed allowing timely gas cylinder replacement without inadvertent loss of therapy. Backup gas cylinders were available at all times. All staff involved in administering NO to participants received training in administration from a company representative. This covered correct set-up and connections, operation and monitoring.

Nitric oxide was given via nasal cannula. The NO was added to air or air/oxygen mix – according to clinical need (set on the air/O₂ blender) and run at approximately 4 L/min. This allowed adequate mixing of NO before delivery to the participant.

Once connected to the NO, participants received 8 hours of uninterrupted flow. If a participant needed to be removed from the NO at any stage then the weaning protocol was implemented (see Appendix 5 study guidance: weaning of NO). NO must be weaned before stopping, as there is a theoretical risk of increased pulmonary hypertension and reduced oxygenation when the NO is stopped suddenly. This data is based on neonates with significant pulmonary hypertension and poor oxygenation receiving doses around 20 ppm. This was thought to be unlikely to cause any significant problems in the population at the dose of 10 ppm, however as it was a clinical trial, the manufacturers guidelines regarding weaning were followed. Participants in the placebo group had “sham” adjustments made to the NO/placebo delivery device at time points equivalent to weaning doses to maintain participant blinding.

Use of NO or placebo was recorded on medical gas cylinder accountability records completed by trial staff. All trial treatment was administered during hospital inpatient visits which ensured accurate monitoring of IMP compliance.

Originally, it had been planned for all participants to receive 7 days of inhaled therapy, however, a substantial amendment was submitted to ethics and approved before the study had started to allow participants to complete a minimum of 5 days of inhaled therapy up to an ideal of 7 days. This was to allow participants who were well enough to be discharged, after 5 days of IV antibiotics, to be able to go home. The T7 assessment (end of inhaled therapy) was then performed at that point (T5-7).

The upper limit of exposure (mean exposure) to nitric oxide for personnel defined by worker's legislation is 25ppm for 8 hours (30mg/m³) and the corresponding limit for NO₂ is 2-3ppm (4-6mg/m³). The concentrations used in this study did not result in any over exposure to research staff.

2.10.3 Placebo

Participants randomised to the placebo arm of the trial received air or 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 were receiving the trial treatment or placebo, including sham weaning procedures. The use of a placebo was felt important in this study to minimize bias. There was no additional risk to participants receiving the placebo (Miller, Wendler et al. 2003).

2.10.4 Antibiotic therapy

Intravenous tobramycin and ceftazidime were used in line with normal clinical care in this study as Non Investigational Medicinal Products (NIMP) in accordance with EU directive 2001/20/EC Article 13 and 14. Tobramycin was given at a dose of 7.5 mg/kg once a day (as a 30 min infusion) for 10 days. Ceftazidime was given at a dose of 2 g three times a day (for adults) and 50 mg/kg three times a day for children (maximum 2 g) for 10 days. Tobramycin levels were monitored according to standard clinical practice. The first dose of IV antibiotics was given before treatment with the IMP was commenced.

The majority of treatment was administered during hospital visits which ensured accurate monitoring of NIMP compliance. For the adult participants who usually administer their own IV antibiotics at home, they were allowed to continue with this (after the 5-7 days of inhaled therapy had ended) and were asked to complete a

diary of dose and time administered and return empty vials at each visit to ensure compliance with the NIMP. As per standard clinical practice, IV antibiotic therapy was reviewed daily by the CF clinician in charge of the participant's clinical care. If the consultant felt at any time that the participant required a change of antibiotics for clinical reasons this was permitted and documented in the participants CRF. This did not affect treatment with the IMP.

2.10.5 Other concomitant treatments

Nebulised antibiotics were continued while participants were receiving IV antibiotics (as is standard clinical practice). These were recorded in the CRF.

All other treatments (including fluids) were given at the discretion of the lead clinician in accordance with standard inpatient management of cystic fibrosis. The name and dose of all concomitant medications was documented on the CRF throughout the study. All participants had twice daily physiotherapy regimes whilst on IV antibiotics. This is standard procedure for CF participants, especially during a pulmonary exacerbation.

2.11 Study procedures

Samples were collected from the participants during the 10 day period of IV antibiotic therapy as detailed in table 2 (study procedures). Additionally, participants were asked to attend a follow-up visit 10-15 days after completing their IV antibiotics for further samples and clinical evaluation.

Table 2 Study procedures

Time	Screening	Randomisation (baseline assessment) T 0	During NO therapy T 5	7 days (end of NO therapy) T 7	10 days (end of IV antibiotic therapy) T10	Follow-up (10-15 days after end of IVs) T 20
Eligibility assessment and informed consent	x	Consent confirmed				
Sputum for microbiology	x	x	x	x	x	x
Clinical assessment	x	x		x	x	x
Bloods		x	x x		x	x
Lung function		x		x	x	x
Exhaled nitric oxide measurements		x	x	x	x	x
HRQOL		x				x
Sputum for storage		x	x	x	x	x
Randomisation		x				
NO levels in sputum		x	x	x		
Additional optional procedures (if consent given)						
Nasal examination		x				
Nasal brushings			x			

2.11.1 Sputum

Sputum was collected as detailed in the study procedures table (see table 2). It was collected in a sterile universal container and labelled with the participants' unique study number and initials as well as sample time point (e.g. T0/T7 etc.). Transport of sputum samples between departments/buildings/labs took place using appropriate secure/safe carriers according to standard operating procedures for transport of microbiological specimens within the Southampton Centre for Biomedical Research.

2.11.2 Blood

In order to investigate the inflammatory pathways and observe any possible side effects of NO therapy, blood samples were obtained at time points T0, T10 and follow-up/T20-25. Only a small sample of blood (10 ml) was required on these three

occasions, designed to coincide with times that participants would usually undergo blood sampling as part of routine clinical care. These volumes of blood were minimal and would not result in any clinical harm. If blood sampling was particularly difficult or became distressing for the participant, the research samples were not obtained and clinically relevant samples referred back to the clinical team to decide on the importance. For safety monitoring and assessment of NO uptake by the lungs, 2 additional blood samples were taken during the first night of inhaled therapy at 1 and 7 hours after starting treatment. Less than 5 ml was required for these 2 samples.

2.12 Laboratory data and methods

Sputum was collected as detailed above for analysis of the primary outcome measure. This included fluorescent in situ hybridisation (FISH) analysis, colony forming units (CFUs) and quantitative PCR (qPCR). Sputum was also used to investigate NO levels in fresh sputum. Blood was used for analysis of safety measures, levels of NO metabolites and other inflammatory markers. Sample processing and laboratory analysis was carried out by Dr Rob Howlin and team as detailed in Appendix 6.

2.12.1 Estimation of NO uptake by lungs

NO levels in sputum

A Unisense Nitric Oxide probe was set up to analyse the amount of NO in sputum samples immediately after ending 8 hours of NO therapy (or placebo) in order to try and assess whether the levels of NO in the sputum were increased as a result of therapy. This method was recognised as potentially flawed, as the diffusion time of NO out of sputum is not known. It was possible that, because NO is so reactive, any NO in the sputum would have dispersed before analysis. However, it was included as an outcome measure to see if any exploratory data could be gathered.

NO metabolites in blood

Analysis of NO metabolites in blood was carried out by Prof Martin Feelisch and Dr Bernadette Fernandez. 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 800xg; 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 (Bryan, Rassaf et al. 2004, Bryan, Rassaf et al. 2004, Levett, Fernandez et al. 2011). 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) (Bryan, Rassaf et al. 2004), and generated NO was quantified by gas phase chemiluminescence as above.

2.13 Clinical data and methods

Clinical outcomes were not suitable as the primary outcome measure for this proof of concept study due to small numbers of participants and a short follow-up period. However, they were important in order to assess the safety of the treatment and to help inform design of any future larger studies.

2.13.1 Lung Function

Forced Expiratory Volume in 1 second (FEV₁) is a well-established predictor of survival and the most widely used surrogate outcome measure for CF lung disease (Gibson, Burns et al. 2003). There are a number of reasons for this, including the established correlation to longer term outcomes, widely available equipment with standardised procedures and accepted standards. Hence, it is reliable and inter and intra operator variability is reduced (Ramsey, Pepe et al. 1999, Miller, Crapo et al. 2005). It was not expected in this pilot study (with a small sample size) that any significant differences in FEV₁ would be detected between the 2 groups, but it was important to include it as a secondary outcome measure a) for safety monitoring and b) so that any differences between the 2 groups could be used for statistical power calculations to estimate sample size in a future large phase 3 randomised controlled trial (RCT).

During routine treatment of pulmonary exacerbations in patients with CF, lung function is measured on admission and completion of IV antibiotics (as a marker of

success of treatment). 2 additional measurements of lung function were carried out as part of the study.

Lung function testing was carried out using the Koko spirometer (see figure 10) and portable laptop computer. This enabled bedside testing for CF patients who were all confined to cubicles during their inpatient stay. This spirometer is widely used in clinical practice and research studies and used frequently in the department where the research was conducted. The nurses using the spirometer underwent the appropriate training and were using it frequently to maintain their skills. They followed the standard operating procedure within the Biomedical Research Unit for the use of this spirometer.



Figure 10 Koko spirometer and portable laptop

Measurements were made at time points T0, T7, T10 and T20 and these included 3x FEV₁ and 3x FVC. The 'best' value was taken for analysis as is common practice in both clinical work and research studies. The percentage predicted value was also recorded. Percentage predicted FEV₁ or FVC allows comparison of changes in measurements between patients of different ages and heights and is often used in reporting of clinical trials and in clinical practice.

2.13.2 Quality of life

This was assessed using the Cystic Fibrosis Questionnaire (CFQ-UK). This is a disease specific, developmentally appropriate, quality of life (QOL) questionnaire designed to measure the physical, emotional and social impact of CF on participants and their families. There is a growing recognition that the measurement of health-related QOL (HRQOL) provides unique information about the impact of an illness and the effectiveness of current and newly developed treatments. This is

particularly true of chronic illnesses like CF, where an important goal for treatment is to improve daily functioning and participant well-being. Although more conventional measures of efficacy (such as lung function) are important, often they do not fully gauge the broader impact of the disease on the participant's physical, social and psychological functioning.

There are different versions of the CFQ-UK questionnaire for different ages; for children aged 12-13 and for teen/adults (14 years or older). Both versions are completed by the participant with no input from parents/family. They are scored according to the CFQ-UK scoring system (Quittner, Sweeny et al. 2000). The questions refer to the previous 2 weeks, so subjects were asked to complete the questionnaire when treatment for a pulmonary exacerbation was initiated (at randomisation, T0) and at the follow-up visit (T20-25). This allowed comparison of scores before and after treatment between groups (see Appendix 7 for full versions of the CFQ-UK).

Participants completed the questionnaire on their own with no external input at the start of the study period (T0 - time of exacerbation) and at follow-up (T20-25). Researchers were available to answer any specific queries the participants had regarding the completion of the questionnaire, but were not permitted to be involved in the answering of any questions. The CFQ-UK is an easily understandable questionnaire and no participants required input from researchers to achieve completion. The questionnaire comes with software for entry of the data and compilation of scores (higher scores indicate a better quality of life). The scores were then exported into the main study dataset for analysis.

2.13.3 Length of antibiotic treatment

Most participants received 7-10 days IV antibiotics, as per standard clinical practice, however, if their CF clinician felt at the end of 10 days they needed further treatment, they were able to extend the course of antibiotics, or change the antibiotics if necessary.

For all participants the length of course of antibiotics was recorded on the CRF as well as any changes in antibiotic therapy. This was to assess whether NO had any impact on length of antibiotic course (as determined by the clinicians looking after the patient who were blinded to therapy). It was also an important safety measure to

ensure those receiving NO did not require longer courses of antibiotics compared to those who received placebo.

2.13.4 Exhaled Nitric Oxide levels

Participants with CF are known to have reduced exhaled nitric oxide levels; this has been shown in a number of studies (Balfour-Lynn, Lavery et al. 1996, Dotsch, Demirakca et al. 1996, Lundberg, Nordvall et al. 1996, Grasemann, Michler et al. 1997). The aim of administering low dose iNO in this study was to increase airway NO levels to cause biofilm dispersal. Exhaled NO levels were measured at T0 and T7 to assess effect of treatment on increasing eNO. Exhaled nitric oxide levels were measured using a Niox Mino (see figure 11).



Figure 11 Niox mino machine for exhaled nitric oxide measurement

The Niox mino was familiar to staff as it is used regularly within the department. All staff were trained and followed the standard operating procedure for this piece of equipment within the Biomedical Research Unit. Two readings were taken and an average of the two recorded on the case report form (CRF).

2.14 Monitoring

Participants were monitored closely during the administration of inhaled therapy to assess safety and tolerability of therapy as documented in Table 3.

Table 3 Monitoring during inhaled therapy

Parameter	Method of monitoring	Frequency	Recorded on chart	Action to be taken
Heart rate	3 point ECG/cardiac monitor	Continuous monitoring	Every 10 mins for first and last 30 minutes of therapy (weaning) each day. Hourly for rest of the 8 hours.	Referred to study guidance for weaning NO for changes during the last 30 minutes of therapy.
Oxygen saturations	Pulse oximetry on finger or toe	Continuous monitoring	Every 10 mins for first and last 30 minutes of therapy (weaning) each day. Hourly for rest of the 8 hours.	Paediatric participants: increased O ₂ if sats <92% for more than 5 minutes continuously or <85% for over 30 seconds continuously. Adult participants: referred to individual guidance for each patient (provided by the clinical team)
Respiratory Rate	Observation and manual counting by staff	Every 10 minutes for the first and last 30 minutes of therapy each day. Once after 4 hours of therapy.	Every 10 minutes for the first and last 30 minutes of therapy (weaning) each day. Once after 4 hours of therapy.	Referred to study guidance for weaning NO for changes during the last 30 minutes of therapy.
Blood pressure	Automated or manual BP cuff	Every 10 minutes for the first and last 30 minutes of therapy each day. Once after 4 hours of therapy.	Every 10 minutes for the first and last 30 minutes of therapy (weaning) each day. Once after 4 hours of therapy.	Referred to study guidance for weaning NO for changes during the last 30 minutes of therapy.
metHb levels	Blood gas sample from finger prick or venous sample (from central line), taken to gas machine in ITU/PICU	At 1 hour following the start of NO therapy on the first night and just before weaning (after 7 hours of therapy).	At 1 hour following the start of NO therapy on the first night and just before weaning (after 7 hours of therapy).	If levels were <5% then there was no need for further monitoring of metHb after the first period of therapy. If metHb levels were >5% then the NO dose was halved and the levels checked again after 1 hour (this did not occur in any participant). If the levels were to remain >5% then NO was weaned and stopped (according to the study guidance: weaning of NO). If levels of metHb continued to rise, despite stopping the NO, then treatment for methaemaglobinaemia was considered. Staff were advised to refer to the SPC and standard management in this event; however this did not occur during the study.
Inspired oxygen concentration	Read off NO machine	hourly	hourly	
NO level	Read off NO machine ('sham' recording if in placebo group)*	hourly	hourly	Minor fluctuations in NO concentration (<2ppm) were acceptable. If large fluctuations (>2ppm) occurred staff referred to the INOvent operating manual and performed checks and if necessary contacted customer support, this did not occur during the study.
NO₂ level	Read off NO machine ('sham' recording if in placebo group)*	hourly	hourly	Minor fluctuations in NO ₂ concentration (anything <1ppm) were acceptable. If large fluctuations occurred (>1ppm) staff referred to the INOvent operating manual and performed checks and if necessary contacted customer support. If NO ₂ levels were raised above 1ppm then the NO dose was halved. This did not occur during the study.

2.14.1 Methaemaglobin monitoring

iNO therapy has been reported to increase methaemaglobin (metHb) in neonates where the use of much higher iNO doses up to 80ppm led to an increase in metHb above 5% in a minority of cases. However, this recovered as soon as the concentration of NO was reduced (Group 1997, Clark, Kueser et al. 2000).

Raised levels of metHb were not expected due to the low dose (10ppm) used in the study, however levels were monitored at 1 and 7 hours after the start of therapy during the first 8 hour period of treatment to monitor for safety. Levels of methaemaglobin (metHb) were measured as shown in Table 3. Normal levels of metHb are <1% (higher in smokers). Symptoms from methaemaglobinaemia are very rare below 5%. Methaemaglobinaemia results in a reduced ability of the red blood cells to release oxygen to the tissues and therefore leads to tissue hypoxia and cyanosis. There is an increased risk of methaemaglobin formation if substances with a known tendency to increase methaemaglobin concentrations are administered concomitantly with NO (e.g. alkyl nitrates and sulphonamides). Prilocaine, whether administered as oral, parenteral or topical formulations (EMLA cream) may cause methaemaglobinaemia therefore such products were not used during this trial (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000337/WC500032784.pdf INOtheapeutics 2006).

2.15 Clinical Data Collection

Data was collected on all participants through the course of the study. Screened patients had basic data collected on a screening Case Report Form (CRF). Those who were randomised had more detailed data recorded including demographic data, clinical data; including outcome measures, laboratory results and monitoring data, as well as information regarding concomitant therapies, duration of antibiotic treatment and monitoring during inhaled therapy. The data was collected on a Case Report Form (CRF from randomisation) that included the participant's individual study number and initials, but was otherwise anonymised. See Appendix 8 for both CRFs.

2.16 Withdrawal of participants

Participants could be withdrawn from the study for any of the following reasons:

1. Participant or parent withdrew consent.
2. Any change in clinical condition preventing further treatment.
3. Unacceptable toxicity measured by metHb at 1 hour and after 7 hours continuous therapy.
4. Any change in the participant's condition that justified the discontinuation of treatment in the clinician's opinion.

Participants were free to withdraw their consent at any time without having to provide a reason. If a participant wanted to withdraw from the trial treatment but not sample follow-up, then further microbiological analysis would have been carried out and analysed as a separate group. General follow-up would also have continued unless the participant also withdrew consent for follow-up. Participants who withdrew completely from the trial would have had anonymised data collected up to the point of withdrawal included in the analyses. Analysis would have been carried out on an intention to treat basis. No patients withdrew from the study after randomisation. 2 patients who had screened positive and been enrolled into the study withdrew before randomisation, and did not to proceed to randomisation when they were admitted with a pulmonary exacerbation.

2.17 Statistical considerations

2.17.1 Method of randomisation

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. The randomisation list was run by an online randomisation service (Tenlea) using a 24 hour web-based randomisation service. The randomisation strategy was not known to the trial team during the study. It was set up by the trial statistician.

An emergency telephone randomisation service was available for use should a temporary problem have been experienced with the web based randomisation system, however, this was not required during the study.

2.17.2 Sample size

Consideration was given to sample size in order to be able to show significance if a difference between the groups was found. A power calculation was attempted,

however, there was a lack of suitable preliminary data to complete this accurately and hence a decision was made to look for trends in the data instead. In addition, data was collected in this study to allow for an accurate power calculation in advance of any further studies looking at the use of NO in CF. A feasible sample size of 20 participants (10 in each group) was selected and felt to be adequate to show trends of differences between the groups. It was felt that it would be possible to enrol enough participants (30-40 estimated) to lead to randomisation of 20 (at the time of pulmonary exacerbation) in a year, the approximate time available to conduct the study due to staff and physical resources. Unfortunately, as will be discussed in detail in chapter 6, a total of only 12 participants were randomised into the study.

In order to demonstrate that the treatment with NO was better than the control the probability of observing the correct ordering of the proportion of bacteria in biofilms for each group was calculated, for a given sample size and effect size (estimated taking into account the results observed from the laboratory experiments) (Piantadosi 2005).

It was estimated that the proportion of bacteria in biofilms in the patients treated would be 0.7 for placebo and 0.4 for patients treated with NO (based on a priori discussion). A sample size of 10 participants in each treatment group was felt to be sufficient to determine that the NO treatment arm was superior to the control group (by reducing the proportion of bacteria) with 90% probability assuming a change from 0.7 to 0.4 (Piantadosi 2005).

It was recognised that this study would have limited ability to detect important but rare treatment-related adverse events which would be identified in a future larger RCT.

2.17.3 Analysis

The primary analysis was carried out on an intention-to-treat basis including all randomised participants. The change in proportion of bacteria in biofilms between baseline and follow-up (T20-25) was calculated for each treatment group, and the mean difference of this change between the groups was estimated with 95% confidence intervals. This analysis was then repeated for day 5, 7 and 10. Further analysis of secondary outcomes comparing the two treatment groups consisted of

estimating the difference in means (where appropriate) with 95% confidence intervals. The use of appropriate data transformations (such as log transformation) was individually assessed for each endpoint. The treatment effect for the microbiological outcomes FISH, CFU and q-PCR was estimated by calculating the mean difference between groups of the change from baseline on the natural log scale. To make full use of repeated measurements of outcomes over time, the mean difference between groups was also estimated by conducting linear regression using generalized estimating equations (GEE) analysis (Zeger and Liang 1986). GEE models estimate average treatment effect while accounting for longitudinal dependence. Residuals were examined to assess model assumptions. Analyses were performed in Stata software, version 11 by Dr Victoria Cornelius (study statistician).

2.18 Assessment of Safety

Participants who had entered this study were monitored closely for safety reasons, and to be able to alert the co-investigators and local ethics committee about any event that seemed unusual, whether the event was considered an unanticipated benefit or harm to the participant. Assessment of safety of trial participants was carried out using standard procedures for reporting adverse events and reactions according to the principles of Good Clinical Practice for CTIMPs. Full details are described in the study protocol in Appendix 9.

2.19 Quality control and quality assurance

This study was conducted in accordance with The Medicine for Human Use (Clinical Trial) Amendment Regulations 2006 and subsequent amendments; the International Conference for Harmonisation of Good Clinical Practice (ICH GCP) guidelines and the Research Governance Framework for Health and Social Care. The study was monitored and audited in accordance with University Hospital Southampton (UHS) NHS Foundation Trust procedures. Full details are included in the study protocol in Appendix 9.

2.20 Ethical considerations

This study was performed with full Research Ethics Committee (REC) and local Research and Development (R&D) approval. In particular, the following ethical issues specific to this study were considered.

2.20.1 The use of minors in a Controlled trial of an Investigational Medicinal Product (CTIMP)

The inclusion of children aged 12 or over in this study was considered and felt essential. For lung diseases that start in childhood, there may be windows of opportunity in which intervention with novel therapeutic options may reduce or prevent progression to chronic lung infection or inflammation that might otherwise occur. Chronic PA infection in the lungs of participants with CF becomes more problematic the older the participant. If the use of nitric oxide was found to be beneficial to participants it could become a key new therapy for children to try and prevent the lung damage caused by chronic PA infection.

2.20.2 Confidentiality

The study required trained researchers not directly involved with the participants' medical care to have access to confidential information. It was necessary to gain consent from the participant and/or their parents (if they were <18 years) to link anonymised clinical information with the laboratory data generated. This was done by using study numbers to identify specimens and clinical data.

2.20.3 Risk to participants

As this was a trial of an Investigational Medicinal Product (IMP) there were some unknown risks to the participants. This new use of NO was outside the current license for NO gas and therefore the exact risks were unknown. However, at the low doses used, there were no anticipated significant side effects or increased risk to participants. Participants were monitored closely to identify any potential problems, side effects or additional risk.

2.20.4 Informed consent process

Informed consent is a process initiated prior to an individual agreeing to participate in a study and continues throughout that individual's participation. Informed consent is a requirement for all participants participating in a CTIMP. During the informed

consent process, the investigator or their delegate must comply with all the applicable regulatory requirements and adhere to Good Clinical Practice (GCP) and to ethical principles that have their origin in the Declaration of Helsinki.

Patients and/or parents were given the opportunity to discuss the objectives, risks and inconveniences of the study. Staff with experience in obtaining informed consent explained to potential participants the details of how the study was going to be conducted. Age appropriate information sheets were provided to participants and/or their parents. These described in detail the study interventions, procedures and risks. Once the individual had had time to read the document there was opportunity for the investigator to explain the study to the individual. At all times staff emphasised that participation in the study was completely voluntary and that the participant could withdraw from the study at any time and for any reason. (See Appendix 3 for information sheets).

All potential participants were given the opportunity to ask questions and had time (ideally 24 hours or longer) to consider the information they have received and discuss it with their family prior to consent being given. All participants were provided with the appropriate contact details for further information about the trial.

If, after these discussions, the patient and/or parent were happy to take part, they were asked to sign and date the informed consent document. This was signed and dated by both the participant and the person receiving consent. The participant and/or parent were given a copy of the consent form to keep for their own records. The original was filed in the participant's clinical notes and another copy was kept in the Investigator Site File (see Appendix 4 for consent forms).

Assent in minors

All children aged 12-17 were asked to give their assent to the study in addition to parental consent. They were approached by a member of the research team with experience in paediatrics and communicating with children. They received age-appropriate information regarding the study, procedures and risks, and had the opportunity to have any questions answered. If they agreed to take part, they were then asked to write/sign their name on the consent form, which was signed by their parent and the researcher. Children aged 12 and above were approached so that they could fully understand the study themselves and give informed assent to take part.

2.21 Data handling and record keeping

Participants were identified by their trial number to ensure confidentiality. All electronic and personal data was stored in accordance with the Data Protection Act 1998. Data handling and record keeping was carried out in accordance with the standards set out in Good Clinical Practice for CTIMPs and detailed in the study protocol (see Appendix 9).

2.22 Financial and insurance matters

Details about participant payment and indemnity are included in the study protocol in Appendix 9.

Chapter 3: Image Analysis Methods

3.1 COMSTAT

In the preliminary study (see introduction) COMSTAT was the image analysis programme used by Dr Rob Howlin for quantification of biofilm. However, as will be discussed below, this was not found to be suitable for the clinical study analysis which required the use of Fiji. The work described in this chapter is the authors own and was carried out as part of this thesis. Problems described with COMSTAT were discovered during the analysis of the trial data and an alternative image analysis programme was then sought as detailed below.

3.1.1 COMSTAT methods and problems

COMSTAT was designed by Arne Heydorn as a script in MATLAB 5.1 (MathWorks). COMSTAT comprises ten features that can quantify 3 dimensional image stacks (Heydorn, Nielsen et al. 2000). (The programme is available free of charge from the COMSTAT homepage at <http://www.im.dtu.dk/comstat>).

Heydorn et al reported the first description of the use of COMSTAT in the literature (Heydorn, Nielsen et al. 2000). Pseudomonal biofilms were grown in flow cells and imaged using a Confocal Scanning Laser Microscope (CLSM).

COMSTAT has 10 possible functions for measuring various properties of the biofilm.

1. Bio-volume

Total biomass (pixels) in the stack, multiplied by the voxel size (biomass volume) and divided by the area of the substratum. This value can represent the total biovolume of the biofilm and can be an estimate of biofilm biomass.

2. Area occupied by bacteria in each layer

This is the proportion of each image in the stack that is occupied by biofilm. The most useful of these being the substratum layer demonstrating how well the bacteria have colonised the substratum.

3. Thickness distribution

This looks at the highest point above each pixel in the base layer and can be used to look at biofilm roughness and mean thickness.

4. Mean thickness

This is a measure of the size of the biofilm and is the most commonly reported in the biofilm literature. It is based on calculation from thickness distribution above.

5. Identification and area distribution of micro-colonies in the substratum

This identifies micro-colonies that are in the substratum. Only micro-colonies above a certain size (as decided by the user) are measured. This function looks at the total number of colonies, area of each colony and mean colony area.

6. Volumes of micro-colonies identified in the substratum

Looks at the volume of colonies counted above.

7. Fractal dimension

Calculated using 2 different methods, the Minkowsky sausage method or the cross correlation function method.

8. Roughness coefficient

Calculated using the thickness distribution and provides an idea of the variability in the thickness of the biofilm indicating its heterogeneity.

9. Distribution of diffusion distances, average and maximum diffusion distance

The diffusion distance for a pixel in the biofilm is the minimum distance to a non-biofilm containing pixel. The average diffusion distance is calculated from all the diffusion distances throughout the biofilm and maximum is the furthest of the diffusion distances within the biofilm. These can give an indicator of the distance that nutrients have to diffuse to reach bacteria in the micro-colony.

10. Surface to volume ratio

The surface is identified as pixels where at least one neighbour is a background pixel. This calculates surfaces that are exposed to nutrient. Surface to volume ratio is calculated from the surface area divided by the biovolume, this shows what proportion of the biofilm is exposed to nutrient.

COMSTAT analyses image stacks from the substratum. A threshold is applied by the user which sets the level of fluorescence of the image above which the programme will count a value of **one**. Once the threshold has been set, the image stack becomes a 3D matrix with pixels containing either the value **one** or **zero**. Anything originally above or equal to the set threshold value (and therefore assumed to be biofilm biomass) is counted as **one** and anything below this is counted as **zero** (assumed to be the background). There is no automatic way to threshold the images therefore this has to be done manually. This can lead to operator bias and between user variability, particularly if the image stacks have

been acquired by different users of the CLSM as this can result in variability of the background grey-level pixel value.

In COMSTAT after the image stack has been thresholded, the programme removes any biomass pixels that are not connected to the substratum. This is done by starting at the substratum layer and moving through the stack of images to find connected pixels. Any pixels that aren't identified as connected are then set to **zero**.

COMSTAT was used to analyse the laboratory work from the preliminary study (results described in the introduction). This produced good results in this group of highly selective sputum samples from patients with a high growth of PA and showed a positive effect on treatment of PA biofilms with NO.

Initially COMSTAT was used to analyse the clinical study results. However, a number of problems were encountered (work done as part of this thesis). The task of preparing all the files and thresholding each image stack was laborious and required a second observer to check the threshold values to ensure reliability and reduce bias. Overall 20% of images were checked (120 stacks) which demonstrated that thresholding was highly subjective and could not be reproduced reliably. Table 4 shows the threshold set by 2 different users for the images. It shows clearly that selection of thresholds for the images was not reproducible. It highlighted the difficulty in setting a threshold to exclude background noise in the sample, but still pick up all fluorescing bacteria in microcolonies.

Table 4 Thresholds set by 2 observers independently in COMSTAT

Observer 1	Observer 2	Observer 1	Observer 2	Observer 1	Observer 2
100	90	150	140	240	140
120	110	80	140	240	140
70	110	100	160	240	140
100	110	100	160	250	160
80	120	100	160	200	140
60	100	150	160	200	160
200	150	130	150	200	160
150	130	140	160	200	160
200	150	100	130	180	140
200	160	140	150	180	140
200	150	150	160	200	140
200	150	150	160	200	140
250	160	200	170	220	140
250	170	170	160	200	140
250	150	170	180	100	55
200	160	150	160	100	60
100	150	200	160	80	40
100	120	200	170	80	90
100	130	80	160	100	120
100	110	100	160	100	80
100	140	100	130	100	65
120	140	100	130	100	60
220	140	100	110	60	80
250	180	120	95	100	60
150	180	100	80	120	80
150	180	100	130	100	70
150	190	120	130	70	120
150	180	200	140	100	120
200	180	120	130	100	110
150	160	240	140	100	130
150	120	240	200	100	115
50	120	20	40	40	60
40	60	50	80	20	60
40	45	50	40		

Figure 12 shows a representative image that demonstrates the background, single cells and colonies. Achieving this balance reliably and reproducibly was very difficult as shown by the big differences between thresholds selected by 2 independent observers in table 4. As a result, calculation of values relating to the size/volume of the biofilm were thrown into question because these calculations are all based on the initial threshold set. In the pre-clinical studies thresholds had been set by one observer only, however, for robust analysis of the results from the clinical trial a second observer had independently selected thresholds for 20% of image stacks. The aim had been to show reproducibility and agreement between the observers, but as shown, this did not occur, and therefore a new image analysis method was sought for the trial results.

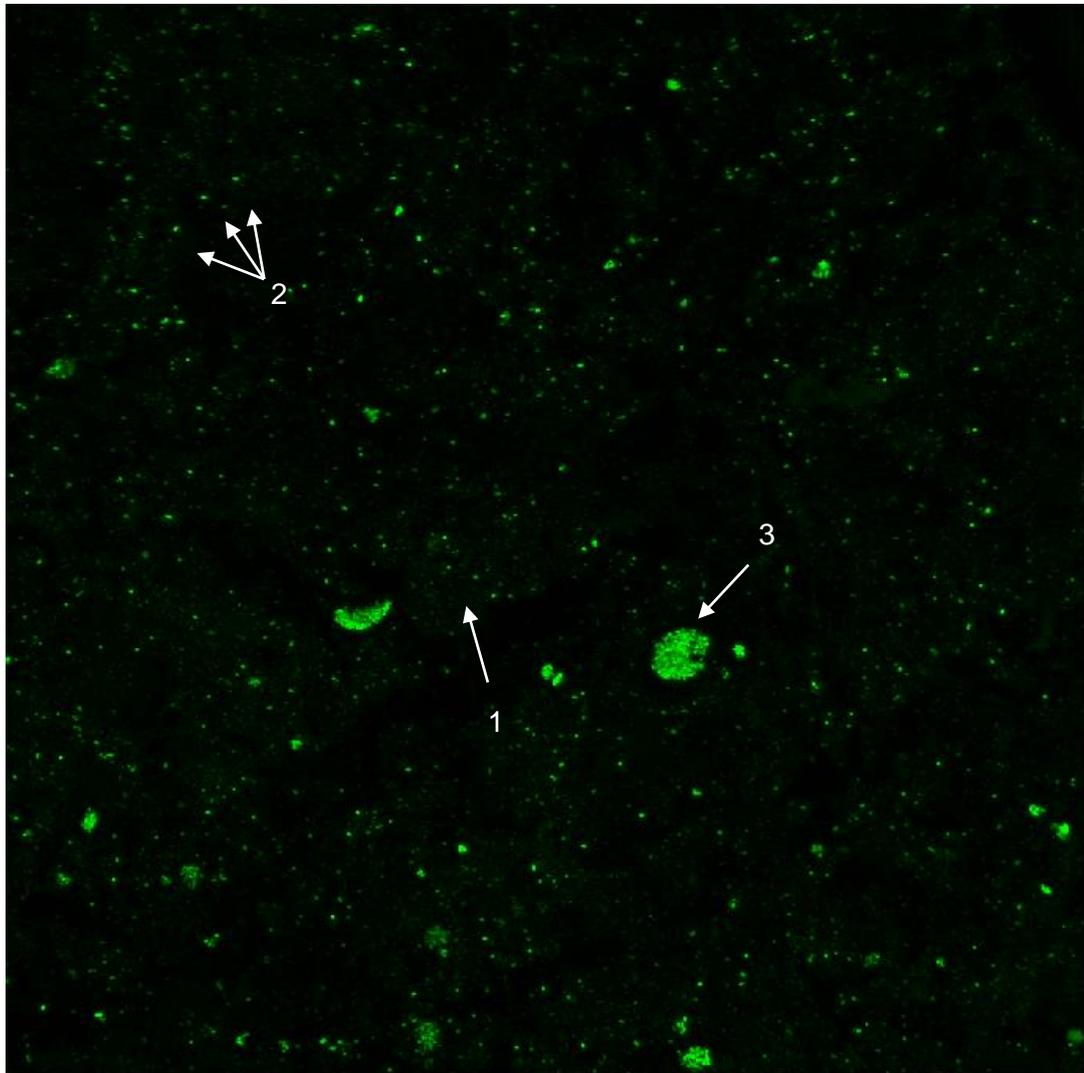


Figure 12 CLSM image of PA bacteria fluorescing.
1=background noise, 2=single cells, 3=microcolonies

In addition to the thresholding difficulties, the reliance on the base layer (or substratum) as the basis for all but one of the calculations in COMSTAT raised a number of concerns for the validity of the study results if analysed this way. The sputum samples provided by the participants did not demonstrate biofilm 'growing' from a base layer, rather biofilm clusters were suspended throughout the sputum. Therefore, any colonies that were above the base layer were not counted in the COMSTAT analysis.

Figure 13 shows representative images from one image stack. These images show many microcolonies above the base layer that were excluded from the COMSTAT analysis and hence would have caused error by these microcolonies not being included in the overall results of biofilm biovolume. One variable in COMSTAT that does not rely on biofilm being identified in the base layer is biomass. This variable includes everything that fluoresces in the whole image stack. However, as mentioned above, due to the difficulties with thresholding, background fluorescence was included in addition to the single cells and clusters of bacteria, resulting in an unrepresentative result which could be misleading in further analysis.

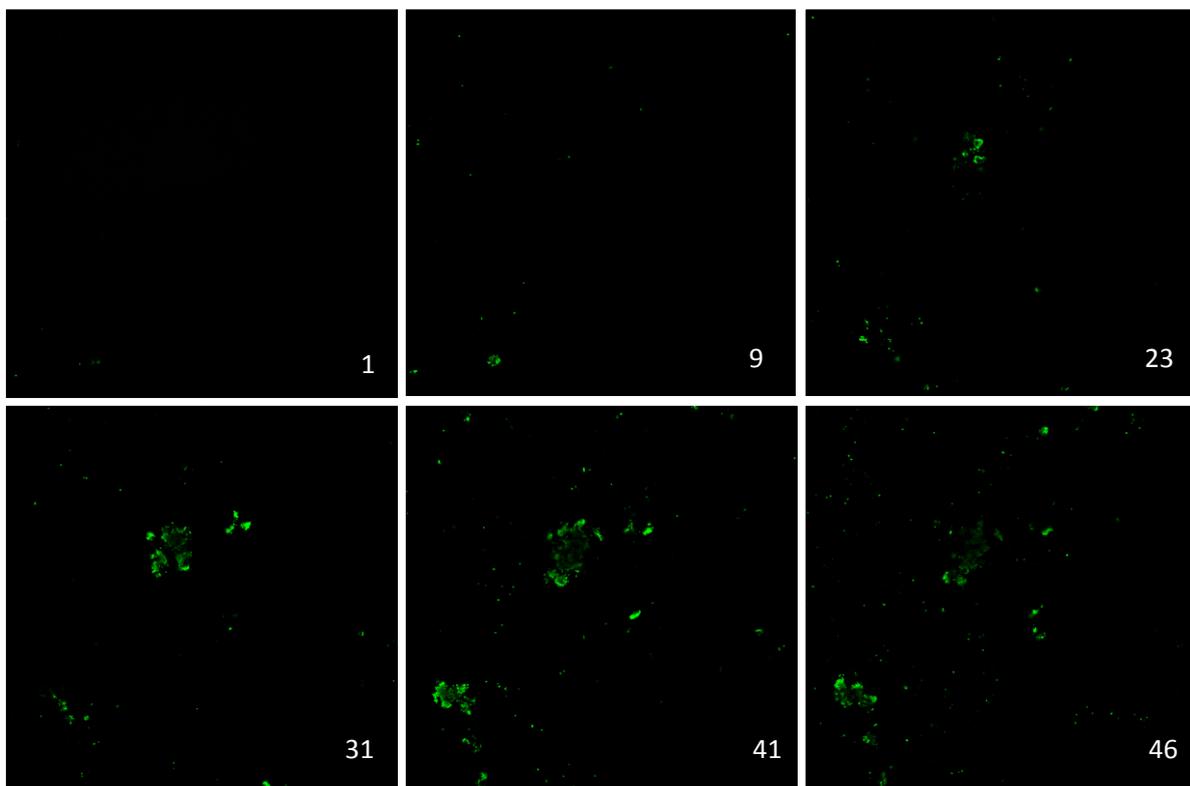


Figure 13 Representative images from one image stack (numbers show the image number in the stack 1=base layer).

Because of the difficulties and limitation encountered with COMSTAT an alternative method for analysing the images was sought. It was important that this method was able to exclude background noise (fluorescence) and include all microcolonies throughout the sample (image stack), even if not associated with the base layer.

3.2 Fiji (Image J)

After extensive research and discussion with biofilm image analysis experts (Dr Jeremy Webb, Prof Paul Stoodley, Dr Luanne Hall-Stoodley) the programme that was selected was Image J, more specifically the 3D version of Image J – Fiji (**Fiji Is Just Image J**). It is a Java based application designed for analysing scientific images. Fiji is particularly useful for 3D analysis, reconstruction and visualisation of images (Schindelin, Arganda-Carreras et al. 2012). Images from the confocal microscope are saved as TIF files which preserves any calibrations applied to the images and does not compress them allowing accurate analysis of structures. It can be freely downloaded at <http://fiji.sc/Fiji>. The Fiji programme applies the same threshold to all images and therefore removes the variability in this step of the image analysis process. Fiji is widely used for 3D image analysis and reported in the scientific literature, but has not previously been reported in the literature for use analysing biofilm in sputum.

The study (despite the small number of participants) generated a huge amount of data. The scale of the data (numbers of images generated) for analysis is shown in Table 5.

Table 5 Numbers of sample, image stacks and images to be analysed from the study

	Per stack	Per Sample	Per patient	Total for study
Patient				12
Samples			5	60
Image Stacks		10	50	600
Images	15-70 (average 40, depends on size of sample)	400	2000	24,000

A function in Fiji called 3D object counter was used to analyse the image stacks (see full process in section 3.2.2). However, to be able to further analyse the results it was necessary to know the size of a PA cell (in order to separate out single cells and colonies later in the analysis process).

3.2.1 Single cell size analysis

Initially, single cells were identified in the image stacks. Criteria for selection were based on the size, shape and orientation of the object fluorescing, taking account of the position when scrolling through the image stack. Selected objects that were thought to be single cells were reviewed with colleagues until consensus was reached. Figure 14 shows an example image where single cells were identified (circled).

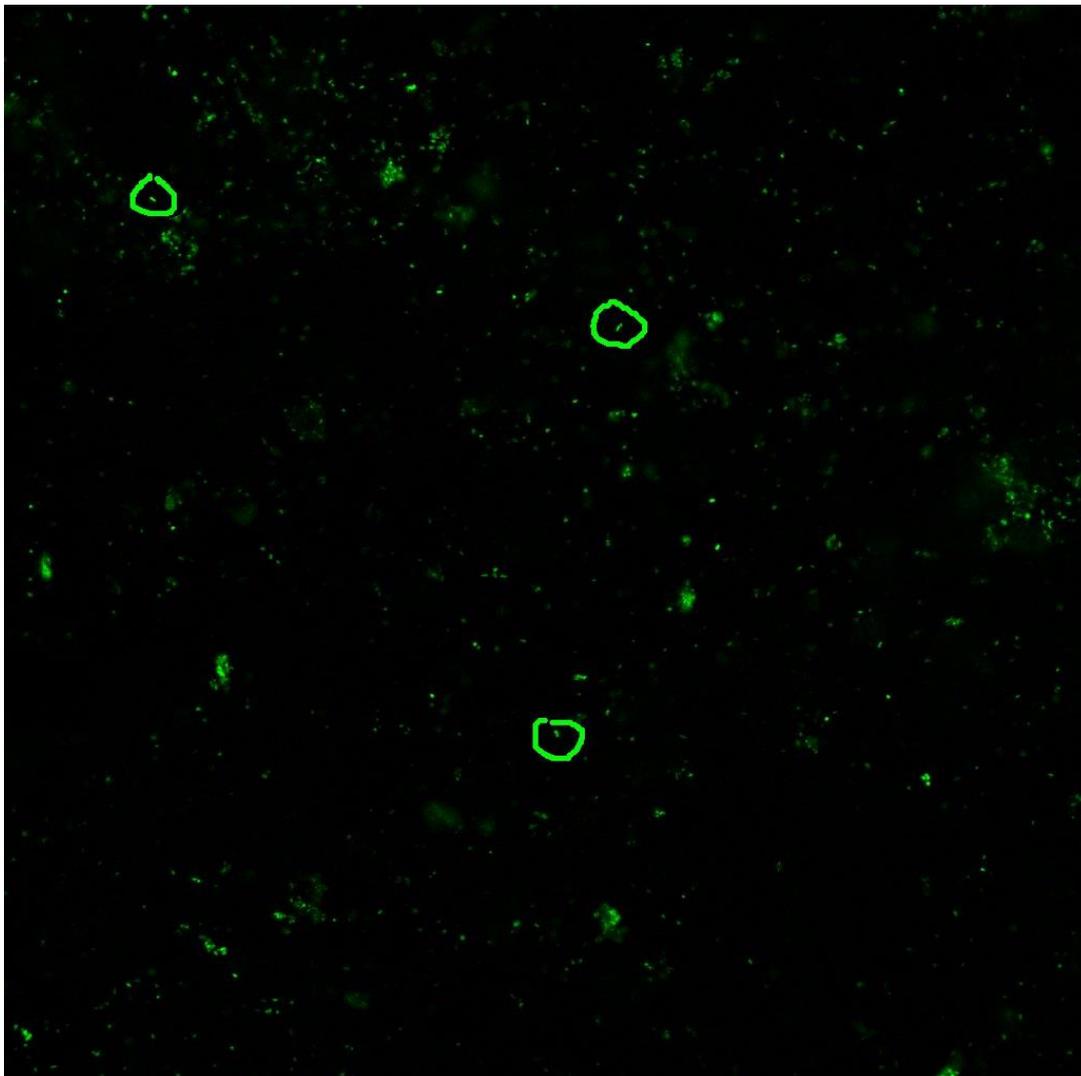


Figure 14 Example of object identified in image likely to be single cell

The identified single cells were then located on the objects map (see Figure 15) produced in Fiji from the image (after thresholding – see following section 3.2.2 for complete Fiji analysis sequence). The corresponding volume in the data generated was found by identifying the object number of the relevant cell.

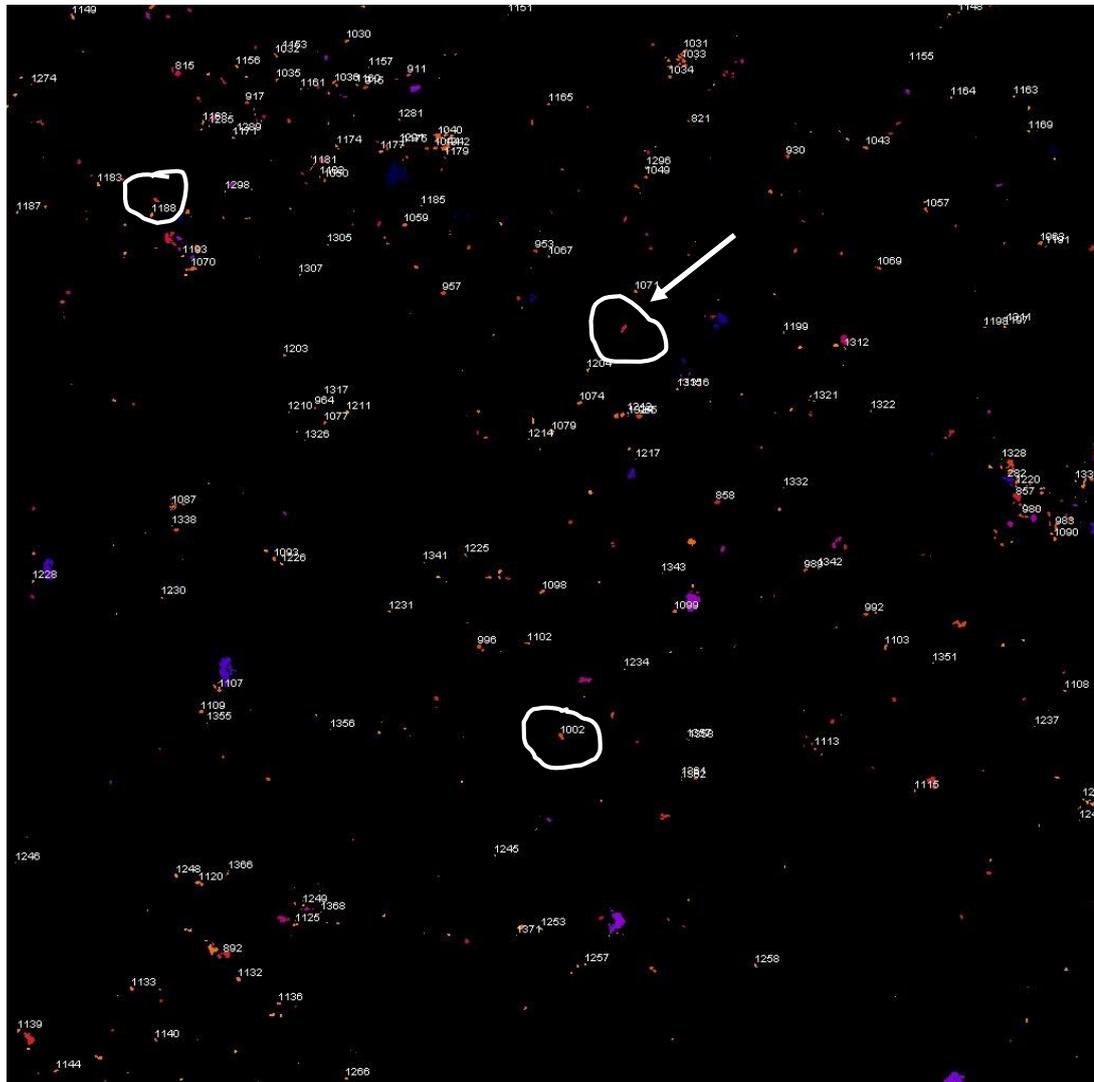


Figure 15 Same image as in Figure 14, shows object map with identified single cells and corresponding object numbers (note the top right cell shape shown by an arrow does not have a number in this image, but was identified in a different image in the stack (either above or below this one).

For each patient an image stack was selected at random and then 10-12 objects thought to be individual cells were identified. Images were reviewed at research group meetings and discussion took place separately with a group of experienced biofilm scientists from both within and outside the core project group in order to achieve agreement on which objects were likely to represent single cells. Following

this consensus, cell volumes were analysed. The volume was recorded and 95% confidence intervals calculated for each patient for the size of a single cell. Literature values of the size of a *Pseudomonas* rod were also calculated based on 0.5-1µm width and 1-5 µm length (Garrity 2007). These were used to calculate literature values for the minimum and maximum volumes of a PA cell as shown in Figure 16 which shows where these measurements relate to the shape of a pseudomonas rod.

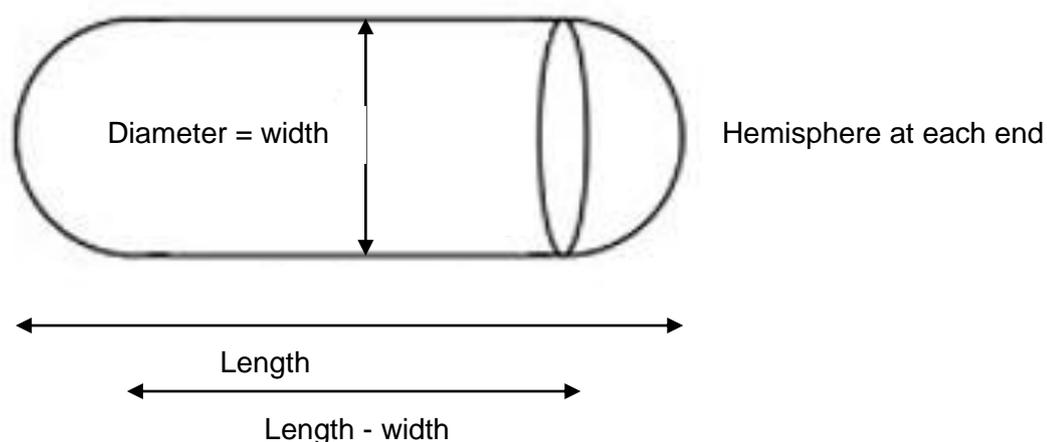


Figure 16 Details for calculating the volume of a *Pseudomonas aeruginosa* cell

To calculate the volume of a PA cell using literature values:

$((\text{Length} - \text{width}) \times \text{area of a circle with diameter} = \text{width}) + \text{volume of a sphere with diameter} = \text{width}$

Area of a circle = πr^2 ($\pi = 3.14$, $r = \text{radius} = \text{diameter}/2 = \text{width}/2$)

Volume of a sphere = $4/3\pi r^3$ ($\pi = 3.14$, $r = \text{radius} = \text{diameter}/2 = \text{width}/2$)

Table 6 shows the volumes calculated using the above formulae.

Table 6 PA cell volumes calculated from literature values for maximum and minimum length and width

PA width (µm)	PA length (µm)	PA cell volume (µm ³)
0.5	1	0.164
0.5	1.5	0.262
1	1	0.524
1	2	1.309
1	5	3.665

This gives a minimum possible cell volume of $0.164 \mu\text{m}^3$ using minimum values for length ($1 \mu\text{m}$) and width ($0.5 \mu\text{m}$) and maximum possible cell volume of $3.665 \mu\text{m}^3$, using maximum possible values for length ($5 \mu\text{m}$) and width ($1 \mu\text{m}$).

The data generated from the cell volume analysis in the images obtained from the study samples is shown in table 7.

Table 7 Cell volume analysis results from images taken from each participant samples at random

Participant randomisation number	N (number of cells analysed)	Minimum volume (μm^3)	Maximum volume (μm^3)	Mean volume (μm^3)	Standard error (μm^3)	95% confidence interval (μm^3)
1	12	.792	3.281	1.579	.181	1.225 - 1.933
2	12	.933	4.498	2.360	.313	1.747 - 2.972
3	10	1.358	4.441	2.752	.372	2.023 - 3.482
4	10	1.725	3.310	2.563	.165	2.240 - 2.886
5	12	.990	3.225	2.060	.170	1.727 - 2.394
6	10	.679	4.582	2.272	.356	1.574 - 2.969
7	10	.622	1.754	1.171	.126	0.924 - 1.418
8	10	1.895	4.102	2.979	.246	2.496 - 3.461
9	10	2.037	4.158	3.072	.272	2.539 - 3.604
10	12	1.273	3.592	2.086	.198	1.698 - 2.474
11	10	1.075	5.205	2.560	.416	1.745 - 3.375
12	10	1.443	3.112	2.368	.207	1.961 - 2.774
Total	128	.622	5.205	2.300	.086	2.132 - 2.468

The above results show that the 95% confidence interval for cell volume for each participant fell within the calculated literature values for the minimum and maximum volumes of a PA cell ($0.164\text{-}3.665 \mu\text{m}^3$). Further analysis of the results of the study (primary outcome measure) was carried out using the minimum and maximum calculated literature values for volume of a cell as the values from the participants images corresponded well (minimum 95% CI $0.924 \mu\text{m}^3$ and maximum 95% CI $3.604 \mu\text{m}^3$). The literature values were felt to be broader and more 'inclusive' so would definitely capture all single cells, whereas using a narrower range would potentially exclude objects that were actually (small) single cells. The drawback to

using a broader range was that it might capture some 'noise' (fragments of PA cells below single cell size) and some 2-3 cell clusters of smaller cells, but after discussion amongst the research team, it was felt more important to be inclusive in the single cell group. Other debris including neutrophils, epithelial cells and other extra cellular substances should not be fluorescing due to the PA specific probe being used in the FISH analysis. The upper limit that was taken for a single cell volume was $3.665 \mu\text{m}^3$ and this value was used for all further calculations based on the volume of a cell in analysis of the primary outcome measure.

3.2.2 Sequence for analysing the images in Fiji

The formal process for analysis of image stacks and 3D objects was as follows:

1. **The stack of images was loaded in Fiji** – as shown in table 5, there were a total of 600 image stacks for this study.
2. **A threshold was set** – above which anything fluorescing was counted and anything below was not counted. A number of different image stacks were reviewed to look at the threshold and (unlike COMSTAT) a standard threshold could be applied to all images. The images and thresholds were reviewed with all the investigators before this method was applied. See Figure 17 for an example image with the corresponding threshold image for comparison.

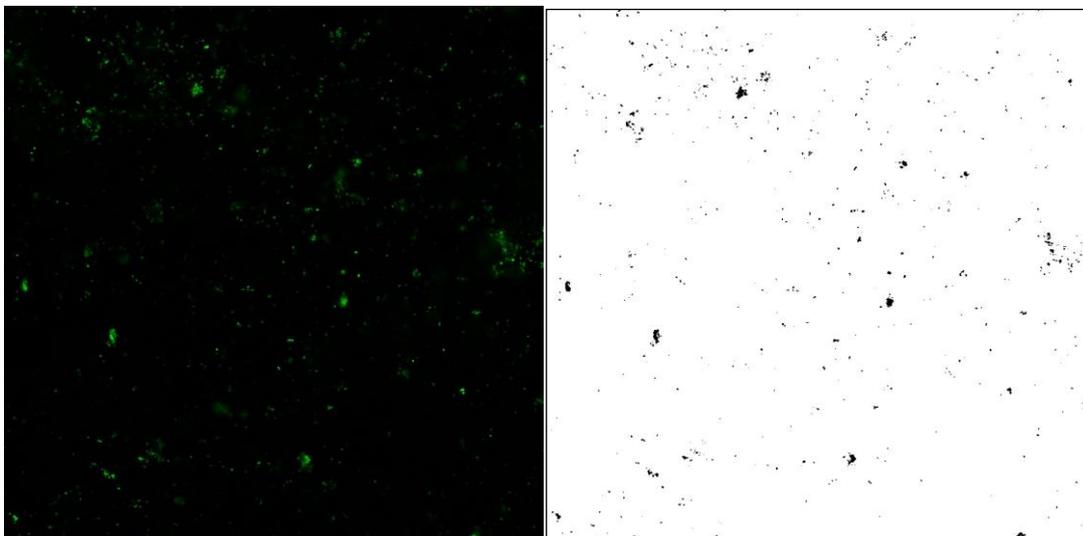


Figure 17 Example image with corresponding threshold image

3. **3D object counter was run** – 3D object counter is a plugin in Fiji that scans through the stack of images and identifies any objects and records their volumes. This uses voxel size which is identified using the measurements taken

with the confocal light microscope when the images were acquired. The size of the image in μm and the distance between the images (in the stack) is registered which allows the programme to know what a pixel and voxel size is and then work out the object size in μm^3 by knowing how many voxels make up each object. For this study the image size was the same (1024×1024 pixels = $246.03 \mu\text{m} \times 246.03 \mu\text{m}$) and the 'step' size between images in the stack was the same for each sample ($0.49 \mu\text{m}$), so these measurements were constant. This generated an object map (such as the one seen in figure 15) and each number on the map was one object (or cell/cluster or cells). A list of objects in each stack was generated in a spread sheet with each numbered object and its corresponding volume. This list contained up to 25,000 objects for some image stacks.

4. **Results were collated for the 10 image stacks per sample per patient** – The lists of objects and volumes were transferred into SPSS (IBM, SPSS Statistics, version 21) to allow the object volumes from all 10 image stacks from each sample to be recorded together (each spread sheet was then labelled with the participants randomisation number and sample date e.g. T0/T7). Because of the large numbers of objects in some of the image stacks, these databases contained up to 152,000 objects. This collation then allowed ordering of results and further separation into categories (see below) for each sample per participant.
5. **Results were divided into groups (single cells, noise, colonies etc.)** – Using previously agreed and calculated volumes for a single cell (see section 3.2.1, value used $3.665 \mu\text{m}^3$), the data was split into sections:
 - Objects below single cell size (also termed 'noise', this included any objects below the minimum calculated literature value for a PA cell $<0.164 \mu\text{m}^3$)
 - Objects of single cells size ($0.164 \mu\text{m}^3$ - $3.665 \mu\text{m}^3$)
 - Objects over single cells size ($>3.665 \mu\text{m}^3$)
 - Objects of single cell size and above, to represent total PA biomass in the sample ($>0.164\mu\text{m}^3$ or previous 2 categories added together)
 - Objects over 10 cell size ($>36.65 \mu\text{m}^3$)
 - Objects over 20 cell size ($>73.3 \mu\text{m}^3$).

In multiplying the maximum possible volume of a cell (based on literature values) of $3.665\mu\text{m}^3$ there was recognition that some clusters would have larger numbers of smaller cells. There was also no allowance within the calculation for extra cellular polymeric substance (EPS); however, with the limit for calculations

being the upper size for a cell it was felt that there was enough flexibility to be confident these were clusters of cells.

All samples had clusters of greater than 20 cell size at the start of therapy (using the upper limit of a cell size and recognising that in fact some of these clusters may be well over 20 cell size if the cells within them were smaller). Any changes seen were assumed to be due to treatment effect. The data was also split above 20 cells; over 30 cell size and over 100 cell size. However, these were not used for any further analyses as there were lots of samples with no clusters over this size at T0 and therefore the effect of treatment could not be assessed. In a study with larger numbers, even if some samples had no clusters over this size at baseline, this analysis could be possible if there were enough samples to analyse the effects on this size of biofilm. At clusters over 100 cell size only 4 patients had biofilms of this size in their samples at T0, so there were not enough data points to perform any type of analysis or draw any conclusions. Again, in a larger study, it might be possible to look at these larger clusters in more detail, and the effects of treatment on them if there were more patients with clusters of this size in their sputum at baseline.

Chapter 4: Results - Screening and baseline data

4.1 Screening

4.1.1 Methods

Patients aged 12 or above with cystic fibrosis who were known to have chronic PA infection (50% or more of all cultures in the last 12 months positive for PA), and regularly produced sputum, were approached about the study during routine clinic visits. They were given information sheets and the study was explained to them. Once informed consent had been given, screening took place. A sputum sample was collected for confirmation of PA biofilm growth. As per protocol (Appendix 9) if PA could not be isolated from the sample in the lab to grow biofilm, the participant was informed that they could not proceed any further with the study. Screening was crucial for this small proof of concept clinical trial. The primary outcome measure looked at changes in the proportion of PA bacteria. As such, it was important that any patients who were randomised had enough detectable PA in their sputum to enable changes to be seen (if present) as a result of the treatment given.

If PA biofilm was grown from the sputum, the participant was enrolled. In addition the following assessments were performed:

1. Confirmation (check with NHS laboratory) of CF diagnosis (sweat test +/- genotype confirmation of disease causing mutations)
2. Confirmation of PA infection
3. Fulfilment of inclusion/exclusion criteria

Once enrolled, the participant and the participant's CF physician were asked to contact the research team if the participant presented with an acute exacerbation requiring IV antibiotic therapy. At this stage, consent was confirmed, and if the participant agreed, they were randomised to receive either inhaled NO therapy or placebo in addition to standard antibiotic therapy.

4.1.2 Participants

79 patients were approached about the study. 35 patients age 16 and above were screened. Patients were eligible for inclusion if they were 12 years old or above, however, there were no suitable patients aged between 12-16 years identified during the study period. 21 patients screened positive for PA in their sputum and were enrolled into the study. 12 patients were randomised at the time of exacerbation during the study period. Figure 18 shows the consort diagram with details of participants from initial approach to the end of the study.

44 patients were excluded for a variety of reasons. 21 did not meet the inclusion criteria, due to a lack of chronic pseudomonas colonisation. 15 declined to participate when approached about the study and given time to read the information sheet. These were primarily because of the requirement for additional blood tests in needle phobic patients and/or the need to be admitted to hospital for a 5-7 night stay in patients who would usually receive their IV antibiotics at home. 8 patients were excluded for other reasons, mainly due to hypersensitivity to the study antibiotics. One was excluded due to the need for non-invasive ventilation at night and another due to a planned admission for treatment of non-tuberculosis mycobacteria.

4.1.3 CONSORT Flow Diagram

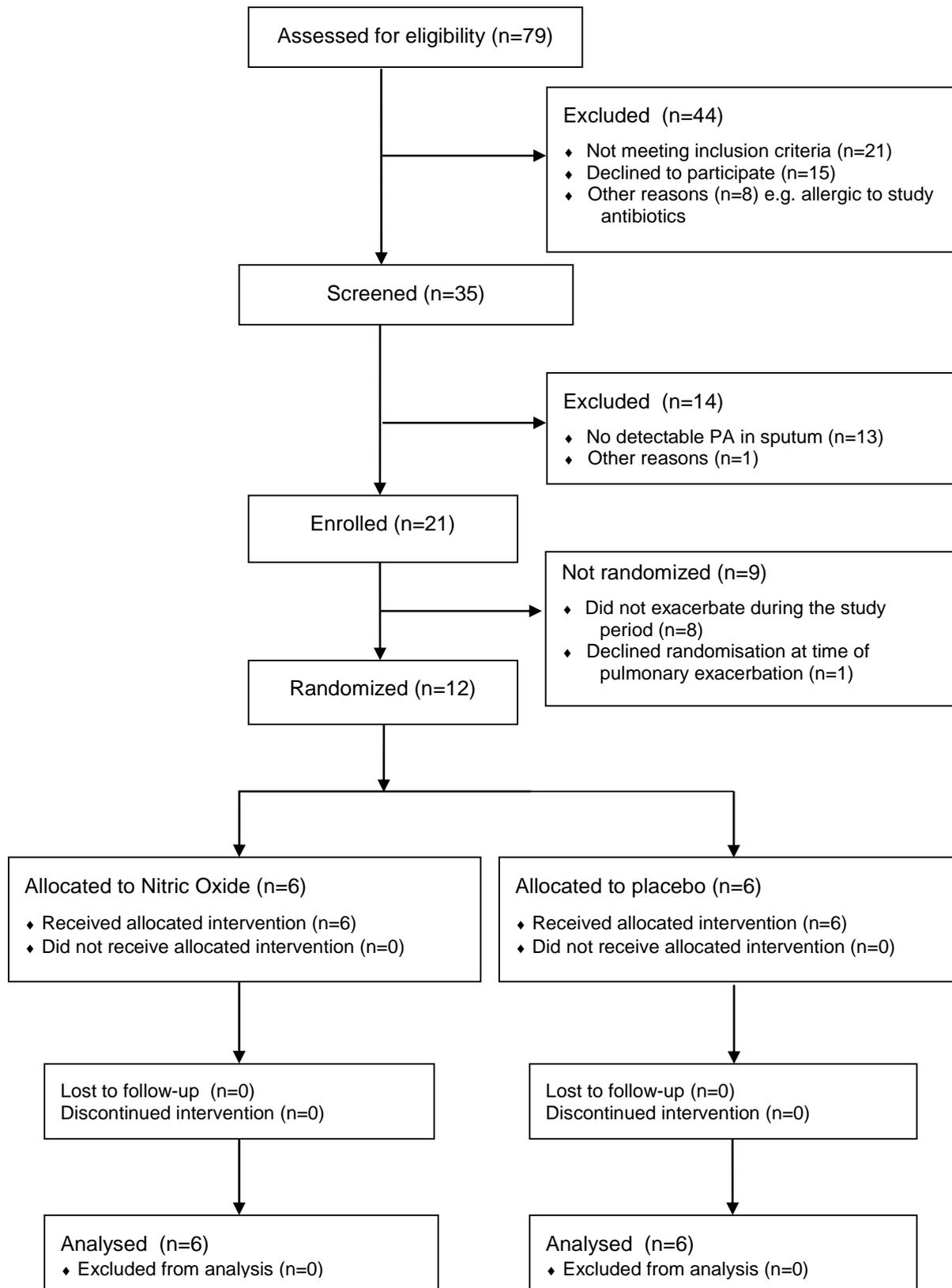


Figure 18 Consort diagram

4.2 Baseline data

Table 8 shows the baseline characteristics of the 2 groups. Mean values are shown for the variables that are normally distributed. Standard deviations and standard error of mean are shown as well as the mean difference between the groups and the 95% confidence interval for this difference. These values have been compared using a T test and the p values are shown in the far right column. None of the differences are significant at a p value of <0.05. Blood results are presented as medians and interquartile ranges as these parameters are not normally distributed (Table 8). There is no difference between the 2 groups at baseline. Because of the small numbers of participants it is recognised that these comparisons have little power to detect true differences, but they are included to show that the groups have been compared at baseline for information only. In such a small study, there was not option for stratification of the groups, however, in larger studies, this may be considered depending on the design of the study.

Heights and weights were converted to z scores. $Z \text{ score} = (\text{observed value} - \text{median value of the reference population}) / \text{standard deviation value of reference population}$. However, as z scores are used in paediatric nutritional assessments, the age had to be set to 240 months for all participants over 20 years (all except one). It is assumed that they would have reached their growth potential by this age. However, this is not a common parameter reported for adults, instead BMI is used more commonly and hence this was also calculated and reported.

Table 9 shows the baseline bloods of the two groups. Medians and interquartile ranges are presented as the data are not normally distributed.

Table 8 Baseline characteristics of groups

	Treat- ment Group	N	Mean	Std. Devia tion	Std. Error Mean	Mean difference	95% CI lower	95% CI upper	T test (p)
Age in years	NO	6	30.0	13.99	5.71	0.67	-18.39	19.73	0.94
	Placebo	6	29.3	15.60	6.37				
Height in cm	NO	6	162.8	9.45	3.86	-3.17	-15.21	8.88	0.57
	Placebo	6	166.0	9.27	3.79				
Height z score	NO	6	-1.03	1.25	0.51	-0.51	-2.37	1.35	0.55
	Placebo	6	-.052	1.61	0.65				
Weight in kg	NO	6	56.4	9.61	3.93	-6.6	-18.17	4.97	0.23
	Placebo	6	63.0	8.32	3.40				
Weight z score	NO	6	-0.99	1.35	0.55	-0.82	-2.41	0.77	0.28
	Placebo	6	-0.17	1.11	0.46				
BMI	NO	6	21.2	2.55	1.04	-1.88	-6.27	2.5	0.36
	Placebo	6	23.0	4.09	1.67				
Heart rate in bpm	NO	6	89.3	18.62	7.60	-1.83	-24.88	21.22	0.86
	Placebo	6	91.2	17.19	7.02				
Systolic BP (mmHg)	NO	6	107.3	13.84	5.65	-13.67	-32.21	4.87	0.13
	Placebo	6	121.0	14.97	6.11				
Diastolic BP (mmHg)	NO	6	64.2	9.37	3.82	-11.67	-26.79	3.45	0.12
	Placebo	6	75.8	13.73	5.61				
Oxygen saturation (in air)	NO	6	95.2	2.23	0.91	0	-3.59	3.59	1
	Placebo	6	95.2	3.25	1.33				
Resp rate (per min)	NO	6	20.0	1.10	0.45	1.5	-0.71	3.71	0.16
	Placebo	6	18.5	2.17	0.89				
Temp (degC)	NO	5	36.8	.31	.14	-0.05	-0.62	0.52	0.85
	Placebo	6	36.9	.48	.20				
% of predicted FEV ₁	NO	6	40.2	20.14	8.22	-5.52	-30.26	19.22	0.63
	Placebo	6	45.7	18.28	7.46				
% of predicted FVC	NO	6	54.4	17.60	7.19	-17.08	-42.08	7.92	0.16
	Placebo	6	71.5	21.11	8.62				
Average eNO levels (ppb)	NO	6	12.7	9.46	3.86	3.42	-8.37	15.20	0.53
	Placebo	6	9.3	8.86	3.62				

Table 9 Baseline blood results

	Treatment Group	N	Median	Interquartile Range
Haemaglobin	NO	6	132	118.5 - 138.8
	Placebo	6	141	129.5 - 151.8
White cell count	NO	6	7.9	6.6 - 11.7
	Placebo	6	9.4	7.3 - 11.9
Neutrophils	NO	6	6.2	3.6 - 8.8
	Placebo	6	6.3	4.8 - 8.5
Lymphocytes	NO	6	1.9	1.1 - 2.2
	Placebo	6	2.0	1.4 - 2.4
Platelets	NO	6	259	202 - 277
	Placebo	6	248	228 - 297
Sodium	NO	5	138	134.5 - 139
	Placebo	6	138.5	136 - 139.2
Potassium	NO	5	4.2	3.6 - 4.5
	Placebo	6	3.6	3.3 - 4.3
Urea	NO	5	5.2	4 - 6.1
	Placebo	6	3.7	3.0 - 4.4
Creatinine	NO	5	59	43.5 - 73.5
	Placebo	6	57.5	46 - 72.5
Total protein	NO	5	76	66.5 - 76.5
	Placebo	6	73.5	69.3 - 81.5
Albumin	NO	5	36	34 - 37.5
	Placebo	6	37	34 - 40.3
Bilirubin	NO	5	9	7 - 11.5
	Placebo	6	9.5	7.5 - 11.8
ALT	NO	5	20	19 - 28
	Placebo	6	22.5	17.8 - 31
ALP	NO	5	86	76.5 - 194.5
	Placebo	6	83.5	68 - 128
CRP	NO	5	37	13.5 - 41.5
	Placebo	6	21	12 - 49
Calcium	NO	5	2.4	2.3 - 2.4
	Placebo	5	2.3	2.2 - 2.4
Corrected calcium	NO	5	2.4	2.3 - 2.4
	Placebo	5	2.3	2.3 - 2.4

Overall, there are no significant differences between the groups at baseline for all the above characteristics. In addition, no participants were smokers in either group. There were 3 male and 3 females in each group. N=5 in some cases due to missing data.

Table 10 shows the total number of medications being taken by participants at baseline. The range was 8-22. Both median and mean data is presented as it is not clear whether this variable is normally distributed in the population. Number of medications could be seen as a marker of severity of disease, and although there are, on average, slightly fewer medications being taken in the placebo group at baseline, there is no significant difference between the 2 groups.

Table 10 Total number of medications being taken by participants at baseline

Treatment group	Median	Interquartile Range	Mean	Std. Deviation	Std. Error Mean	Mean Difference	95% Confidence Interval of the Difference	T-test
Nitric Oxide	15.5	14-19	16	4.15	1.69	2.5	-2.7, 7.7	.309
Placebo	13.5	10-17	13.5	3.94	1.61			

All participants except for one (who was in the Nitric Oxide group) were being treated regularly with DNase. 9 of the 12 participants (5 in the placebo group and 4 in the NO group) were receiving regular nebulised antibiotics. For 8 of these, it was nebulised colomycin and for one participant (placebo group) this was nebulised tobramycin. 10 participants were on regular prophylactic Azithromycin (6 in the placebo group and 4 in the NO group).

Chapter 5: Results - Microbiological

5.1 Introduction

A proof of concept study with experimental outcomes was carried out in order to investigate the effect of NO on biofilms when given to CF patients. The primary outcome measure was between group differences in proportion of bacteria in biofilms (as determined by live/dead staining, direct visualisation of the biofilm and image analysis) between T0 and T20-25. In addition, secondary outcome measures included looking at differences between groups at all time points (T0, T5, T7, T10 and T20).

The results were all analysed before unblinding.

5.2 Primary outcome measure assessment

The primary outcome measure was between group differences in proportion of bacteria in biofilms (as determined by colony forming units, live/dead staining, direct visualisation of the biofilm and image analysis) between T0 and T20-25.

5.2.1 Fluorescent In Situ Hybridisation (FISH)

The number and total biovolume of PA in microcolonies (in μm^3) were important measures to analyse. A sample could contain a few very large colonies or a larger number of smaller colonies, so just an analysis of number of colonies could be misleading if there was a large variability in size of colony. In addition, as the size of sputum sample varied between patients, the results were calculated per cm^3 . As described in section 3.2.2 the data generated from the image analysis was split into groups for further analysis. These groups were:

1. Objects below single cell size (also termed 'noise', this included any objects below the minimum calculated literature value for a PA cell $<0.164 \mu\text{m}^3$)
2. Objects of single cells size ($0.164 \mu\text{m}^3$ - $3.665 \mu\text{m}^3$); objects over single cells size ($>3.665 \mu\text{m}^3$)
3. Objects of single cell size and above, to represent total PA biomass in the sample ($>0.164 \mu\text{m}^3$ or previous 2 categories added together)
4. Objects over 10 cell size ($>36.65 \mu\text{m}^3$)

5. Objects over 20 cell size ($>73.3 \mu\text{m}^3$)

In multiplying the maximum possible volume of a cell (based on literature values) of $3.665 \mu\text{m}^3$ there was recognition that some clusters would have larger numbers of smaller cells. However, there was no allowance within the calculation for extra cellular polymeric substance (EPS), so it was decided by the investigator group that using the upper limit of a cell size for calculations would result in enough flexibility to be confident these were clusters of cells.

5.2.1.1 Selection of image analysis criteria for biofilm size

In order to analyse the data using the image analysis software a decision was made regarding size of biofilm for analysis. All samples had clusters of greater than 20 cell size at the start of therapy (using the upper limit of a cell size as discussed in chapter 3 and recognising that in fact some of these clusters may be well over 20 cell size if the cells within them were smaller). The data was also split above 20 cells; over 30 cell size and over 100 cell size to look at larger colonies. However, these were not used for any further analyses as there were lots of samples with no clusters over this size and therefore the effect of treatment could not be assessed.

At over 30 cell size there were 9 out of 56 samples with no clusters plus 4 samples with missing data which totals 22% of samples without data for analysis using clusters of over 30 cells. With such a small number of participants in the study, this would mean there were too many missing data points for analysis. At clusters over 100 cell size only 4 patients had biofilms of this size in their samples at T0, so there were not enough data points to perform any type of analysis or draw any conclusions. A total of 20 samples (out of the 56 obtained) contained clusters over 100 cell size, (in addition to the 4 samples with missing data) this represents only 33% of the samples and as such was not possible to analyse for any treatment effect.

Hence, over 20 cell size was selected as the image analysis criteria for initial analysis of results using FISH, but data was also compared for greater than 10 cell size colonies as well as single cells and total PA biomass as assessed by FISH and image analysis.

5.2.1.2 Data transformation using the natural log scale

Before starting statistical analysis of the data, the distribution of the data was reviewed to assess the need for any transformations. Figure 19 shows histograms of the raw FISH data showing number and volume of microcolonies (graphs a and c). Both of these are negatively skewed. The best transformation for this data to a 'normal' distribution curve was the natural Log (Ln) (graphs b and d).

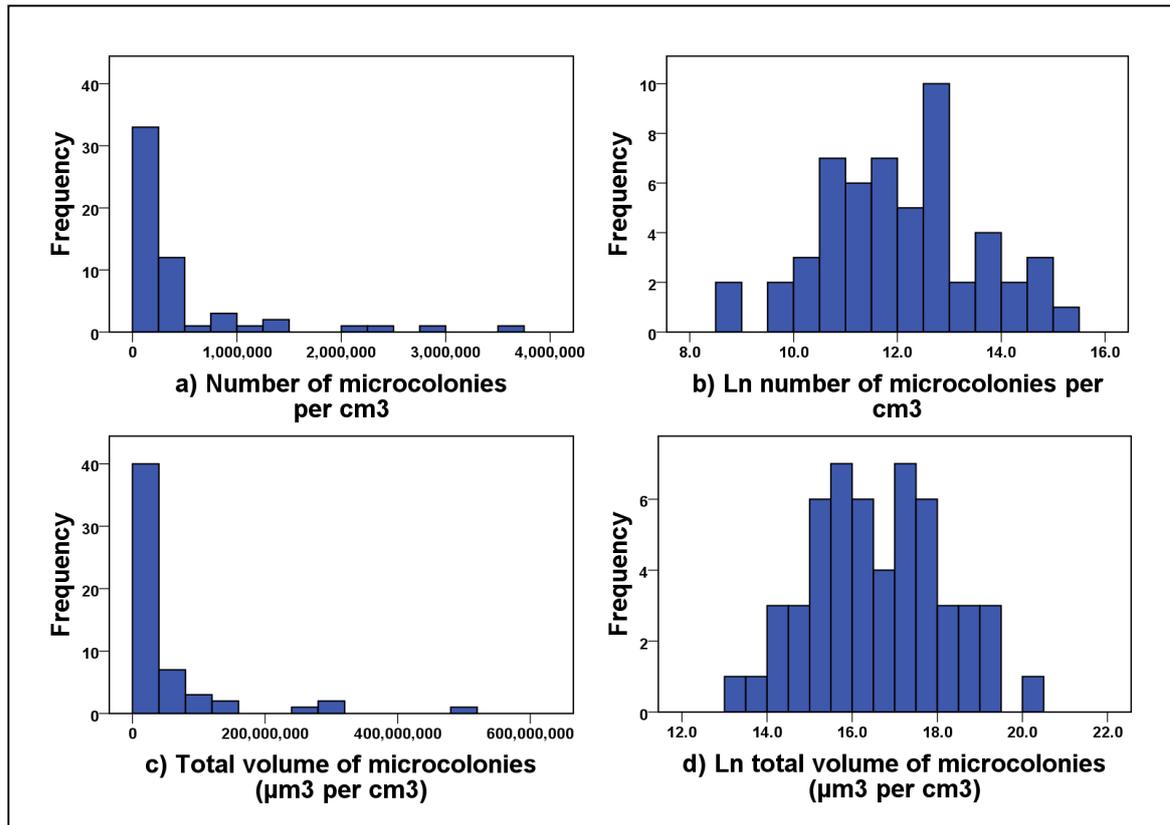


Figure 19 Histograms showing number and volume of microcolonies where data is negatively skewed and histograms of data with Ln transformation where data is normally distributed

Analysis of all the FISH data using histograms and Ln transformation showed that this was the appropriate transformation for this dataset. All FISH results were then analysed using Ln transformations (histograms not shown for each individual group of data). Following discussion with the statistician, values of 0 (in the original data) were changed to 1 to allow the Ln transformation to take place across the dataset. This then resulted in a Ln value of 0 for these data points.

5.2.1.3 Over 20 cell cluster analysis

Table 11 shows the number of objects over 20 cell size per cm³ as well as the total biovolume of these objects at T0 and T20. Unfortunately there were 4 missing samples at T20 where patients were unable to produce enough sputum for analysis. The results show no difference in mean Ln number or volume of microcolonies over 20 cell size between T0 and T20 in either group. The 95% confidence intervals overlap greatly between the two groups and show no difference in treatment effect on clusters of PA over 20 cell size between T0 and T20.

Table 11 Ln number and volume of objects over 20 cell size at T0 and T20

	Ln number of clusters over 20 cell size per cm ³		Ln volume of clusters over 20 cell size (µm ³ /cm ³)	
	T0	T20	T0	T20
Nitric Oxide	11.68	12.24	16.55	17.40
	10.16	*	15.79	*
	10.75	10.84	15.30	15.65
	11.54	12.22	16.25	17.18
	12.14	13.05	17.37	18.22
	9.84	*	14.80	*
mean	11.017	12.09	16.012	17.111
95%CI	10.058	10.631	15.047	15.406
	11.976	13.547	16.976	18.815
SD	0.914	0.916	0.919	1.071
Placebo	8.93	*	14.05	*
	14.00	13.37	19.25	18.35
	10.61	10.76	15.29	15.45
	9.97	*	15.38	*
	11.98	11.82	17.18	16.96
	11.56	10.51	16.71	15.66
mean	11.176	11.61	16.310	16.604
95%CI	9.322	9.543	14.401	14.474
	13.030	13.686	18.219	18.734
SD	1.767	1.302	1.819	1.339

* Missing data (no sample received)

Figure 20 shows box plots of this data and demonstrates that there is no difference in Ln number or volume of clusters > 20 cells between the treatment groups and between T0 and T20.

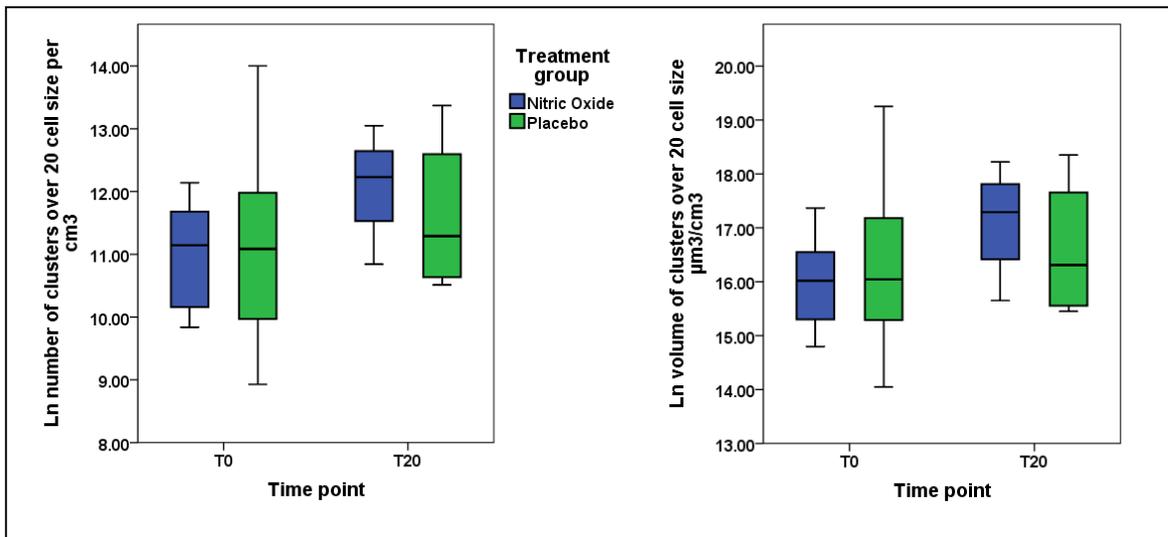


Figure 20 Box plots showing Ln number and volume of clusters >20 cell size from T0 to T20

5.2.2 Colony forming units

Colony forming units (CFUs) are used extensively in clinical microbiology and are one way of looking at the amount of bacteria in a sample. Depending on the growth agar used, growth of different bacterial colonies can be enhanced or inhibited. In this study CFUs were important to investigate the amount of planktonic PA in the sample, especially to address concerns that giving NO might increase planktonic bacteria by releasing bacteria from biofilms. This in turn could raise concerns about safety and result in increased symptoms for patients. CFU's were measured on 3 types of agar plate: Tryptic soy agar (TSA) – a general growth agar which gives a representation of the whole colony, *Pseudomonas* isolation agar (PIA) – for the growth of all pseudomonads and Cetrimide agar (Cet) – specific growth agar for PA. The primary outcome measure was relating to PA CFUs and therefore the growth on Cetrimide agar.

Colony forming units are reported here using the Natural log transformation as this successfully transformed the data from negatively skewed to a normal distribution.

Figure 21 shows the histograms of the raw data for the 3 types of agar (a, b, c) and histograms of the Ln data (d, e, f) showing the transformation to a normal distribution curve (data shown is NO and placebo groups combined).

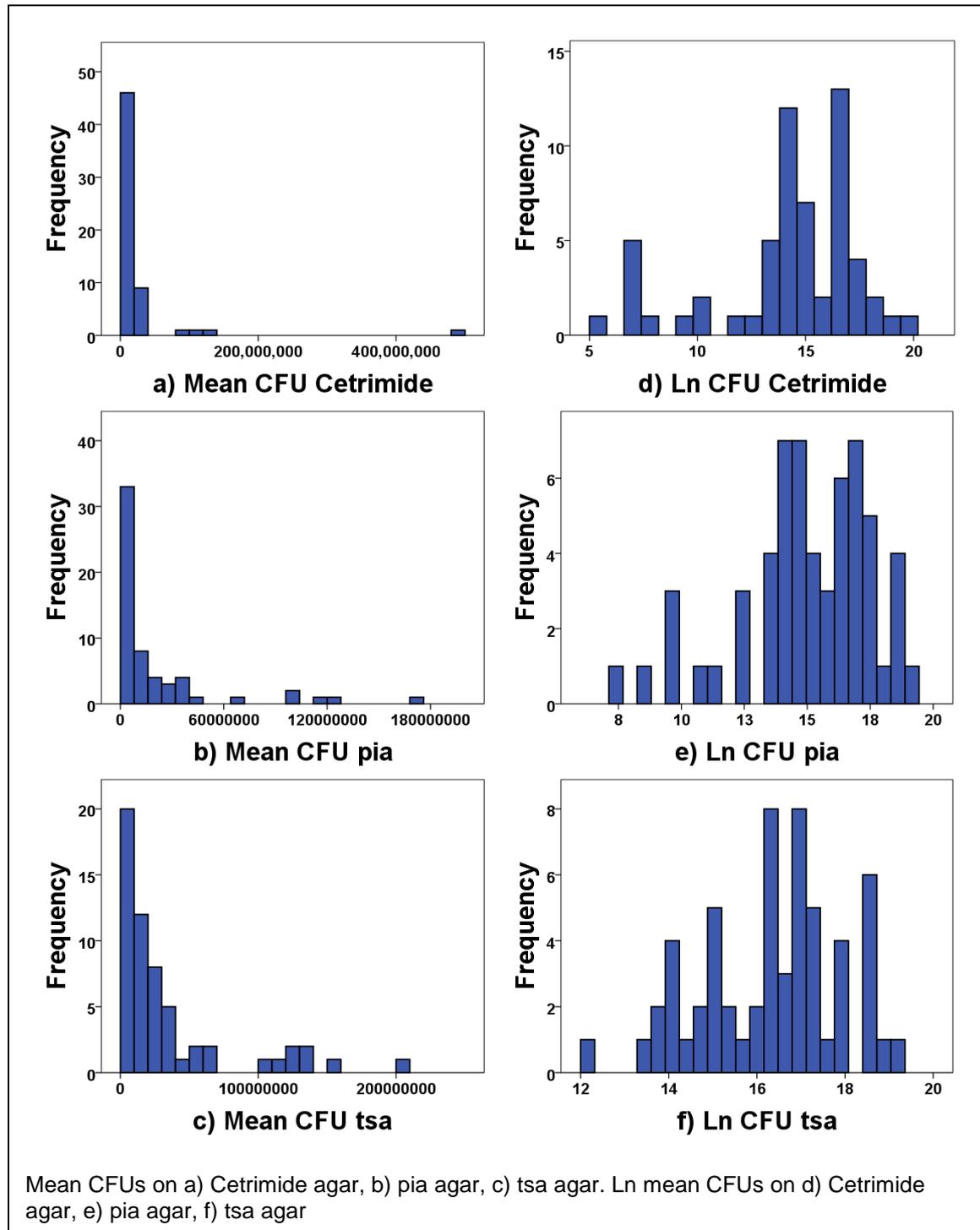


Figure 21 Histograms of CFUs and Ln transformation

Following discussion with the study statistician, it was felt that these distributions were near enough normal to use the Ln transformation for all further analysis of the CFU data. Because of the small number of patients there were not a large number of values for each of the above histograms, but the overall spread of the data when transformed using the natural log was that of a normal distribution.

Table 12 shows Ln CFUs on Cetrinide agar which is specific for growth of PA. This shows no significant difference in CFUs between T0 and T 20 in either group. There was one missing sample and 2 patients in the placebo group whose CFUs reduced by a lot more than the other participants. Figure 22 shows the wide spread of the data and there were no overall significant differences seen. One of the reasons for including CFUs in the primary outcome was because of safety concerns. These results show that there was no increase in CFUs in the NO group compared to the placebo group. The wide variation in CFUs before and after treatment is in keeping with the knowledge that CFUs are highly variable both within and between patients (McMenamin, Zaccone et al. 2000, van Belkum, Renders et al. 2000).

Table 12 Ln CFUs on Cetrinide agar

	T0	T20		T0	T20
Nitric Oxide	15.58	16.3	Placebo	13.01	*
	18.57	16.92		14.33	18.36
	17.41	18.66		16.83	14.52
	14.61	14.01		12.44	6.8
	14.47	16.22		11.44	6.8
	17.34	17.13		15.12	16.49
mean	16.33	16.54	mean	13.86	12.59
95% CI	14.56	14.95	95% CI	11.80	5.81
	18.10	18.13		15.92	19.37
SD	1.68	1.519	SD	1.96	5.46

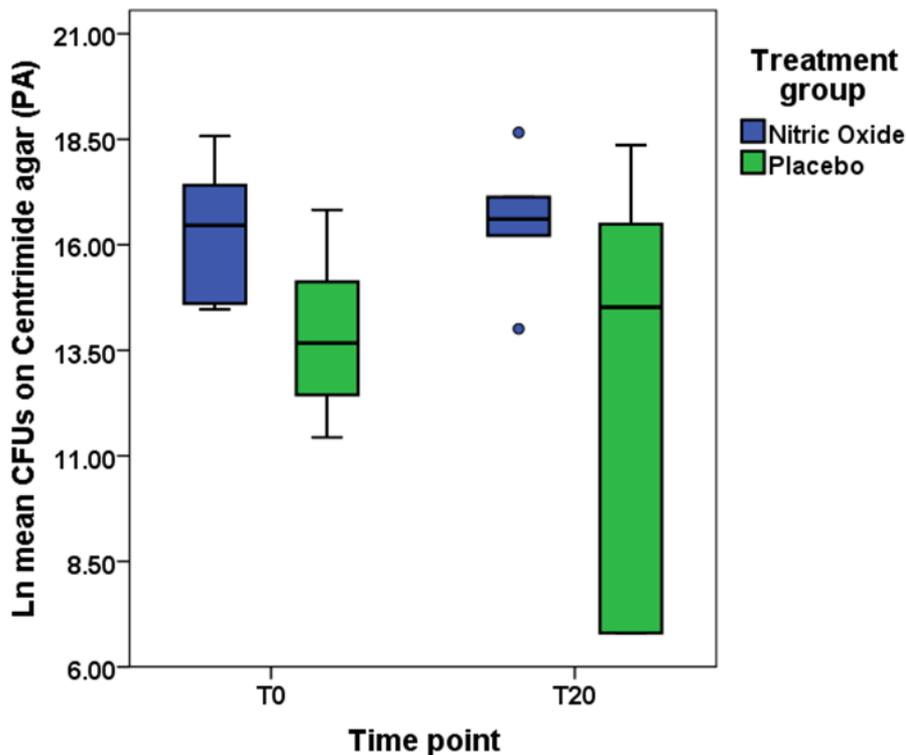


Figure 22 Box plots of PA CFUs at T0 and T 20.

Middle bars represent the median, boxes the interquartile range and whiskers 1.5 IQR. Outliers >1.5 IQR are shown by dots.

Figure 22 shows that the NO group had higher CFUs at baseline than the placebo group, although the interquartile ranges overlap. The median CFUs in the NO group did not change following treatment. The median CFUs in the placebo group has increased, although the mean has decreased. This highlights the difficulty in drawing conclusions with such a small number of data points as the mean is pulled down by the 2 patient's whose CFUs decreased quite a lot following treatment, but the median is pulled up as more patients had higher CFUs following treatment. Again, this shows the wide variation in CFUs and the problems with relying on this as an outcome measure. One result that is helpful is that there has been no increase in CFUs in the NO group. This is reassuring from a safety perspective as it has not shown an increase in planktonic bacteria (those typically measured by CFUs) following treatment with NO that would cause release of bacteria from biofilms.

5.2.3 Quantitative Polymerase Chain Reaction (qPCR)

qPCR is a way of quantifying the amount of viable PA in a sample. This was another important measure for this study to ensure that the viable PA did not

increase as a result of NO therapy and release of bacteria from biofilms. qPCR was carried out by a collaborator at Kings College Hospital, London (Dr Geraint Rogers).

Ln transformation was used as the data was negatively skewed and using the Ln transformation resulted in a near normal distribution (see figure 23).

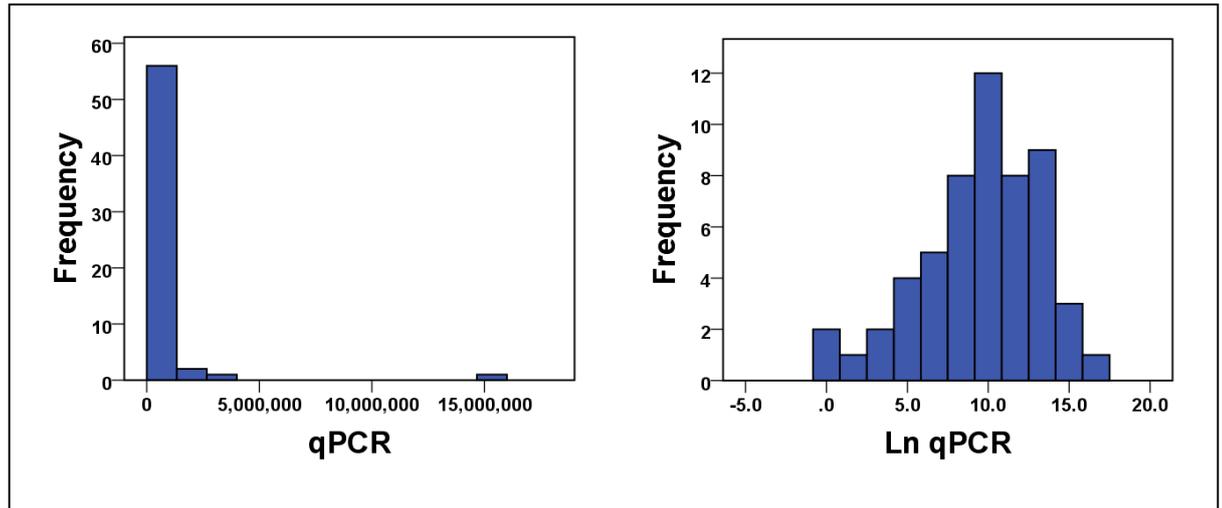


Figure 23 Histograms of qPCR and Ln qPCR

Table 13 shows Ln qPCR from T0 to T20. There is one missing sample. One patient in the placebo group had no detectable PA by qPCR at T20 and there were others with much less detected at T20, although interestingly some of the patients in the placebo group had much less PA detected by qPCR at baseline too.

Table 13 Ln qPCR T0-T20

	T0	T20		T0	T20
A Nitric Oxide	6.95	5.52	B Placebo	13.32	*
	12.41	10.34		13.27	14.43
	16.55	11.71		13.29	7.53
	11.57	8.44		3.72	0.00
	10.54	5.88		8.03	1.02
	14.98	10.70		13.72	12.05
mean	12.166	8.766	mean	10.895	7.00
95%CI	8.610	6.035	95%CI	6.569	-0.989
	15.722	11.496		15.220	14.996
SD	3.389	2.60	SD	4.122	6.437

* missing sample

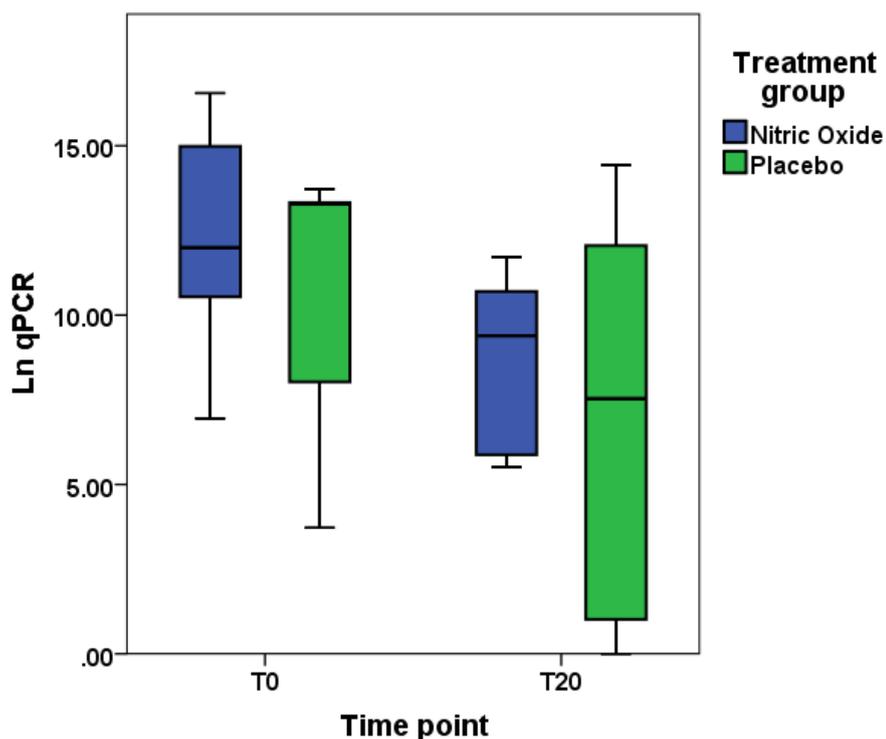


Figure 24 Box plots showing qPCR at T0 and T 20.

Middle bars represent the median, boxes the interquartile range and whiskers 1.5 IQR.

Figure 24 shows that there is a wide variation in qPCR both at baseline and following treatment. Both groups show an average decrease in qPCR following treatment as would be expected following IV antibiotics. The NO group start with higher average qPCR, but the interquartile ranges overlap between both groups and timepoints and any differences are not significant.

5.3 Secondary outcome measure assessment

- Between group differences in proportion of bacteria in biofilms (as determined by colony forming units, live/dead staining, direct visualisation of the biofilm and image analysis) at all time points (T0, T5, T7, T10 and T20).
- Between group differences in 'change in PA colony forming units' (as determined by plate counts and intensity of qPCR) between T0, T5, T7, T10 and T20-25.
- Between group differences in whole colony CFU's at T5, T7, T10 and T20-25.
- Between group differences in NO levels in fresh sputum at T7.
- Between group differences in the characteristics of the wider microbial community as assessed by microbial community analysis/molecular analysis.

- Between individual and between group differences in the level of nitric oxide metabolites in the blood.

The assumptions made for primary outcome assessment regarding transformation of the data also apply to these analyses. All data was transformed using the natural log scale unless otherwise stated as explained in section 5.2.

5.3.1 Fluorescent in situ hybridisation (FISH)

For this analysis data was split into a number of groups (as discussed in section 5.2.1.1 and 5.2.1.2). This secondary outcome measure assessment was looking at the effects of treatment across all timepoints of the study, in comparison to the primary outcome assessment which was assessing the effects of treatment between T0 and T20-25.

5.3.1.1 Over 20 cell cluster data

The following tables and graphs show the FISH data for biofilms in clusters over 20 cells ($20 \times 3.665 \mu\text{m}^3$ or $>73.3 \mu\text{m}^3$). There is considerable debate in the literature about the size of a biofilm, but at this size these clusters can be called biofilms. In addition, this data shows the most impressive changes between the treatment groups. For all the FISH data, these differences between groups are present, but they are more pronounced in the > 20 cell data.

Table 14 and 15 show the Ln total number of microcolonies over 20 cell size and the Ln total biovolume of PA in microcolonies over 20 cell size respectively. This is divided into group A (Nitric Oxide) and group B (Placebo). It can be seen that the NO group shows a reduction in mean Ln number and volume of microcolonies at T5 and T7 with means returning to baseline (or slightly above) by T10 and T20. In the placebo group the mean Ln number and volume of microcolonies shows no real change during the study.

Table 14 Ln number of colonies over 20 cell size per cm³

	T0	T5	T7	T10	T20
A Nitric Oxide	11.68	0.00	11.15	13.11	12.24
	10.16	10.77	10.50	11.97	*
	10.75	9.11	8.99	11.94	10.84
	11.54	10.63	12.40	12.97	12.22
	12.14	9.60	9.87	9.53	13.05
	9.84	0.00	0.00	12.43	*
mean	11.017	10.027	10.581	11.992	12.09
95%CI	10.058	9.184	9.227	10.624	10.631
	11.976	10.871	11.935	13.359	13.547
SD	0.914	0.804	1.290	1.303	0.916
B					
Placebo	8.93	9.75	10.98	*	*
	14.00	13.12	14.13	12.20	13.37
	10.61	9.01	10.04	9.90	10.76
	9.97	14.49	11.26	14.22	*
	11.98	11.74	13.11	12.53	11.82
	11.56	9.58	9.65	11.17	10.51
mean	11.176	11.281	11.528	12.004	11.61
95%CI	9.322	8.968	9.689	10.005	9.543
	13.030	13.593	13.368	14.003	13.686
SD	1.767	2.204	1.753	1.610	1.302

* Missing data (no sample received)

Table 15 Ln total volume of colonies over 20 cell size ($\mu\text{m}^3/\text{cm}^3$)

	T0	T5	T7	T10	T20
A Nitric Oxide	16.55	0.00	16.34	18.29	17.40
	15.79	15.65	15.36	17.01	*
	15.30	13.85	14.08	16.70	15.65
	16.25	15.46	17.25	17.67	17.18
	17.37	14.51	14.86	14.44	18.22
	14.80	0.00	0.00	17.79	*
mean	16.012	14.870	15.578	16.985	17.111
95%CI	15.047	13.987	14.270	15.547	15.406
	16.976	15.752	16.886	18.422	18.815
SD	0.919	0.841	1.246	1.370	1.071
B Placebo	14.05	14.56	15.48	*	*
	19.25	18.27	19.17	17.42	18.35
	15.29	13.41	14.39	14.35	15.45
	15.38	19.87	16.37	19.30	*
	17.18	16.63	18.22	17.35	16.96
	16.71	14.17	14.07	16.43	15.66
mean	16.310	16.152	16.284	16.968	16.604
95%CI	14.401	13.475	14.122	14.738	14.474
	18.219	18.830	18.445	19.198	18.734
SD	1.819	2.551	2.060	1.796	1.339

* Missing data (no sample received)

Figure 25 shows a scatterplot of the data from table 14 (Ln number of microcolonies >20 cell size per cm^3 over time). The interpolation lines show the mean values for each group. This shows that the nitric oxide group have a large reduction in mean Ln number of microcolonies during the period of treatment (day 5-7) which then returns to the same as the placebo group once treatment with the study drug has ended, despite the ongoing antibiotic treatment until day 10 (or longer in some cases). The placebo group shows no real variation in mean values throughout the study period.

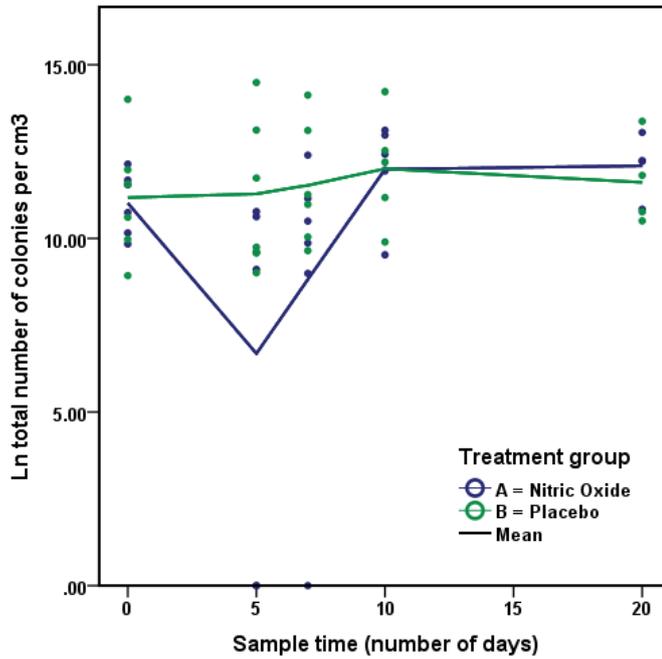


Figure 25 Scatterplot showing Ln number of microcolonies >20 cell size per cm³ over time

Figure 26 shows the same effect as figure 25, but for Ln total volume of microcolonies over 20 cell size (μm^3 per cm³). The effect is seen in the intervention group during the period of treatment, again returning to similar values as the placebo group after the period of inhaled therapy has finished.

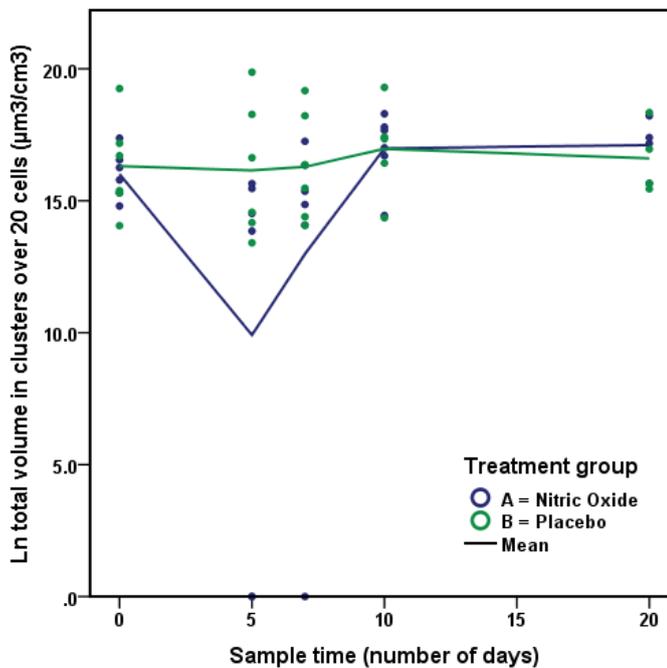


Figure 26 Scatterplot showing Ln total volume of microcolonies over 20 cell size (μm^3 per cm³) over time

Figure 27 shows representative FISH images from a CF patient being treated with NO adjunctive to conventional antimicrobial agents (ceftazidime and tobramycin) compared to a patient on antibiotics alone. Almost no PA biofilms were detectable in the treatment group compared to placebo.

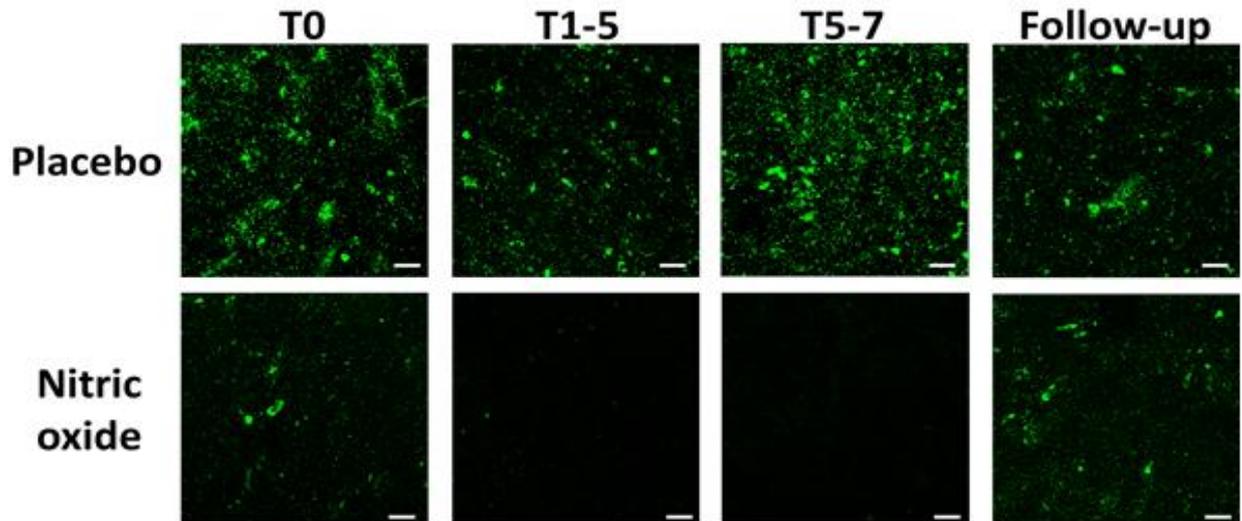


Figure 27 Reduction in PA biofilm with NO adjunctive therapy: representative FISH images from CF patient being treated with NO adjunctive to conventional antimicrobial agents (ceftazidime and tobramycin) compared to a patient on antibiotics alone. Scale bars = 25 μ m

The study was designed to detect trends in the data in order to see if further research in this area was warranted. As a result, simple statistical tests of significance were felt to not be appropriate because the data was complex and changes over time within each group needed to be accounted for. In addition, due to the fact that for practical reasons patients received between 5-7 days of treatment or placebo, combining these 2 time points for analysis of the data had the additional benefit of increasing the number of data points for analysis.

After discussion with the study statistician (Dr Victoria Cornelius), it was agreed to use generalized estimating equations (GEE) to look at the trends in the data. One benefit of GEE is that it produces quite accurate standard errors and therefore the confidence intervals have the correct coverage rates. (Hanley, Negassa et al. 2003, Hardin and Hilbe 2008). GEE is a method that allows analysis of repeated events over time, taking account of the correlation between observations within a participant.

To assess the association of outcomes by treatment group, the mean change from baseline on the natural log scale was compared between groups along with a 95% confidence interval for the estimate. This was achieved using 2 approaches. First the mean difference between the 2 groups was calculated at day 7 (end of treatment, table 16) and day 20 (follow-up only).

Table 16 Mean values of change from baseline in number and volume of clusters > 20 cells (FISH) at day 7 by treatment group.

7 day difference from baseline	Nitric Oxide				Placebo			
	n	mean	range	SD	n	mean	range	SD
Ln FISH: no. of aggregates containing > 20 cells	6	-2.19	-9.84, 0.86	3.93	6	0.35	-1.91, 2.05	1.44
Ln FISH: volume of aggregates containing > 20 cells	6	-3.03	-14.8, 1.0	5.88	6	-0.26	-2.64, 1.43	1.54

Then, in order to take account of measurements over time, a GEE analyses was conducted including treatment group as a co-variate (tables 17 and 18). GEE employs robust standard errors to account for longitudinal dependence. Residuals were examined to assess the validity of model assumptions. Patients with missing outcome data were not included in the analysis.

Adjunctive NO used in combination with tobramycin and ceftazidime demonstrated a significant reduction in PA clusters over 20 cells in size compared to those receiving placebo over the 7 days of treatment (GEE analysis, $p=0.031$, table 17 and figure 25). Data suggested less PA biofilm as quantified by both number and volume of aggregates greater than 20 cells in the NO group than placebo through day 7 while on NO therapy.

Table 17 Results of regression and GEE analysis showing mean difference of change between nitric oxide therapy compared to placebo in aggregates > 20 cells (FISH) at 7 days

Variable	Analysis	No. of patients (no. of observations)	Time points (change from baseline)	Mean difference	95% CI	p value
FISH: Ln no. of aggregates > 20 cells	Regression	12 (12)	day 7	2.55 ¹	-1.25, 6.36	0.166
	GEE	12 (24)	days 5 & 7	3.49 ²	0.32, 6.67	0.031
FISH: Ln volume of aggregates > 20 cells	Regression	12 (12)	day 7	3.00 ¹	-2.53, 8.54	0.253
	GEE	12 (24)	days 5 & 7	4.47 ²	-.04, 8.98	0.052

¹For the number and volume of aggregates >20 cells, the estimated difference in Ln mean between the nitric oxide and placebo groups where a high value indicates lower absolute numbers in NO group compared to placebo (estimate in direction of benefit for NO compared to placebo).

² For the number and volume of aggregates containing >20 cells, The GEE estimate takes into account all data in individual patients up to day 7, showing less PA biofilm in the NO group compared to placebo.

This effect was not maintained when treatment was stopped (Table 18).

Table 18 Results of regression and GEE analyses showing mean difference of change from baseline between nitric oxide therapy compared to placebo in FISH, CFU and qPCR up to day 20.

Variable	Analysis	no. of patients (no. of observations)	Time points (days from baseline)	Mean difference	95% CI	p value
FISH: Ln no. of aggregates > 20 cells	Regression *	-	-	-	-	-
	GEE	12 (43)	5, 7, 10, 20	1.35 ¹	-0.58, 3.27	0.170
FISH: Ln volume of aggregates > 20 cells	Regression *	-	-	-	-	-
	GEE	12 (43)	5, 7, 10, 20	2.35 ¹	0.08, 4.63	0.043

* estimates not calculated due to missing observations (4 available in each treatment group)

¹ For the number and volume of aggregates containing >20 cells, The GEE estimate takes into account all data in individual patients up to day 20, showing less PA biofilm in the NO group compared to placebo.

The analysis for this data was quite challenging due to few participants, great variation observed in outcome data and missing values. As a consequence it was hard to assess model assumptions and draw any robust conclusions. However, there is evidence for a significant treatment effect seen with FISH, indicating that

5.3.1.2 Over 10 cell cluster data

The following tables and graphs show the FISH data for biofilms in clusters of over 10 cells ($10 \times 3.665 \mu\text{m}^3$ or $>36.65 \mu\text{m}^3$). The over 10 cell cluster data shows the same pattern as the over 20 cell cluster data. Tables 19 and 20 show a reduction in mean Ln number and volume of microcolonies (over 10 cell size) at T5 and T7 in the NO group with means returning to baseline (or slightly above) by T10 and T20. The placebo group shows no real change in the mean Ln number and volume of microcolonies (over 10 cell size) during the study.

Table 19 Ln number of colonies over 10 cell size per cm^3

	T0	T5	T7	T10	T20
A Nitric Oxide	12.58	10.20	11.47	14.06	12.76
	10.95	11.33	11.27	12.89	*
	11.95	9.80	8.99	12.51	11.98
	12.26	11.57	13.48	13.79	12.83
	12.83	10.86	10.56	11.78	13.74
	10.75	8.84	0.00	12.88	*
mean	11.887	10.432	9.295	12.984	12.83
95%CI	10.986	9.353	4.279	12.106	11.681
	12.788	11.511	14.312	13.861	13.975
SD	0.859	1.028	4.780	0.836	0.721
B Placebo	9.62	10.85	12.08	*	*
	14.52	13.87	14.89	12.82	14.13
	12.07	10.96	10.33	11.28	11.57
	11.07	15.13	11.96	14.68	*
	12.82	12.59	13.57	13.12	12.47
	12.10	10.50	10.90	11.69	11.46
mean	12.035	12.315	12.289	12.718	12.41
95%CI	10.306	10.340	10.512	11.058	10.447
	13.763	14.289	14.066	14.377	14.371
SD	1.647	1.882	1.693	1.336	1.233

* Missing data (no sample received)

Table 20 Ln total volume of colonies over 10 cell size $\mu\text{m}^3/\text{cm}^3$

	T0	T5	T7	T10	T20
A Nitric Oxide	17.01	13.87	16.43	18.66	17.58
	15.99	15.89	15.73	17.41	*
	16.08	14.14	14.08	17.00	16.30
	16.65	15.95	17.82	18.13	17.43
	17.63	15.13	15.15	15.88	18.47
	15.22	13.09	0.00	17.92	*
mean	16.431	14.681	13.201	17.500	17.449
95%CI	15.541	13.462	6.288	16.470	16.033
	17.322	15.900	20.115	18.531	18.864
SD	0.848	1.162	6.588	0.982	0.889
B Placebo	14.26	15.20	16.17	*	*
	19.42	18.56	19.50	17.63	18.68
	16.25	14.92	14.63	15.31	15.87
	15.73	20.07	16.60	19.47	*
	17.49	17.07	18.39	17.63	17.18
	16.89	14.72	15.04	16.58	16.04
mean	16.673	16.754	16.723	17.323	16.944
95%CI	14.846	14.444	14.731	15.421	14.886
	18.500	19.065	18.715	19.225	19.003
SD	1.741	2.201	1.898	1.532	1.294

* Missing data (no sample received)

Figure 29 shows scatterplots of the Ln total number of microcolonies and total volume of PA in microcolonies >10 cell size over time. The interpolation lines show the mean values for each group. These scatterplots show that the mean number and volume of microcolonies decreases in the NO group during the period of treatment, returning to similar levels as the placebo group at the end of NO therapy, while the placebo group shows little change throughout the study period. This is the same as the effect seen with the >20 cell microcolonies.

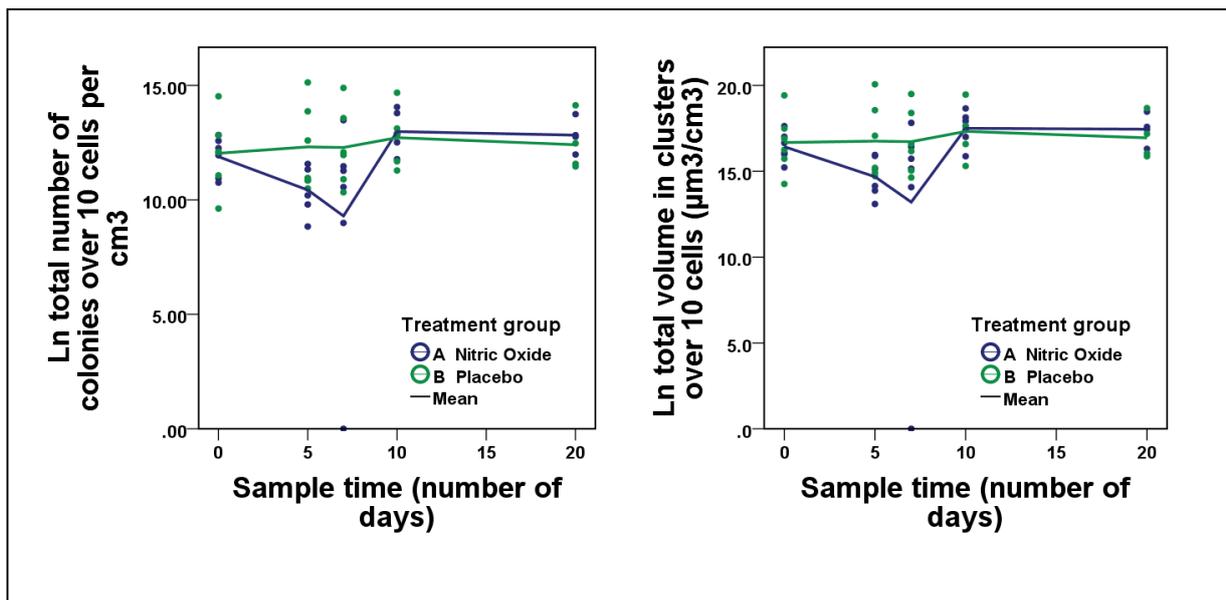


Figure 29 Scatterplot showing Ln number of microcolonies and Ln total volume of microcolonies >10 cell size per cm³ over time

5.3.1.3 Total *Pseudomonas aeruginosa* biomass data

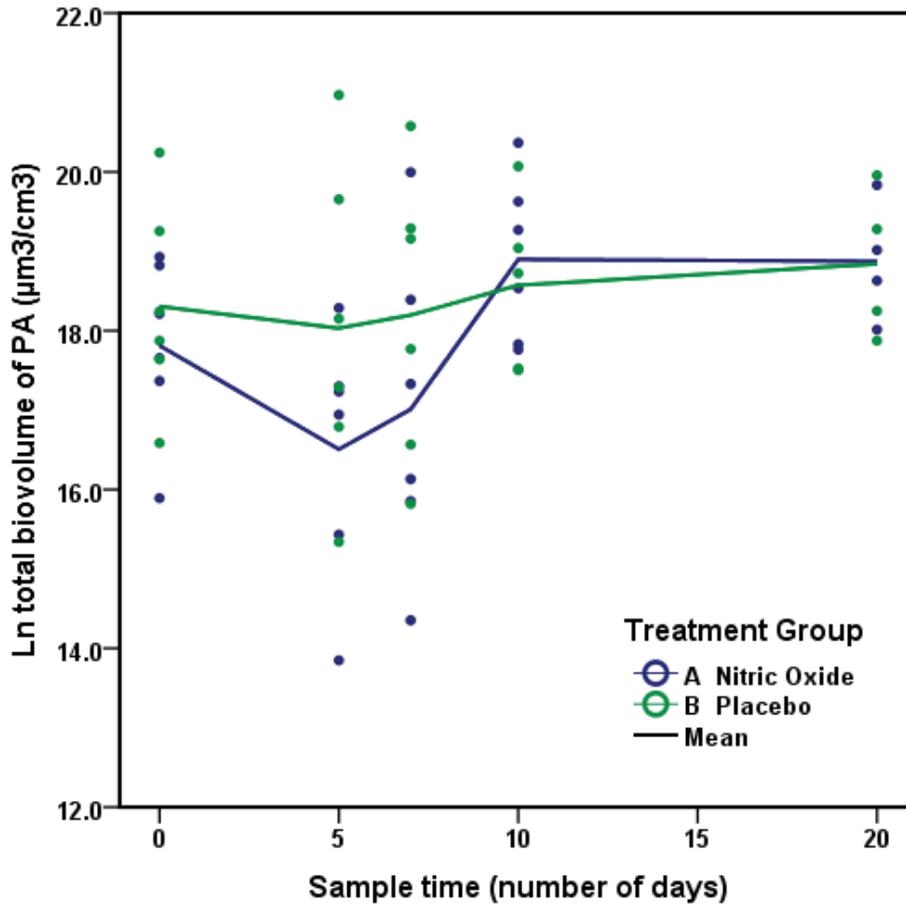
This split of the data takes into account all PA that is single cell size or above (using the lower limit of cell size; 0.164µm³ as described in section 3.2.2). It excludes any 'noise' in the sample below the size of a cell, but includes everything else and hence is a representation of the total biomass/biovolume of PA in the sample. Table 21 shows the Ln total PA biovolume for the 2 groups over time (number of microcolonies is not an appropriate measure here as the data includes single cells and small clusters).

Table 21 Ln total biovolume in sample (all single cells and above) in μm^3 per cm^3

	T0	T5	T7	T10	T20
A Nitric Oxide	18.93	17.23	18.39	20.37	19.02
	17.37	17.30	17.33	19.27	*
	17.66	15.43	15.85	17.83	18.01
	18.21	18.29	20.00	19.63	18.63
	18.82	16.94	16.13	17.76	19.83
	15.89	13.85	14.35	18.53	*
mean	17.813	16.507	17.009	18.898	18.874
95%CI	16.632	14.832	14.902	17.807	17.660
	18.995	18.182	19.115	19.990	20.085
SD	1.126	1.596	2.005	1.038	0.762
B Placebo	16.59	17.29	17.77	*	*
	20.24	19.65	20.58	18.72	19.96
	17.87	16.79	16.57	17.50	18.25
	18.24	20.97	19.16	20.07	*
	19.25	18.15	19.29	19.04	19.28
	17.64	15.34	15.82	17.53	17.87
mean	18.307	18.032	18.197	18.572	18.840
95%CI	16.960	15.901	16.305	17.223	17.320
	19.650	20.162	20.092	19.921	20.360
SD	1.284	2.031	1.804	1.087	0.953

* Missing data (no sample received)

In this data a similar pattern to the >20 cell and >10 cell data is seen. In the NO group the total biovolume of PA reduces during the period of treatment (T5-7) and then increases, with an overall increase in PA biovolume during the study period. The placebo group also shows an overall increase in PA biovolume during the study period, but with no real reduction in values during the period of inhaled therapy. Figure 30 shows the scatterplot of this data over time. The interpolation lines show the difference between the 2 groups (non-significant) with the NO group demonstrating a reduction in mean total PA biovolume during the period of treatment and no real change seen in the placebo group. This trend is the same as that seen in the previous FISH data for PA in clusters >10 and >20 cells.

Figure 30 Scatterplot showing Ln total biovolume of PA ($\mu\text{m}^3/\text{cm}^3$)

5.3.1.5 Single cell data

The single cell data (shown in tables 22 and 23) takes account of all objects in the images that are between the lower and upper limits of the pre-determined cell size; $0.164 \mu\text{m}^3 - 3.665 \mu\text{m}^3$ (as described in section 3.2.2). There is undoubtedly some inclusion of noise (at the lower end) and cells in clusters of 2-3 small cells (at the upper end), but this cut off allowed confidence in the inclusion of all single cells in the samples.

Table 22 Ln number of single cells (per cm³)

	T0	T5	T7	T10	T20
A Nitric Oxide	18.06	15.60	18.28	18.30	18.37
	16.96	17.14	16.91	17.46	*
	17.55	15.35	15.87	17.38	17.88
	18.10	18.61	20.14	19.56	18.32
	18.34	17.00	15.78	17.72	19.25
	15.12	13.67	14.56	17.59	*
mean	17.357	16.229	16.923	18.003	18.46
95% CI	16.097	14.424	14.815	17.134	17.547
	18.617	18.035	19.031	18.873	19.367
SD	1.201	1.72	2.01	0.83	0.57
B Placebo	16.81	17.25	17.71	*	*
	18.77	18.63	19.10	18.01	19.07
	17.96	16.93	16.88	17.92	18.46
	17.15	18.57	18.75	18.42	*
	18.96	18.06	17.86	18.94	19.12
	16.98	14.58	15.17	17.03	17.70
mean	17.770	17.337	17.578	18.064	18.59
95% CI	16.788	15.748	16.087	17.189	17.536
	18.752	18.926	19.069	18.938	19.639
SD	0.935	1.514	1.421	0.704	0.661

* Missing data (no sample received)

Table 23 Ln total biovolume of single cells ($\mu\text{m}^3/\text{cm}^3$)

	T0	T5	T7	T10	T20
A Nitric Oxide	17.74	16.18	18.04	18.90	18.40
	16.76	16.69	16.78	17.85	*
	17.05	14.75	15.23	16.92	17.48
	17.66	17.93	19.61	19.00	17.79
	18.13	16.32	15.11	17.34	19.22
	14.56	12.88	14.11	17.39	*
mean	16.983	15.792	16.481	17.898	18.223
95% CI	15.632	13.953	14.310	16.989	17.006
	18.333	17.631	18.651	18.807	19.440
SD	1.287	1.752	2.068	0.866	0.765
B Placebo	16.22	16.94	17.35	*	*
	19.01	18.67	19.39	17.95	19.04
	17.20	16.13	16.02	17.08	17.95
	17.49	19.10	18.78	18.67	*
	18.79	17.50	18.14	18.52	18.94
	16.61	14.03	14.55	16.64	17.38
mean	17.554	17.062	17.369	17.771	18.328
95% CI	16.361	15.125	15.467	16.669	17.055
	18.748	18.999	19.272	18.872	19.601
SD	1.137	1.846	1.813	0.887	0.800

* Missing data (no sample received)

The Ln number and total volume of single cells in the NO group decreases during the treatment period, returning to above baseline levels by the end of follow-up. The placebo group shows a similar picture with a small decrease during initial therapy, again increasing above baseline levels by the end of follow-up. It is important to note that there is not an increase in single cells during treatment in the NO group. This shows that despite NO causing release of bacteria from biofilms, the concurrent antibiotic therapy can effectively remove the planktonic cells. This data is reassuring and important for safety reasons that the number of single cells did not increase during treatment with NO.

Figure 31 shows the Ln number and volume of PA single cells over time. As mentioned above, there is a more marked decrease in the mean Ln number and volume of single cells in the NO group compared to the placebo group during the period of inhaled therapy, and both groups show a slow steady increase from the end of treatment to follow-up.

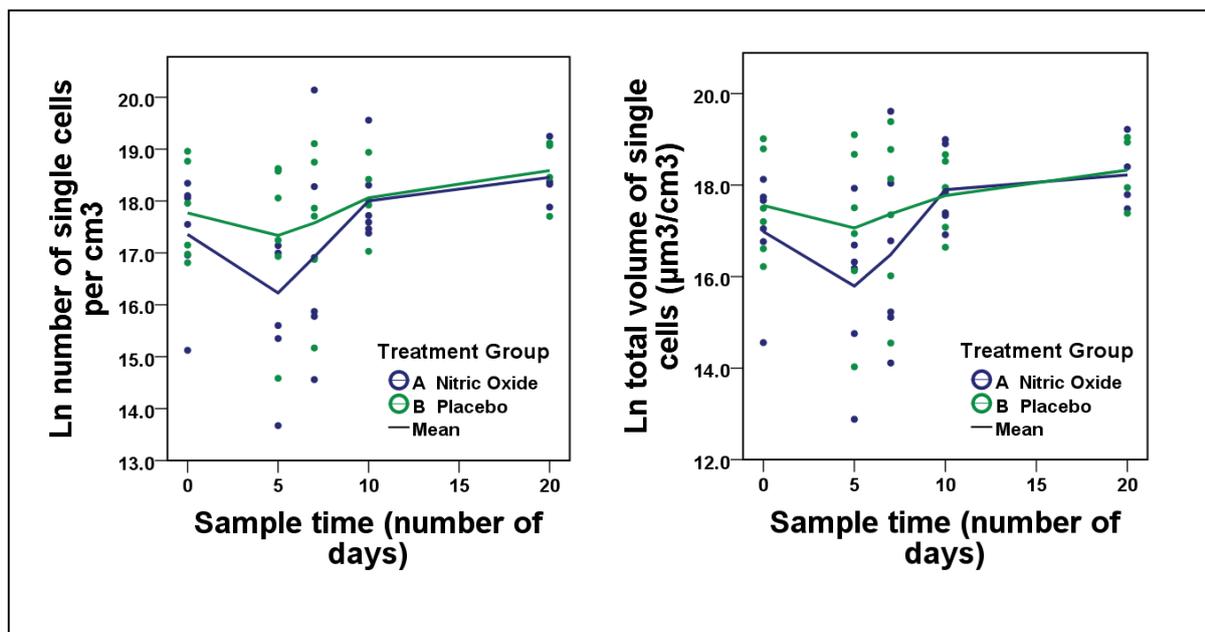


Figure 31 Scatterplot showing Ln total number and Ln total volume of single cells per cm³

5.3.1.6 Over single cell data

This group of data shows all PA in the samples above the size of a single cell (>3.665µm³). Undoubtedly, this will exclude some small clusters that are made up of cells smaller than the largest estimate of cell size; 3.665µm³ as described in section 3.2.2.

Tables 24 and 25 show the Ln number and volume of objects (clusters) over single cell size in each sample. They show a similar pattern to the single cell data with a greater decrease in number and volume in the NO group than the placebo group during the period of therapy (T5-7), with both groups showing a slight increase at follow-up.

Table 24 Ln number of objects (clusters) over single cell size per cm³

	T0	T5	T7	T10	T20
A Nitric Oxide	16.34	14.78	14.51	17.87	15.48
	13.79	13.54	13.77	16.83	*
	14.21	11.94	11.94	14.02	14.48
	14.52	14.53	16.36	16.11	15.09
	15.06	13.43	12.48	13.99	16.27
	12.14	10.22	10.81	14.55	*
mean	14.345	13.076	13.310	15.560	15.33
95%CI	12.880	11.269	11.223	13.861	14.138
	15.809	14.882	15.397	17.260	16.527
SD	1.395	1.721	1.989	1.619	0.751
B					
Placebo	12.92	13.54	14.03	*	*
	16.94	16.38	17.51	15.14	16.66
	14.33	13.44	13.02	13.85	14.43
	15.50	18.07	15.81	16.55	*
	15.67	14.16	16.07	15.20	15.52
	13.79	11.72	12.39	13.81	14.32
mean	14.859	14.554	14.804	14.908	15.23
95%CI	13.333	12.154	12.730	13.500	13.485
	16.385	16.954	16.879	16.316	16.980
SD	1.454	2.287	1.977	1.134	1.098

* Missing data (no sample received)

Table 25 Ln total biovolume of objects (clusters) over single cell size ($\mu\text{m}^3/\text{cm}^3$)

	T0	T5	T7	T10	T20
A Nitric Oxide	18.57	16.80	17.17	20.10	18.24
	16.57	16.52	16.47	18.99	*
	16.87	14.72	15.09	17.32	17.13
	17.35	17.08	18.85	18.87	18.06
	18.13	16.18	15.68	16.70	19.06
	15.59	13.37	12.81	18.15	*
mean	17.181	15.779	16.013	18.356	18.123
95%CI	16.044	14.268	13.872	17.064	16.861
	18.317	17.290	18.154	19.648	19.385
SD	1.083	1.439	2.040	1.231	0.793
B Placebo	15.41	16.06	16.70	*	*
	19.90	19.19	20.21	18.11	19.45
	17.16	16.07	15.71	16.44	16.91
	17.60	20.80	18.01	19.79	*
	18.26	17.41	18.91	18.14	18.04
	17.20	15.03	15.49	16.99	16.92
mean	17.588	17.425	17.505	17.893	17.829
95%CI	16.041	15.128	15.540	16.542	16.569
	19.136	19.722	19.469	19.244	19.090
SD	1.475	2.189	1.872	1.288	1.201

* Missing data (no sample received)

Figure 32 shows scatterplots of Ln number and volume of objects (clusters) over single cell size. They show little change in the placebo group (slight increase overall) over the whole study period, but a reduction in the amount of PA (both in number of clusters and volume) during the period of NO therapy with an increase following the end of therapy to follow-up.

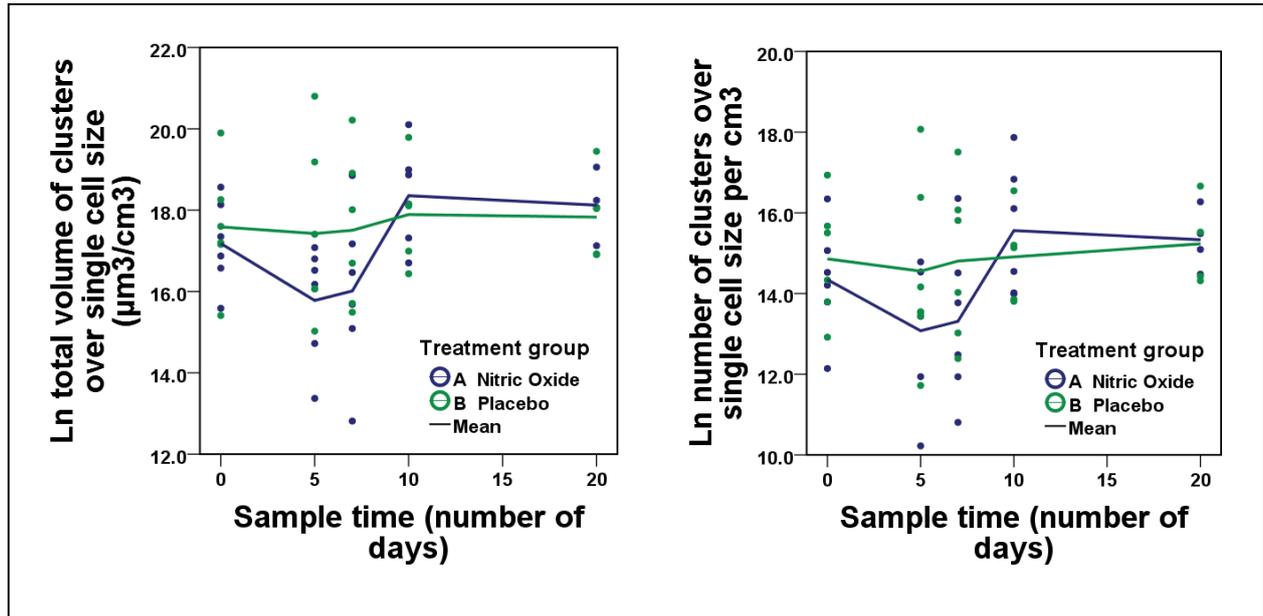


Figure 32 Scatterplots showing Ln total number and volume of objects (clusters) above single cell size per cm^3

5.3.2 Colony Forming Units

This secondary outcome measure assessment was looking at the effects of treatment on PA specific CFUs across all timepoints of the study, in comparison to the primary outcome assessment which was assessing the effects of treatment between T0 and T20-25.

The following table shows the Ln data over time in each patient for Cetrimide agar (PA specific CFUs, table 26), It can be observed that the treatment group start with higher mean CFUs than the placebo group. There is large variability in CFUs between patients and CFU load does not correlate to clinical status. The NO group do not show an increase in CFUs during treatment with NO which is reassuring from a safety perspective. Both groups show a decline in CFUs during antibiotic treatment.

Table 26 Ln mean CFUs on Cetrимide agar over time (PA specific)

	T0	T5	T7	T10	T20
A Nitric Oxide	15.58	9.81	9.99	16.55	16.3
	18.57	16.66	20	14.65	16.92
	17.41	16.52	13.92	16.21	18.66
	14.61	14.77	15.82	13.52	14.01
	14.47	14.15	13.85	14	16.22
	17.34	14.23	16.88	15.25	17.13
mean	16.330	14.357	15.077	15.030	16.540
95%CI	14.562	11.748	11.541	13.767	14.945
	18.098	16.965	18.613	16.293	18.135
SD	1.684	2.485	3.369	1.203	1.520
B Placebo	13.01	14.13	14.91	16.95	*
	14.33	15.12	16.87	17.13	18.36
	16.83	13.82	13.87	13.74	14.52
	12.44	7.4	5.2	6.8	6.8
	11.44	9.23	6.8	6.8	6.8
	15.12	13.78	13.55	16.43	16.49
mean	13.862	12.247	11.867	12.975	12.594
95%CI	11.804	8.954	6.916	7.794	5.814
	15.919	15.539	16.817	18.156	19.374
SD	1.961	3.138	4.717	4.937	5.461

* Missing data (no sample received)

Figure 33 shows the data in graphical form. The graphs show the mean CFUs on Cetrимide agar (*pseudomonas aeruginosa* specific agar). CFUs in the NO group are much higher than the placebo group even at baseline. In the second graph this difference at baseline has been removed by showing the change in CFUs from baseline. Both groups show a reduction in CFUs during antibiotic treatment, with the NO group returning to baseline values at the end of follow-up and the placebo group remaining slightly below baseline. Due to small numbers, these differences are not significant; however, it is important to note that CFUs for PA do not increase in the NO group during treatment during release of bacteria from biofilms.

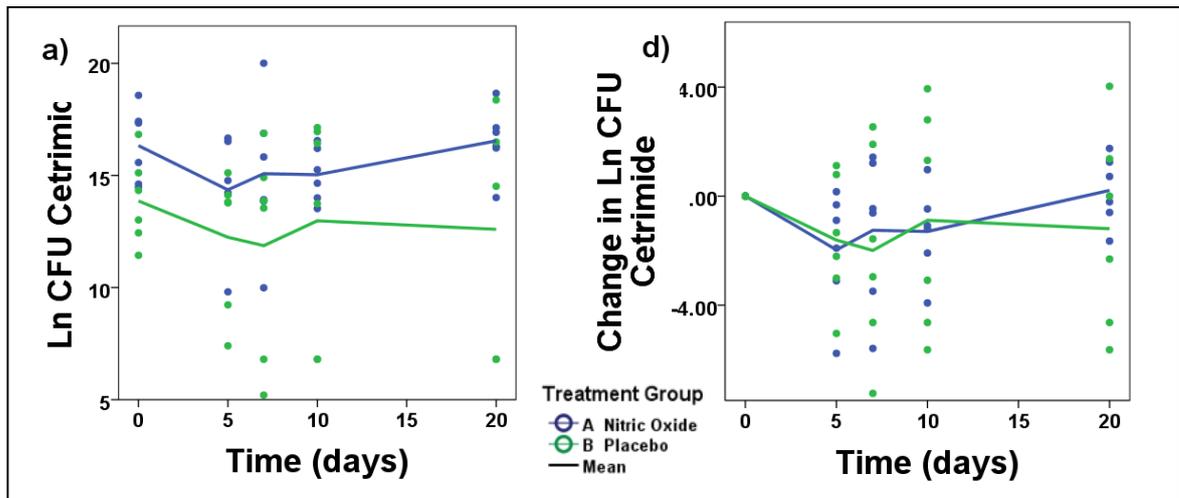


Figure 33 Scatterplots of PA CFUs showing Ln values and change in Ln values over time

The mean difference from baseline at day 7 (end of treatment) was calculated for both groups for CFUs on Cetrimide agar (table 27).

Table 27 Mean values of change from baseline in CFUs at day 7 by treatment group

7 day difference from baseline	Nitric Oxide				Placebo			
	n	mean	range	SD	n	mean	range	SD
Ln CFU	6	-1.25	-5.59, 1.43	2.76	6	-2	-7.24, 2.54	3.77

The regression and GEE estimates of mean difference (taking into account measurements within individuals over time) showed a marginal decrease at day 7 and day 20 in viable planktonic PA in the nitric oxide group compared to placebo. But all estimates were close to the value of no difference and were not significant as shown in table 28. From an individual participant safety perspective, there was no evidence that the biofilm dispersal increased the amount of PA detected in planktonic phase by CFUs.

Table 28 Results of regression and GEE analysis showing mean difference of change between nitric oxide therapy compared to placebo in CFUs

Variable	Analysis	No. of patients (no. of observations)	Time points (change from baseline)	Mean difference	95% CI	p value
Ln CFU	Regression	12 (12)	day 7	-0.74	-5.00, 3.51	0.706
	GEE	12 (24)	days 5 & 7	-0.19	-2.95, 2.56	0.891
Ln CFU	Regression	11 (11)	20	-1.64	-5.59, 2.30	0.37
	GEE	12 (47)	5, 7, 10, 20	0.03	-2.53, 2.59	0.98

CFUs for individual patients over time are shown in figure 34. This graph shows that some patients in each group have a reduction in CFUs over the course of the study. There is no particular pattern to this and a number of patients in both groups who show no/little change in CFUs despite antibiotic treatment. This will be discussed in more details in the chapter 7.

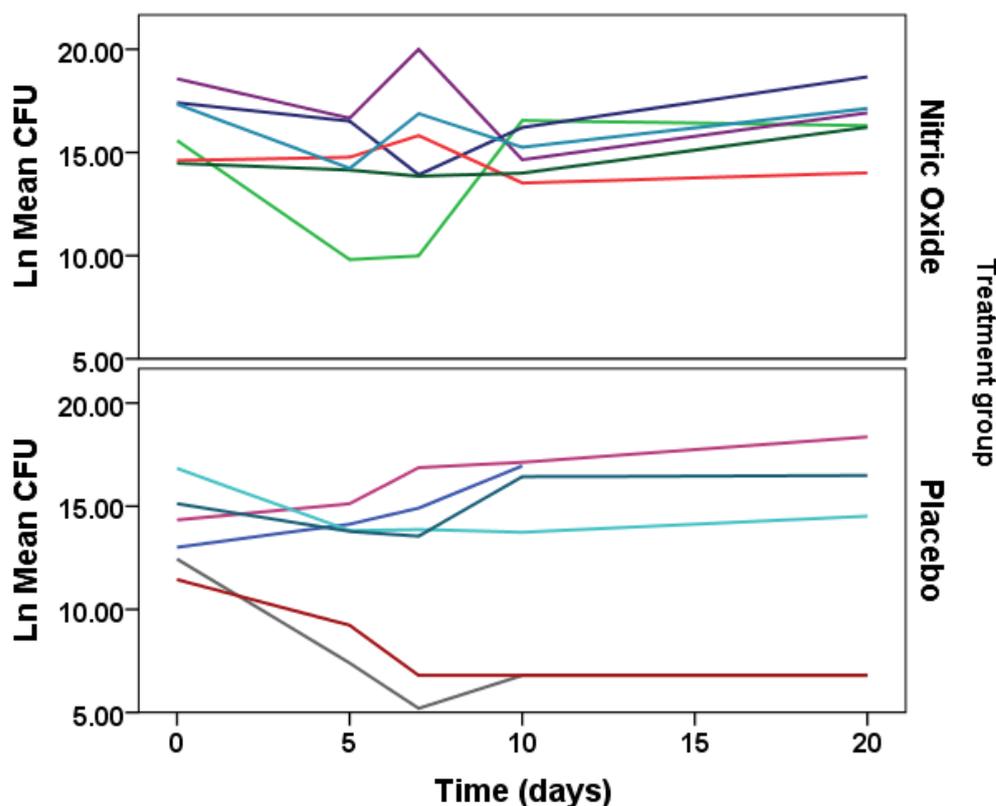


Figure 34 Ln CFUs over time per patient

5.3.3 Quantitative PCR (qPCR)

This secondary outcome measure assessment was looking at the effects of treatment on viable PA measured by qPCR across all timepoints of the study, in comparison to the primary outcome assessment which was assessing the effects of treatment between T0 and T20-25.

Table 29 shows the Ln mean qPCR data.

Table 29 Ln mean qPCR over time

	T0	T5	T7	T10	T20
A Nitric Oxide	6.95	4.96	0.51	4.06	5.52
	12.41	7.71	9.24	7.29	10.34
	16.55	14.51	12.93	12.59	11.71
	11.57	11.20	8.56	9.44	8.44
	10.54	10.23	9.51	6.28	5.88
	14.98	13.21	10.20	7.38	10.70
mean	12.166	10.302	8.492	7.841	8.766
95%CI	8.610	6.597	4.093	4.789	6.035
	15.722	14.007	12.891	10.894	11.496
SD	3.389	3.53	4.19	2.91	2.60
B					
Placebo	13.32	9.38	11.56	8.71	*
	13.27	11.18	7.82	13.61	14.43
	13.29	14.04	10.65	9.56	7.53
	3.72	0.00	1.29	0.00	0.00
	8.03	5.38	0.00	4.36	1.02
	13.72	12.43	8.06	10.59	12.05
mean	10.895	8.734	6.563	7.805	7.00
95%CI	6.569	3.265	1.500	2.702	-0.989
	15.220	14.202	11.627	12.908	14.996
SD	4.122	5.211	4.825	4.862	6.437

* Missing data (no sample received)

Figure 35 shows Ln qPCR and change in Ln qPCR over time, showing a general decrease in both groups over the period of treatment, with a gradual increase towards the end of follow-up. The graph with adjusted changes from baseline more clearly shows that the overall change from start to end of the study period is the same in both groups, with slight variability during the period of treatment with inhaled therapy and IV antibiotics. It is important to note that there is no increase in viable PA in the NO group compared to the placebo.

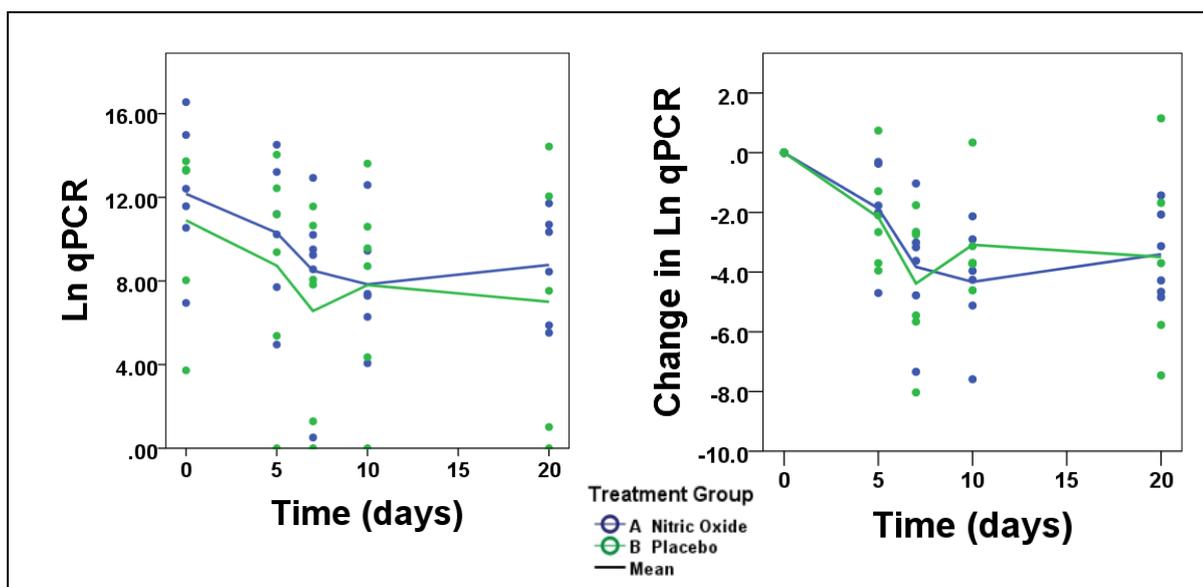


Figure 35 Scatterplots of Ln qPCR and change in Ln qPCR over time

The mean difference from baseline at day 7 (end of treatment) was calculated for both groups (table 30).

Table 30 Mean values of change from baseline in qPCR at day 7 by treatment group

7 day difference from baseline	Nitric Oxide				Placebo			
	n	mean	range	SD	n	mean	range	SD
Ln QPCR	6	-3.67	-6.44, -1.03	1.81	6	-4.33	-8.03, -1.76	2.44

As with the CFUs, the regression and GEE estimates of mean difference (taking into account measurements within individuals over time) showed a marginal decrease at day 7 and day 20 in viable planktonic PA in the nitric oxide group compared to placebo. But all estimates were close to the value of no difference and were not significant as shown in table 31.

Table 31 Results of regression and GEE analysis showing mean difference of change between nitric oxide therapy compared to placebo in qPCR

Variable	Analysis	No. of patients (no. of observations)	Time points (change from baseline)	Mean difference	95% CI	p value
Ln qPCR	Regression	12 (12)	day 7	-0.65	-3.42, 2.11	0.610
	GEE	12 (24)	days 5 & 7	-0.47	-1.91, 0.967	0.519
Ln qPCR	Regression	11 (11)	20	-0.50	-3.66, 2.65	0.73
	GEE	12 (47)	5, 7, 10, 20	-0.37	-1.44, 0.71	0.504

From an individual participant safety perspective, there was no evidence that the biofilm dispersal increased the amount of viable PA cells as detected by qPCR.

Ln qPCR for individual patients over time is shown in figure 36. This shows a general decrease over the study period in both groups, but with some patients showing greater variation over time than others. This will be discussed further in chapter 7.

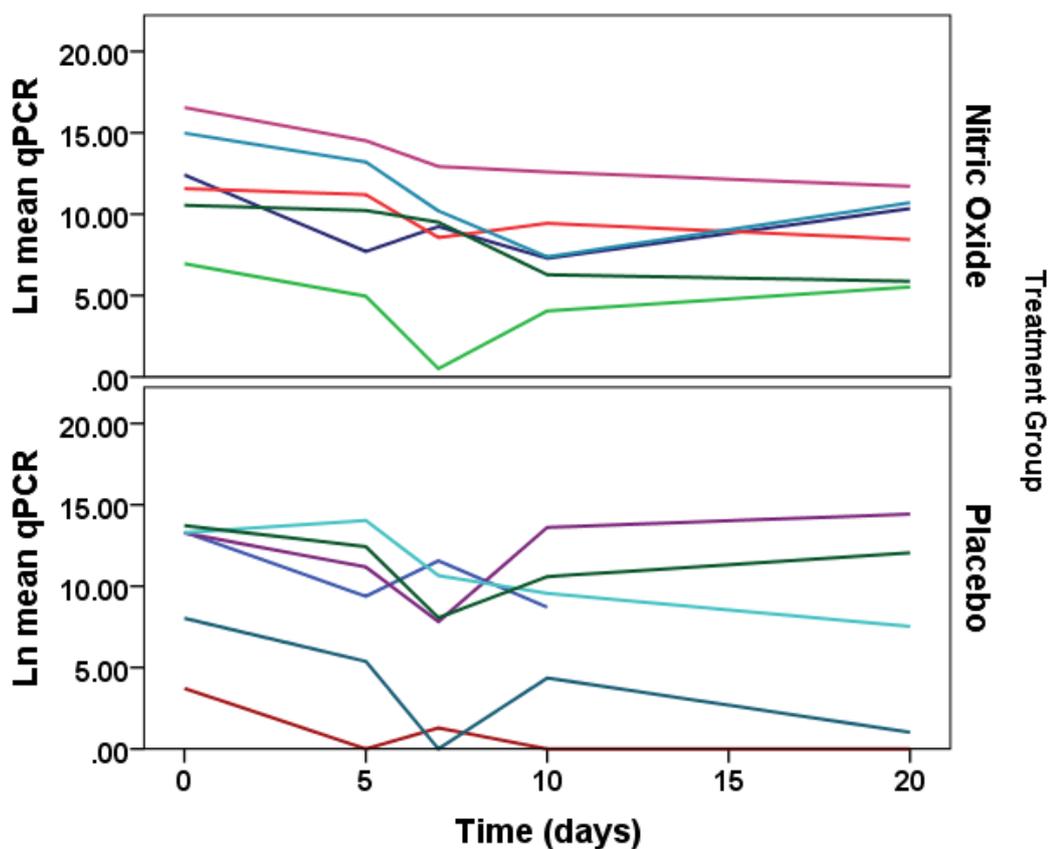


Figure 36 Ln qPCR over time per patient

5.3.4 Wider colony CFUs

CFUs were also measured on PIA (allows growth of all pseudomonads) and TSA (allows growth of the whole colony of bacteria). This was to assess the impact of the NO on the wider bacterial colony within the sputum. Ln mean values are shown in tables 32 and 33.

Table 32 Ln mean CFUs on PIA agar (all pseudomonads)

	T0	T5	T7	T10	T20
A Nitric Oxide	16.25	11.49	10.5	16.95	16.14
	18.56	17.28	16.37	17.3	17.33
	15.92	16.76	13.64	16.87	18.6
	17.15	15.44	17.64	14.64	14.58
	14.85	14.4	14.3	14.21	16.02
	18.05	17.05	15.41	18.42	18.42
mean	16.797	15.403	14.643	16.398	16.848
95%CI	15.338	13.085	12.037	14.686	15.216
	18.255	17.721	17.250	18.110	18.481
SD	1.390	2.209	2.484	1.631	1.556
B					
Placebo	14.18	14.39	16.21	17.02	*
	14.94	15.28	15.14	17.37	18.95
	16.85	13.33	14.37	14.57	13.66
	13.8	8.64	12.51	7.15	9.86
	12.65	14.61	9.8	9.86	12.33
	15.77	14.03	13.98	16.52	16.54
mean	14.698	13.380	13.668	13.748	14.268
95%CI	13.134	10.851	11.297	9.275	9.850
	16.263	15.909	16.039	18.221	18.686
SD	1.490	2.410	2.259	4.262	3.558

* missing data (no sample received)

Table 33 Ln CFUs on TSA (general growth agar)

	T0	T5	T7	T10	T20
A Nitric Oxide	14.74	13.68	14.19	17.11	16.21
	18.6	17.26	18.72	17.28	18.62
	17.06	17.05	15.82	16.59	18.72
	17.39	16.89	17.9	16.85	16.76
	14.96	14.94	14.76	14.21	16.47
	18.42	17.3	17.91	18.57	18.87
mean	16.862	16.187	16.550	16.768	17.608
95%CI	15.113	14.597	14.576	15.269	16.296
	18.611	17.776	18.524	18.268	18.921
SD	1.667	1.515	1.881	1.429	1.251
B					
Placebo	16.93	14.89	16.39	17.1	*
	16.45	16.44	17.78	17.67	19.16
	18	16.44	16.45	15.15	14.36
	15.17	*	13.36	12.14	14.01
	16.14	15.46	15.45	13.85	13.94
	16.04	16.73	16.47	16.84	17.27
mean	16.455	15.992	15.983	15.458	15.748
95%CI	15.456	15.022	14.426	13.198	12.825
	17.454	16.962	17.541	17.718	18.671
SD	0.952	0.782	1.484	2.154	2.354

* missing data (no sample received)

There is large variability in CFUs between patients and CFU load does not correlate to clinical status. The NO group do not show an increase in CFUs during treatment with NO which is reassuring from a safety perspective. Both groups show an overall decline in CFUs during antibiotic treatment, but the results remain highly variable between patients.

In figure 37 graphs a and b show CFUs on PIA agar (Ln mean CFU (graph a) and change from baseline (graph b)). The NO group have higher CFUs at baseline than the placebo group, but both reduce during antibiotic therapy and rise after antibiotic treatment ends. The treatment group show a slightly faster rise in CFUs at the end of inhaled therapy with the placebo group showing a more gradual rise over the period of follow-up, similar to that which the NO group shows after T10.

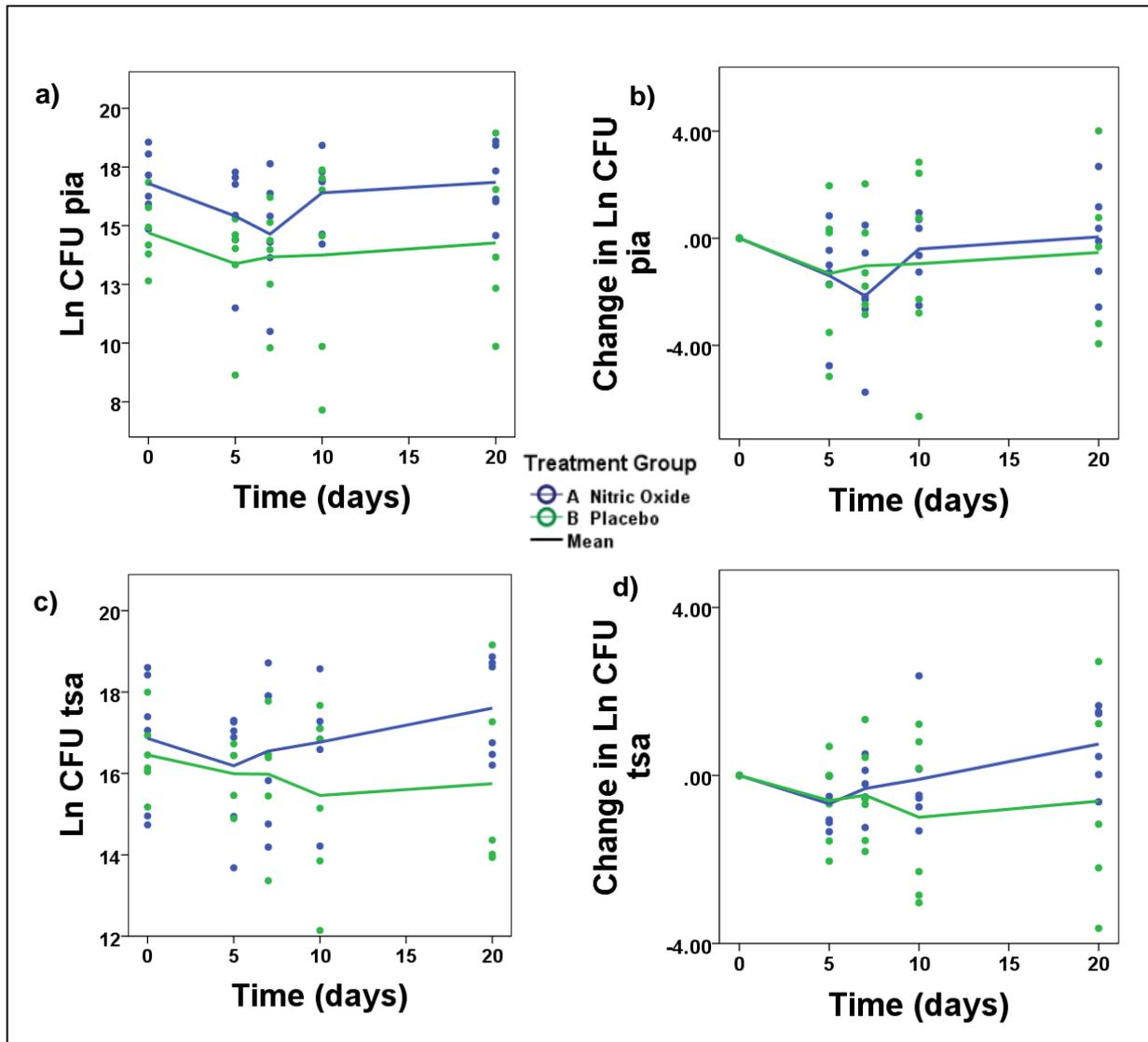


Figure 37 Scatterplots of whole colony CFUs showing Ln values and change in Ln values over time

Graphs c (Ln mean) and d (change in Ln mean) show CFUs on Tryptic soy agar (TSA) which is a non-specific general growth agar allowing growth of colonies of a wide range of bacteria. No significant change is seen between the 2 groups. The trend for the NO group is a slight increase during the follow-up period, but due to the large variability in the results, this is not significant.

5.3.5 NO levels in fresh sputum

NO levels in sputum were planned as part of the study in order to be able to try and estimate the uptake of NO by the lungs.

After researching the NO probes available, the Unisense model was chosen as the most sensitive and specific for NO gas. The probe was complicated to use with a lengthy calibration process that had to be carried out just prior to use. This created difficulties as it was not always known exactly what time of the day the sample would be given. The probe was used in the first participants sample, however, it became apparent that it would not be possible to continue to use it in this study. The probe took a long time to equilibrate in sputum and behaved completely differently to when used in solution, possibly due to the different sample densities. This resulted in an uninterpretable data readout which did not give the 'this sputum sample contains x amount of NO' as had been hoped. In addition, due to the small sputum sample sizes during treatment (as is often the case with CF patients on IV antibiotics), there was very little sample to measure the NO levels in. The tip of the probe is made of fine glass and very delicate and way the probe is set up it has to be lowered into the sample/solution to measure, so lowering it into a very small sample was risky in terms of hitting the bottom of the dish and breaking the probe (£800 to replace). Had the patient been given additional time to produce a larger sample, then the sputum would not have been analysed immediately after completing the 8 hours NO therapy, which had been the original intention for NO measurement. As a result, this measurement was not carried out in the study after the initial tests and trials in the first patient.

5.3.6 Assessment of wider microbial community

This analysis has not yet been carried out as there was no funding or time available within the original study to do this. Samples have been stored to allow this analysis to take place at a later date when researcher time and funding becomes available. This will look in detail at between group differences in the characteristics of the wider microbial community as assessed by microbial community analysis/molecular analyses using methods such as respiratory panel PCR, 16 RNA sequencing, metagenomics and MLST.

5.3.7 NO metabolites in blood

These results were provided by colleagues Prof Martin Feelisch and Dr Bernadette Fernandez from samples taken during the study and are shown in figure 39.

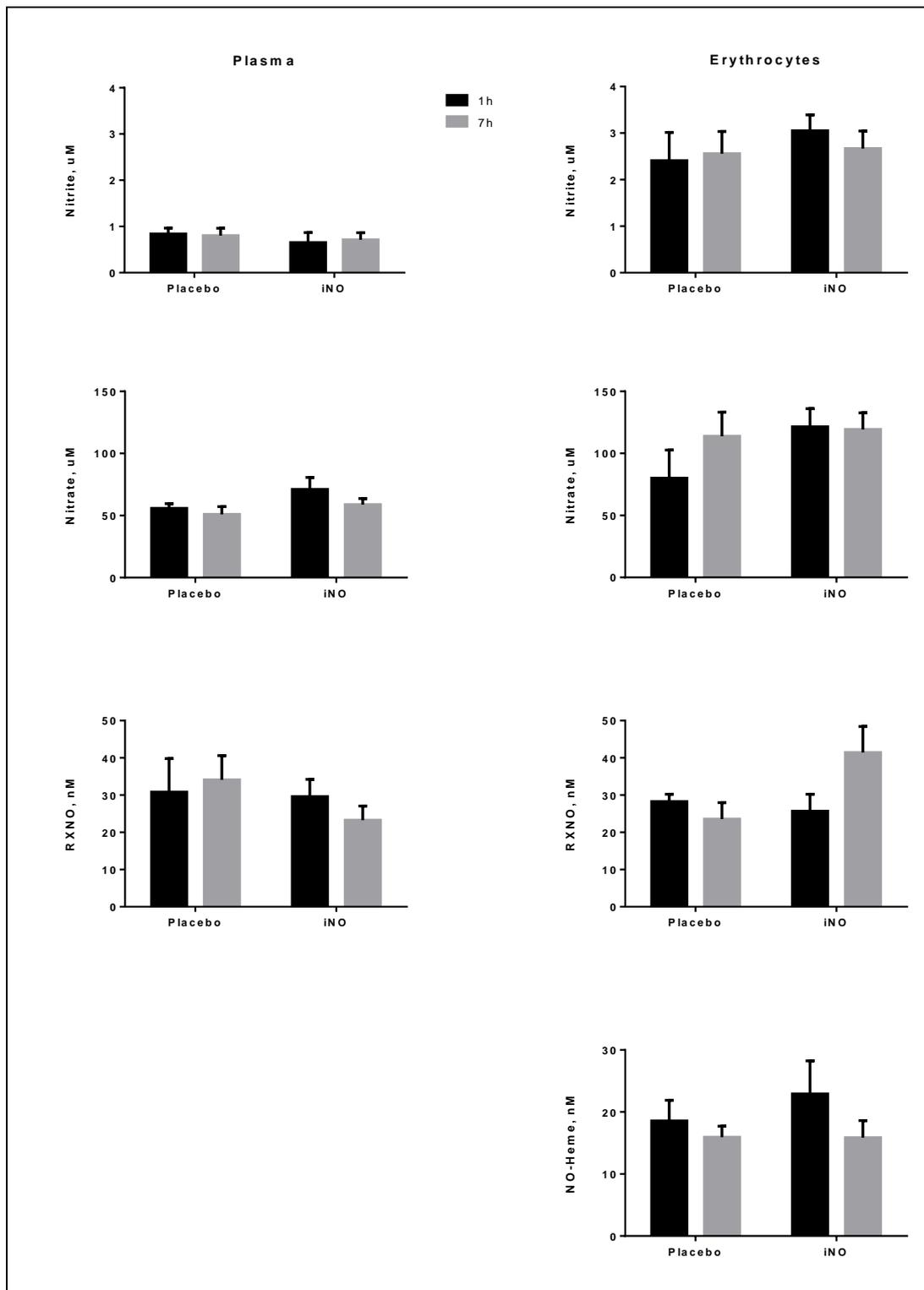


Figure 39 NO metabolite concentrations in blood plasma and erythrocytes for patient groups receiving either placebo or 10 ppm inhaled nitric oxide (means \pm SEM; n=5-6). (Graphs by Prof Martin Feelisch and Dr Bernadette Fernandez)

Determination of plasma NO metabolite changes in response to inhaled NO delivery revealed that the treatment group had higher nitrate levels, but this increase did not reach statistical significance (one way ANOVA $p > 0.05$). Plasma concentrations of nitrite and total nitrosation products paradoxically decreased during inhaled NO application, although this was not significant either. With the exception of unusually high nitrite levels in RBCs compared to plasmatic values there was also no obvious effect of inhaled NO on NO metabolite status in these blood cells, which is surprising given that NO-heme is the most sensitive marker of NO availability *in vivo* and nitrate is the final oxidation product of NO (Nagasaka, Fernandez et al. 2008) (thus, both might be expected to be elevated following prolonged NO inhalation).

Overall, determination of a comprehensive panel of NO metabolites suggested that low-dose inhaled NO did not significantly affect circulating NO metabolites in CF (figure 39). These results are at variance with the effects of inhaled NO on circulating NO metabolite concentrations in infants with pulmonary hypertension (Ibrahim, Ninnis et al. 2012) where a clear increase has been reported to occur with twice the concentration of inhaled NO that was used in this 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 exhaled breath of CF patients while systemic NO production does not appear to be compromised (Grasemann, Ioannidis et al. 1998) suggesting either accelerated degradation secondary to increased oxidative stress in epithelial cells; and/or NO consumption by the bacterial biofilm; or impaired gas exchange due to exaggerated mucus thickness. 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.

Chapter 6: Results - Clinical (safety) outcomes

6.1 Introduction

Clinical outcomes were measured as secondary outcomes for this pilot study for safety monitoring and to detect trends.

The clinical secondary outcomes studied were:

1. Between group differences in FEV₁ at T7, T10 and T20-25.
2. Between group differences in exhaled NO levels at T7.
3. Between group differences in HRQOL scores at T20-25.
4. Between group differences in length of course of antibiotics.

In addition, a variety of clinical monitoring was carried out to be able to assess the safety of the treatment including MetHb levels, clinical observations and adverse incident reporting.

6.2 Lung Function

Lung function was measured as described in chapter 2 using the Koko spirometer. This used the European Coal and Steel Community (ECSC) equations to calculate predicted values (Quanjer, Tammeling et al. 1993, Quanjer, Tammeling et al. 1993). These equations were based on Caucasian males age 18-75 working in the coal and steel industry, but were widely used for many years and were in use at the time of this study. They have since been replaced in 2012 with the Global lung function initiative (GLI) equations (Quanjer, Brazzale et al. 2013) in most clinical settings. These equations are based on data collected from all ages and a wide range of ethnic groups and are hence more widely applicable. However, these equations were not available in the software used by the spirometer when this study was conducted.

6.2.1 Forced expiratory volume in 1 second (FEV₁)

Measured at baseline and time points T7, T10 and T20-25, Table 34 shows the 'best' FEV₁ of 3 attempts. Great variability is observed in baseline values between patients. This is partly due to differing severity of disease, but also differences in age and height. Table 34 also shows the percentage predicted FEV₁ which is a

more sensitive measure of change as it takes into account the patient's age and height. It can be seen that the FEV₁ improves in both groups during treatment and then slowly declines over the follow-up period. This pattern is typical for a patient with CF admitted with a pulmonary exacerbation at which point their lung function has usually deteriorated from their baseline. As a result of antibiotic therapy the lung function usually improves in tandem with clinical signs and symptoms. Once antibiotic treatment is completed the lung function often begins to slowly decline again.

Lung function is normally distributed within the population and therefore did not require any transformation before analysis.

Table 34 'best' FEV₁ and percentage predicted FEV₁

'best' FEV ₁					Percentage predicted FEV ₁				
	T0	T7	T10	T20		T0	T7	T10	T20
A Nitric Oxide	1.19	2.42	2.14	1.31	A Nitric Oxide	37	75	67	41
	0.78	*	0.74	0.75		26	*	25	25
	0.96	1.13	1.00	*		34	39	34	*
	1.58	1.49	1.23	1.40		52	49	41	46
	2.53	2.84	2.51	3.10		74	83	74	79
	0.76	1.93	1.22	1.54		18	47	30	37
mean	1.30	1.96	1.47	1.62	mean	40.2	53.1	45.2	45.6
95%CI	0.59	1.11	0.75	0.53	95%CI	19.0	25.9	23.7	20.5
	2.01	2.82	2.20	2.71		61.3	80.2	66.6	70.7
SD	0.68	0.69	0.69	0.88	SD	20.1	21.9	20.4	20.2
B Placebo	1.01	1.27	1.22	1.02	B Placebo	22	28	27	23
	1.83	1.84	2.23	2.01		52	53	64	58
	1.46	1.91	1.77	1.76		45	59	55	55
	2.49	2.75	2.88	2.78		77	86	87	87
	1.16	1.34	1.36	1.32		39	45	46	45
	1.28	1.41	1.62	1.42		39	43	49	43
mean	1.54	1.75	1.85	1.72	mean	45.7	52.3	54.7	51.8
95%CI	0.97	1.17	1.20	1.06	95%CI	26.5	31.8	33.6	29.6
	2.11	2.34	2.49	2.37		64.9	72.9	75.7	74.0
SD	0.55	0.56	0.62	0.62	SD	18.3	19.6	20.0	21.2

* Missing data

6.2.2 Forced vital capacity

Table 35 shows the 'best' FVC from 3 attempts and the percentage predicted FVC.

Table 35 'best' FVC

'best' FVC					Percentage predicted FVC				
	T0	T7	T10	T20		T0	T7	T10	T20
A Nitric Oxide	2.04	3.48	2.86	2.05	A Nitric Oxide	55	95	78	56
	1.57	*	1.69	1.65		43	*	46	45
	1.23	1.91	1.61	*		42	63	53	*
	2.50	2.02	1.76	1.89		72	58	51	55
	3.10	3.47	3.12	2.35		79	88	80	69
	1.80	3.00	2.05	2.57		36	60	41	51
mean	2.04	2.78	2.18	2.10	Mean	54.4	72.8	58.2	55.2
95%CI	1.33	1.82	1.50	1.65	95%CI	35.9	51.3	40.7	44.2
	2.75	3.73	2.86	2.56		72.9	94.3	75.7	66.2
SD	0.67	0.77	0.65	0.36	SD	17.6	17.3	16.7	8.8
B Placebo	2.51	3.29	3.35	2.68	B Placebo	47	61	62	50
	3.16	2.82	3.27	3.17		79	75	82	80
	2.02	2.34	2.58	2.50		55	64	70	68
	3.72	3.83	3.82	3.89		101	104	104	106
	3.29	3.60	3.58	3.50		88	96	96	94
	2.20	2.18	2.60	2.38		59	58	70	69
mean	2.82	3.01	3.20	3.02	Mean	71.5	76.3	80.7	77.8
95%CI	2.11	2.30	2.66	2.39	95%CI	49.3	56.0	63.4	56.8
	3.52	3.72	3.74	3.65		93.7	96.7	98.0	98.9
SD	0.67	0.67	0.51	0.60	SD	21.1	19.4	16.5	20.0

* Missing data

Figure 40 shows the percentage predicted FEV₁ and FVC over time and shows a clear difference between the 2 groups during the period of inhaled therapy. The NO group show a rapid rise in both FEV₁ and FVC during the period of treatment with NO with a faster decline following the end of inhaled NO therapy, whilst IV antibiotics are ongoing (to T10). This decline then levels out over the follow-up period with final values being slightly higher than baseline as is also seen in the placebo group at follow-up. The placebo group show the classic pattern of a slow rise in lung function during IV antibiotic therapy with a slower decline once antibiotics have stopped during the period of follow-up.

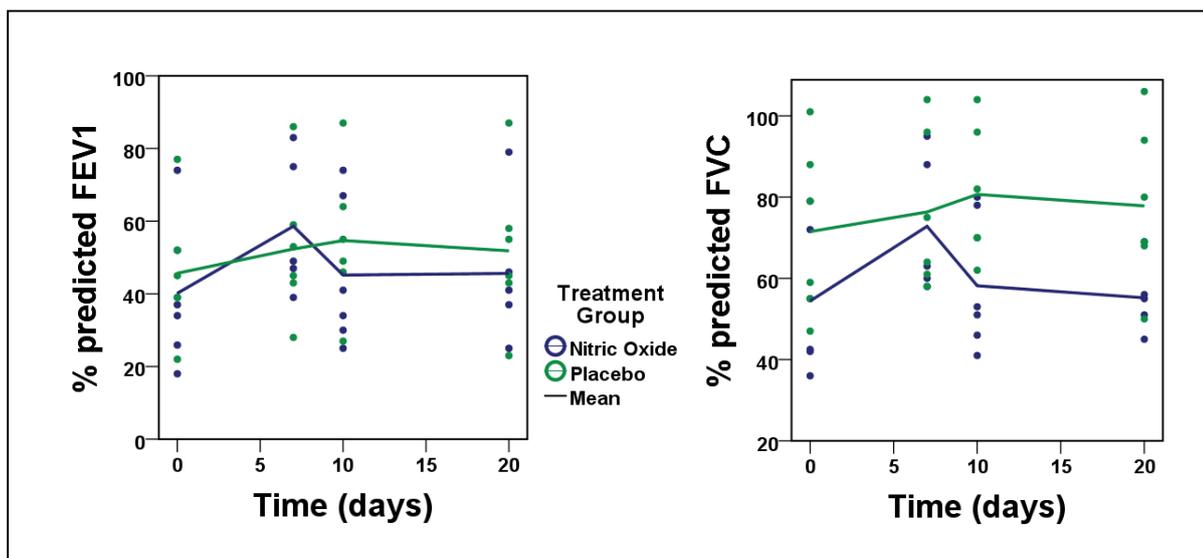


Figure 40 Scatterplots of FEV₁ and FVC over time

Clinical observations were consistent with the observed microbiological effect.

There was a greater absolute increase in FEV₁ and FVC in those treated with NO at day 7 compared with placebo (table 35).

Table 35 Mean values of change from baseline at day 7 by treatment group.

7 day difference from baseline	Group A Nitric Oxide				Group B Placebo			
	n	mean	range	SD	n	mean	range	SD
FEV ₁	5	15.6	-3.0, 38.0	17.2	6	6.67	1.0, 14.0	4.46
FVC	5	16	-14.0, 40.0	20.1	6	4.83	-4.0, 9.0	6.74

This mirrored the favourable changes in bacterial load but did not achieve statistical significance due to the small sample size (see table 36). These differences were not apparent at day 20.

Table 36 Results of regression and GEE analysis showing mean difference of change between nitric oxide therapy compared to placebo in clinical outcomes at day 7 and 20

Variable	Analysis	No. of patients	Timepoints (change from baseline)	Mean difference	95% CI	p value
FEV ₁ (l)	Regression	11	day 7	-8.93 ¹	-25.3, 7.42	0.248
FVC (l)	Regression	11	day 7	-11.60 ¹	-30.7, 8.42	0.229
FEV ₁ (l)	Regression	11	day 20	1.95 ²	-7.31, 11.20	0.645
FVC (l)	Regression	11	day 20	8.03 ²	-4.10, 20.2	0.168

¹ For both FEV₁ and FVC there was a large absolute increase in the NO group compared with placebo at day 7

² FEV₁ and FVC were slightly increased in the placebo group compared to NO at day 20

6.3 Exhaled Nitric Oxide

Exhaled Nitric Oxide levels were measured using the Niox mino machine. The data were plotted on a histogram and found to be negatively skewed, so a natural log transformation (Ln) was performed which resulted in the data resembling a normal distribution (see figure 41). Ln eNO was then used for all further analysis.

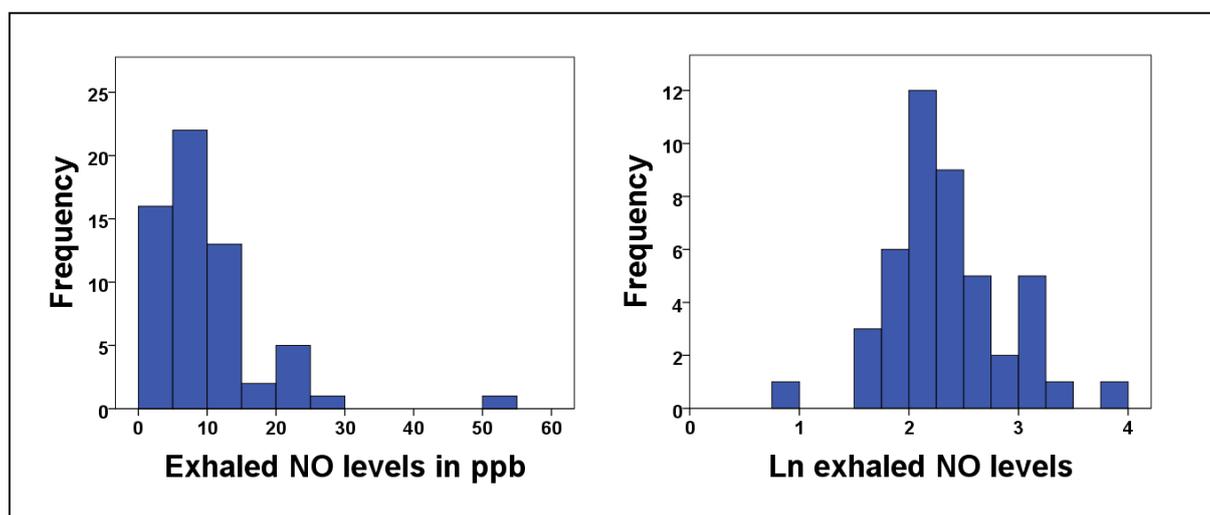


Figure 41 Histograms on eNO and Ln eNO

Because a number of patients registered 0 as their exhaled Nitric oxide level, in order to assess the changes over time with the Ln values it was necessary to add 1 to all eNO values before calculation of the Ln values (because it is not possible to calculate the Ln of 0, but the Ln of 1 is 0). This allowed comparison over time within patients using the Ln values. Table 37 shows the original eNO values, the eNO+1

values and the Ln eNO values. This demonstrates the need for adding 1 to the eNO values before transforming with the natural log.

Table 37 eNO, Ln eNO, eNO +1 and Ln (eNO+1) values

Treatment group	Patient ID	Sample time	eNO	Ln eNO	eNO+1	Ln (eNO+1)
B	1	0	0	*	1	0
B	1	5	21	3.04	22	3.09
B	1	7	8	2.08	9	2.2
B	1	10	51.5	3.94	52.5	3.96
B	1	20	11.5	2.44	12.5	2.53
A	2	0	11	2.4	12	2.48
A	2	5	10.5	2.35	11.5	2.44
A	2	7	7	1.95	8	2.08
A	2	10	8.5	2.14	9.5	2.25
A	2	20	9.5	2.25	10.5	2.35
A	3	0	28.5	3.35	29.5	3.38
A	3	5	0	*	1	0
A	3	7	0	*	1	0
A	3	10	8.5	2.14	9.5	2.25
A	3	20	8	2.08	9	2.2
B	4	0	23	3.14	24	3.18
B	4	5	6	1.79	7	1.95
B	4	7	11	2.4	12	2.48
B	4	10	8	2.08	9	2.2
B	4	20	6.5	1.87	7.5	2.01
A	5	0	7.5	2.01	8.5	2.14
A	5	5	13	2.56	14	2.64
A	5	7	2.5	0.92	3.5	1.25
A	5	10	7	1.95	8	2.08
A	5	20	5.5	1.7	6.5	1.87
A	6	0	14.5	2.67	15.5	2.74
A	6	5	22	3.09	23	3.14
A	6	7	8	2.08	9	2.2
A	6	10	11	2.4	12	2.48
A	6	20	8	2.08	9	2.2
B	7	0	10.5	2.35	11.5	2.44
B	7	5	5.5	1.7	6.5	1.87
B	7	7	0	*	1	0
B	7	10	7	1.95	8	2.08
B	7	20	5.5	1.7	6.5	1.87
B	8	0	14.5	2.67	15.5	2.74
B	8	5	23	3.14	24	3.18
B	8	7	19.5	2.97	20.5	3.02
B	8	10	19.5	2.97	20.5	3.02
B	8	20	23.5	3.16	24.5	3.2
B	9	0	7.5	2.01	8.5	2.14
B	9	5	9	2.2	10	2.3
B	9	7	7.5	2.01	8.5	2.14
B	9	10	10	2.3	11	2.4
B	9	20	14	2.64	15	2.71
A	10	0	14.5	2.67	15.5	2.74

A	10	5	0	*	1	0
A	10	7	0	*	1	0
A	10	10	6	1.79	7	1.95
A	10	20	10.5	2.35	11.5	2.44
B	11	0	0	*	1	0
B	11	5	0	*	1	0
B	11	7	0	*	1	0
B	11	10	0	*	1	0
B	11	20	8	2.08	9	2.2
A	12	0	0	*	1	0
A	12	5	0	*	1	0
A	12	7	0	*	1	0
A	12	10	0	*	1	0
A	12	20	0	*	1	0

* Unable to calculate Ln for 0

Table 38 shows the Ln (eNO+1) with means and confidence intervals. There is great variation in values within participants and over time with no clear trends.

Table 38 Ln (eNO+1) with means and confidence intervals

	T0	T5	T7	T10	T20
A Nitric Oxide	2.48	2.44	2.08	2.25	2.35
	3.38	0.00	0.00	2.25	2.20
	2.14	2.64	1.25	2.08	1.87
	2.74	3.14	2.20	2.48	2.20
	2.74	0.00	0.00	1.95	2.44
	0.00	0.00	0.00	0.00	0.00
	mean	2.247	1.370	0.922	1.835
95%CI	1.015	-0.223	-0.192	0.873	0.874
	3.478	2.963	2.035	2.797	2.813
SD	1.173	1.518	1.061	0.917	0.924
B					
Placebo	0.00	3.09	2.20	3.96	2.53
	3.18	1.95	2.48	2.20	2.01
	2.44	1.87	0.00	2.08	1.87
	2.74	3.18	3.02	3.02	3.20
	2.14	2.30	2.14	2.40	2.71
	0.00	0.00	0.00	0.00	2.20
mean	1.750	2.065	1.640	2.277	2.420
95%CI	0.282	0.853	0.267	0.898	1.901
	3.218	3.277	3.013	3.655	2.939
SD	1.398	1.155	1.308	1.314	0.495

6.4 Health Related Quality of Life

Table 39 shows all the QOL scores for each patient at T0 and T20-25. They are divided into each area covered by the CFQ-UK. Higher scores = better quality of life. There was only 1 patient who had missing data for 2 areas, the rest of the questionnaires were complete.

Table 39 HRQOL scores for CFQ-UK split by area

QOL area	Physical	Physical	Role	Role	Vitality	Vitality	Emotion	Emotion
Time	T0	T20	T0	T20	T0	T20	T0	T20
A Nitric Oxide	33	42	50	42	33	42	60	87
	25	25	67	83	50	50	93	87
	33	33	75	75	50	42	73	87
	79	83	42	33	58	58	100	93
	42	25	17	58	25	33	53	60
	0	0	25	50	8	33	47	40
	Mean	35.4	34.7	45.8	56.9	37.5	43.1	71.1
95%CI	8.4	5.8	21.9	36.7	17.7	32.8	48.3	53.6
	62.5	63.7	69.8	77.2	57.2	53.3	94.0	97.5
SD	25.78	27.59	22.82	19.30	18.82	9.74	21.77	20.93
B Placebo	75	92	75	92	75	83	87	80
	92	100	92	92	50	58	100	100
	25	33	33	42	42	42	87	93
	29	67	33	75	33	58	47	80
	63	75	100	100	75	75	100	100
	21	38	92	75	42	67	80	80
	Mean	50.7	67.4	70.8	79.2	52.8	63.9	83.3
95%CI	19.5	38.6	39.2	57.2	33.9	48.6	62.7	78.4
	81.9	96.2	102.5	101.1	71.7	79.2	104.0	99.4
SD	29.74	27.43	30.16	20.91	18.00	14.59	19.66	10.03

* missing data

QOL area	Social	Social	Body	Body	Eating	Eating	Treatment	Treatment
Time	T0	T20	T0	T20	T0	T20	T0	T20
A Nitric Oxide	67	56	56	56	67	78	56	78
	67	72	67	67	89	100	56	44
	67	78	67	78	78	89	56	56
	72	72	100	100	100	100	67	78
	22	22	33	33	44	44	22	11
	44	56	78	33	67	67	22	67
Mean	56.5	59.3	66.7	61.1	74.1	79.6	46.3	55.6
95%CI	36.1	37.9	43.3	33.8	53.6	57.0	26.2	29.0
	76.8	80.6	90.0	88.5	94.5	102.3	66.4	82.1
SD	19.37	20.38	22.22	26.05	19.45	21.56	19.13	25.33

QOL area	Social	Social	Body	Body	Eating	Eating	Treatment	Treatment
Time	T0	T20	T0	T20	T0	T20	T0	T20
B Placebo	72	78	11	22	100	100	56	44
	94	94	100	100	100	100	78	78
	72	67	100	100	78	89	33	44
	22	50	33	44	44	89	78	78
	89	72	100	100	100	100	78	78
	78	78	67	44	100	100	56	33
Mean	71.3	73.1	68.5	68.5	87.0	96.3	63.0	59.3
95%CI	44.3	57.8	27.8	31.3	63.2	90.3	43.9	37.5
	98.2	88.5	109.2	105.7	110.8	102.3	82.0	81.0
SD	25.67	14.66	38.75	35.43	22.68	5.74	18.14	20.68

QOL area	Health	Health	Weight	Weight	Respiratory	Respiratory	Digestion	Digestion
Time	T0	T20	T0	T20	T0	T20	T0	T20
A Nitric Oxide	56	44	100	100	28	50	67	89
	56	56	33	0	33	39	78	89
	33	56	67	67	50	56	100	100
	67	78	100	100	56	83	100	100
	22	11	0	0	28	44	22	22
	0	11	67	100	22	28	100	100
Mean	38.9	42.6	61.1	61.1	36.1	50.0	77.8	83.3
95%CI	12.6	14.6	20.2	9.6	21.9	30.1	45.6	51.4
	65.2	70.6	102.0	112.6	50.3	69.9	109.9	115.3
SD	25.09	26.67	38.96	49.06	13.49	18.92	30.63	30.42
B Placebo	44	67	0	33	50	83	89	78
	89	100	100	100	78	89	100	100
	33	44	100	100	44	50	100	100
	33	56	67	100	*	94	*	56
	78	89	100	100	89	89	78	78
	33	56	100	100	39	67	100	89
Mean	51.8	68.5	77.8	88.9	60.0	78.7	93.3	83.3
95%CI	25.6	45.9	35.4	60.3	36.9	60.9	82.9	65.6
	78.1	91.1	120.1	117.5	83.1	96.5	103.8	101.0
SD	25.00	21.56	40.36	27.21	22.01	17.00	9.94	16.85

* missing data

The average score for QOL (table 40) was better in the placebo group at the start of the study compared to the treatment group. As a result the difference between baseline and follow-up was used for comparison rather than individual scores.

Table 40 Mean values for total HRQOL score at start and follow-up

	T0	T20		T0	T20
A Nitric Oxide	55.92	63.47	B Placebo	61.16	71.01
	59.39	59.3		89.35	92.59
	62.36	67.87		62.31	67.03
	78.35	81.62		42.02	70.55
	27.59	30.46		87.38	87.96
	40	48.7		67.2	68.82
mean	53.935	58.570	mean	68.237	76.327
95%CI	35.227	40.213	95%CI	49.547	64.790
	72.643	76.927		86.926	87.863
SD	17.827	17.492	SD	17.809	10.993

Comparing the difference between the 2 groups in a participant's average score at T0 compared with T20 using a T test, the mean difference was -3.31 (95% CI - 13.03, 6.41) which is not significant (p=0.466). This is demonstrated in the box plot in figure 42.

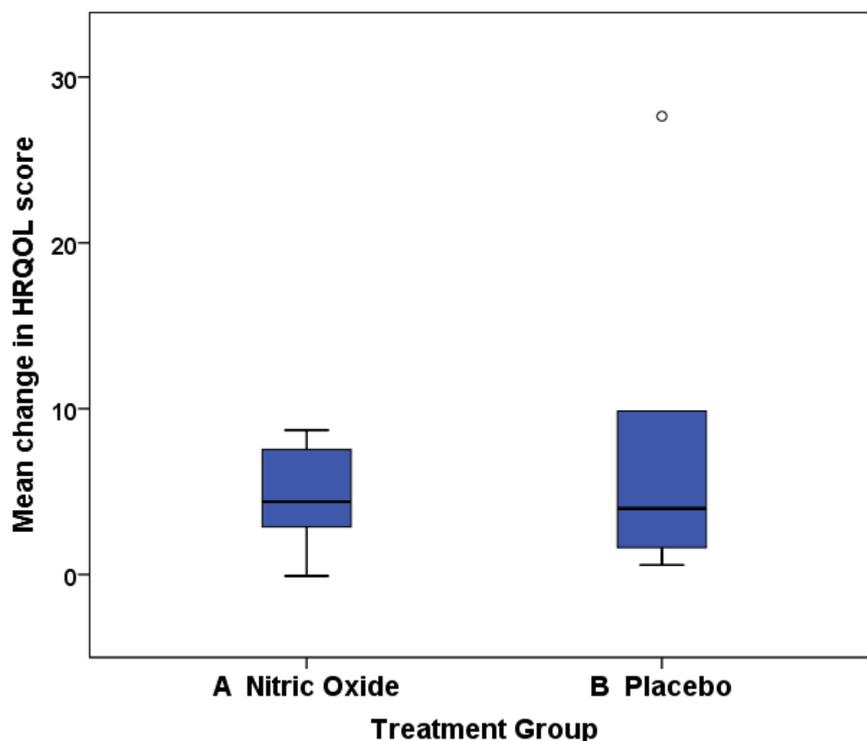


Figure 42 Box plot of mean change in HRQOL score

6.5 Length of course of antibiotics

The length of antibiotic course and inhaled therapy (NO/placebo) is shown in Table 41. Comparing length of antibiotic course between groups we can see that there is a trend towards a shorter length of course of antibiotics in the NO group compared with the placebo group.

Table 41 Length of course of antibiotics and inhaled therapy

	Antibiotics (days)	Inhaled therapy (days)
A Nitric Oxide	8	7
	16	7
	10	5
	6	5
	7	6
	7	6
	median	7.5
mean	9.0	6.0
95%CI	5.130	5.061
	12.870	6.939
SD	3.688	0.894
B Placebo	10	7
	10	5
	21	7
	10	7
	7	7
	17	6
	median	10.0
mean	12.5	6.5
95%CI	6.917	5.622
	18.083	7.378
SD	5.320	0.837

The trend for the length of inhaled therapy was also shorter. The protocol specified a minimum of 5 days, up to a maximum of 7 days for inhaled therapy to allow patients who had improved enough to be discharged from hospital to finish their

therapy at 5 or 6 days rather than having to stay in hospital taking a up a bed that could be used for another patient. Again, the trend is that the NO group had a slightly shorter length of inhaled therapy than the placebo group. Figure 43 shows box plots of the length of course of antibiotics.

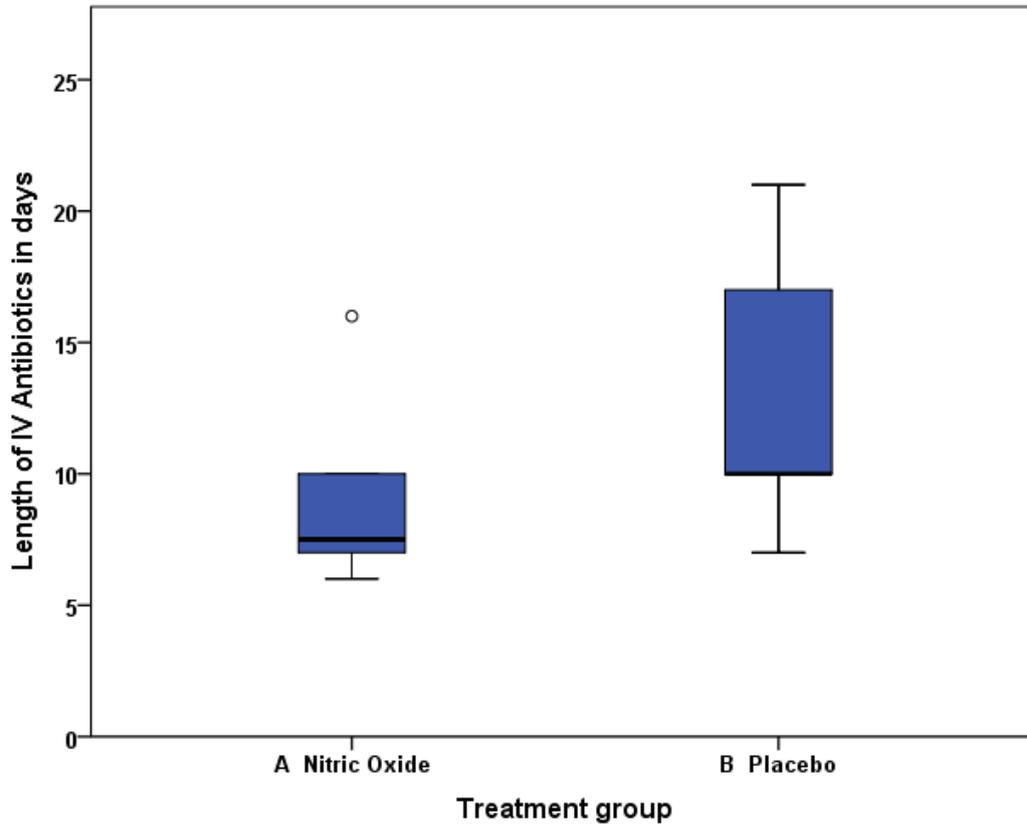


Figure 43 Box plots showing length of IV antibiotic therapy in days

Comparing these values using a Mann-Whitney U test, the differences are not significant. The null hypothesis that the distribution of antibiotic length is the same across both treatment groups is retained ($p=0.132$). In addition the null hypothesis that the distribution of inhaled therapy length is the same across both treatment groups is also retained ($p=0.394$).

6.6 Safety data

One of the major purposes of this study was to investigate the safety of giving NO to patients and monitor patients during administration to ensure that there were no adverse effects from giving NO in this way for this purpose.

6.6.1 Methaemoglobin levels

Levels of methaemoglobin (metHb) were measured at 1 and 7 hours during the first night of inhaled therapy.

Methaemoglobin levels were not raised in any participants. Figure 44 shows a graph of the individual values the highest of which was 2.2%. All participants except one had MetHb levels equal to or above 1% at both measurements.

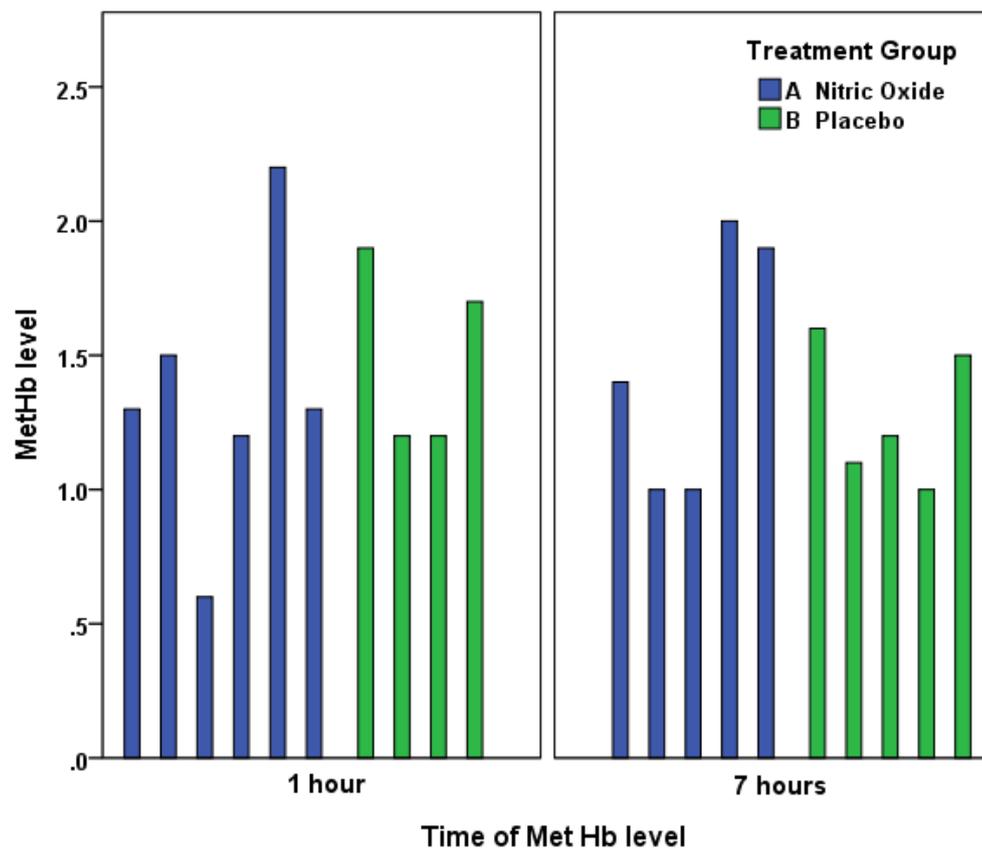


Figure 44 Graph showing MetHb levels for participants at 1 and 7 hours during the first night of inhaled therapy

Comparing the two groups using the T test, there was no significant difference (see table 42).

Table 42 Mean MetHb levels and statistics

	Treat-ment Group	N	Mean	Std. Devia-tion	Std. Error Mean	Mean Differ-ence	Std. Error Differ-ence	95% Confidence Interval	T test (p)
metHb levels day 1, 1 hour	NO	6	1.35	0.517	0.211	-0.150	0.299	-0.839, 0.539	0.63
	Placebo	4	1.50	0.356	0.178				
metHb levels day 1, 7 hours	NO	5	1.46	0.477	0.214	0.180	0.243	-0.380, 0.740	0.48
	Placebo	5	1.28	0.259	0.116				

6.6.2 Clinical parameters

The measurement of a number of clinical parameters including heart rate, blood pressure, oxygen saturations, respiratory rate, temperature and inspired oxygen concentration were measured each night during the period of therapy and recorded in the CRF.

Table 43 shows the parameters that were monitored through the course of the delivery of the inhaled therapy.

Table 43 Monitoring data during period of inhaled therapy

Treatment Group		Max HR, day 1	Min HR, day 1	Max O2 sats, day 1	Min O2 sats, day 1	Max RR, day 1	Min RR, day 1	Max syst BP, day 1	Min syst BP, day 1	Max insp O2, day 1	Min ins O2, day 1
NO	Mean	91.8	69.8	96.0	92.0	21.5	17.5	122.3	97.8	25.7	22.2
	SD	10.7	10.8	1.1	1.7	3.0	2.5	24.0	11.2	5.1	2.4
	Min	77.0	54.0	95.0	90.0	17.0	14.0	98.0	82.0	21.0	21.0
	Max	104.0	81.0	98.0	94.0	24.0	20.0	166.0	115.0	34.0	27.0
Placebo	Mean	101.0	80.7	95.7	91.8	21.0	17.8	126.2	111.0	23.7	22.5
	SD	13.9	14.2	2.3	3.1	3.3	2.6	18.1	19.4	3.1	2.1
	Min	82.0	60.0	93.0	88.0	16.0	14.0	110.0	93.0	21.0	21.0
	Max	120.0	96.0	99.0	96.0	25.0	21.0	161.0	149.0	28.0	26.0
Treatment Group		Max HR, day 2	Min HR, day 2	Max O2 sats, day 2	Min O2 sats, day 2	Max RR, day 2	Min RR, day 2	Max syst BP, day 2	Min syst BP, day 2	Max insp O2, day 2	Min ins O2, day 2
NO	Mean	94.2	69.7	94.8	91.5	20.2	17.5	122.8	103.3	25.3	23.5
	SD	17.1	10.7	1.0	2.4	3.1	2.1	24.6	12.7	3.8	3.9
	Min	73.0	58.0	94.0	89.0	17.0	15.0	101.0	87.0	21.0	21.0
	Max	112.0	84.0	96.0	95.0	24.0	20.0	166.0	119.0	30.0	29.0
Placebo	Mean	92.2	71.5	95.7	92.7	20.5	18.2	122.7	108.7	23.2	22.0
	SD	8.6	7.7	2.0	3.1	3.9	2.9	22.2	14.5	2.8	1.5
	Min	84.0	63.0	93.0	88.0	16.0	15.0	96.0	89.0	21.0	21.0
	Max	106.0	85.0	99.0	97.0	24.0	22.0	157.0	133.0	28.0	24.0
Treatment Group		Max HR, day 3	Min HR, day 3	Max O2 sats, day 3	Min O2 sats, day 3	Max RR, day 3	Min RR, day 3	Max syst BP, day 3	Min syst BP, day 3	Max insp O2, day 3	Min insp O2, day 3
NO	Mean	95.7	68.3	95.2	92.2	22.8	17.5	120.0	100.8	23.3	21.8
	SD	16.3	17.5	1.5	1.9	5.1	2.8	15.5	15.1	3.0	1.3
	Min	78.0	53.0	94.0	90.0	18.0	15.0	108.0	83.0	21.0	21.0
	Max	121.0	91.0	97.0	95.0	32.0	22.0	147.0	127.0	29.0	24.0
Placebo	Mean	94.7	71.8	96.2	92.8	22.7	18.7	126.8	108.0	23.2	22.0
	SD	8.3	7.1	2.0	2.7	3.8	2.4	15.0	14.3	2.8	1.5
	Min	84.0	63.0	94.0	88.0	18.0	16.0	112.0	91.0	21.0	21.0
	Max	105.0	83.0	100.0	96.0	29.0	22.0	151.0	133.0	28.0	24.0
Treatment Group		Max HR, day 4	Min HR, day 4	Max O2 sats, day 4	Min O2 sats, day 4	Max RR, day 4	Min RR, day 4	Max syst BP, day 4	Min syst BP, day 4	Max insp O2, day 4	Min insp O2, day 4
NO	Mean	95.8	68.0	96.7	91.8	22.7	17.7	118.7	98.8	23.2	21.3
	SD	18.5	16.8	1.9	1.9	5.8	2.0	8.8	17.7	3.4	0.8
	Min	82.0	52.0	93.0	89.0	18.0	16.0	110.0	80.0	21.0	21.0
	Max	127.0	92.0	98.0	95.0	34.0	20.0	135.0	124.0	28.0	23.0
Placebo	Mean	92.5	74.7	96.3	92.5	23.2	19.5	131.0	112.7	23.8	22.0
	SD	12.2	11.1	1.8	2.9	3.5	2.3	22.2	20.7	2.4	1.5
	Min	80.0	60.0	94.0	89.0	18.0	16.0	108.0	89.0	21.0	21.0
	Max	108.0	87.0	99.0	97.0	28.0	22.0	170.0	150.0	28.0	24.0

Treatment Group		Max HR, day 5	Min HR, day 5	Max O2 sats, day 5	Min O2 sats, day 5	Max RR, day 5	Min RR, day 5	Max syst BP, day 5	Min syst BP, day 5	Max insp O2, day 5	Min insp O2, day 5
NO	Mean	88.3	68.8	96.3	91.7	19.7	16.8	122.5	99.0	22.3	21.8
	SD	19.0	20.3	2.1	2.3	3.4	3.8	22.8	17.0	2.2	1.3
	Min	74.0	47.0	94.0	89.0	16.0	12.0	103.0	81.0	21.0	21.0
	Max	116.0	98.0	99.0	95.0	24.0	23.0	155.0	126.0	26.0	24.0
Placebo	Mean	92.7	68.0	95.5	92.8	22.7	18.7	126.7	108.3	24.3	22.0
	SD	12.1	10.8	2.3	2.4	2.5	2.5	15.9	19.2	2.3	1.5
	Min	79.0	59.0	94.0	90.0	19.0	16.0	112.0	88.0	21.0	21.0
	Max	106.0	89.0	100.0	97.0	26.0	23.0	149.0	133.0	28.0	24.0
Treatment Group		Max HR, day 6	Min HR, day 6	Max O2 sats, day 6	Min O2 sats, day 6	Max RR, day 6	Min RR, day 6	Max syst BP, day 6	Min syst BP, day 6	Max insp O2, day 6	Min insp O2, day 6
NO	Mean	85.8	64.3	97.3	90.0	20.3	18.0	120.0	102.8	25.8	22.3
	SD	20.5	20.9	1.5	8.0	4.0	5.2	23.5	18.2	8.8	2.5
	Min	67.0	44.0	95.0	78.0	16.0	12.0	98.0	82.0	21.0	21.0
	Max	115.0	93.0	98.0	95.0	25.0	24.0	145.0	119.0	39.0	26.0
Placebo	Mean	85.6	69.8	96.6	92.4	22.2	18.0	133.8	110.8	23.0	21.6
	SD	7.2	8.8	1.7	2.2	1.8	2.0	16.3	15.4	2.5	1.3
	Min	76.0	60.0	95.0	89.0	20.0	16.0	114.0	100.0	21.0	21.0
	Max	96.0	84.0	99.0	95.0	24.0	20.0	157.0	138.0	27.0	24.0
Treatment Group		Max HR, day 7	Min HR, day 7	Max O2 sats, day 7	Min O2 sats, day 7	Max RR, day 7	Min RR, day 7	Max syst BP, day 7	Min syst BP, day 7	Max insp O2, day 7	Min insp O2, day 5
NO	Mean	85.5	56.5	94.5	92.0	19.0	16.0	126.0	101.0	23.5	22.5
	SD	4.9	6.4	3.5	2.8	1.4	2.8	25.5	22.6	3.5	2.1
	Min	82.0	52.0	92.0	90.0	18.0	14.0	108.0	85.0	21.0	21.0
	Max	89.0	61.0	97.0	94.0	20.0	18.0	144.0	117.0	26.0	24.0
Placebo	Mean	91.0	69.3	96.3	92.3	22.3	16.8	124.5	96.3	23.5	21.8
	SD	10.2	9.9	1.5	2.9	2.6	1.5	16.4	8.8	1.7	1.5
	Min	76.0	60.0	95.0	90.0	20.0	15.0	114.0	90.0	21.0	21.0
	Max	98.0	83.0	98.0	96.0	26.0	18.0	149.0	109.0	25.0	24.0

There was no difference in any of these between the 2 groups when compared using a T test (see table 44).

Table 44 P values for monitoring during inhaled therapy ($p < 0.05$ = significant)

	Day	Max heart rate	Min heart rate	Max O2 sats,	Min O2 sats	Max resp rate	Min resp rate	Max syst BP	Min syst BP	Max insp O2	Min insp O2
T-test significance (p value)	1	0.23	0.17	0.76	0.91	0.79	0.82	0.76	0.18	0.43	0.80
	2	0.80	0.74	0.38	0.48	0.87	0.65	0.99	0.51	0.29	0.40
	3	0.90	0.66	0.35	0.64	0.95	0.46	0.46	0.42	0.92	0.85
	4	0.72	0.44	0.76	0.65	0.86	0.17	0.23	0.24	0.70	0.37
	5	0.65	0.93	0.53	0.41	0.12	0.35	0.72	0.39	0.15	0.85
	6	0.99	0.60	0.56	0.54	0.36	1.00	0.33	0.50	0.52	0.63
	7	0.53	0.18	0.41	0.92	0.19	0.68	0.93	0.71	1.00	0.63

6.6.3 Adverse Event Reporting

All formal reported AEs are shown in table 45.

Table 45 Adverse events and adverse reactions

Subject number	Treatment group	Description of event	Occurred during therapy?	AE/AR	SAE	Intensity	Expectedness	Causality assessment
R01	Placebo	Epistaxis	Follow-up	AE	N	Mild	Not expected	Unlikely to be related
R02	NO	Cough and cold	Follow-up	AE	N	Mild	Not expected	Unlikely to be related
R03	NO	Increased cough, reduced appetite	Follow-up	AR	N	Mild	Not expected	Possibly related
R03	NO	Reduced O2 levels during therapy	Therapy	AE	N	Mild	Not expected	Unlikely to be related
R01	Placebo	Epistaxis	Follow-up	AE	N	Mild	Not expected	Unlikely to be related
R05	NO	Epistaxis	Therapy	AR	N	Mild	Not expected	Possibly related
R05	NO	Haemoptysis	Therapy	AR	N	Mild	Not expected	Possibly related
R06	NO	Tobramycin (clinical error, extra dose given at t=12 on day 1 and then 24 hourly thereafter)	Therapy	AE	N	Mild	Not expected	Not related

There were 14 reported adverse events for the study none of which were severe.

The 8 detailed in table 45 relate to patients. 5 other adverse events related to

supply of medical gases which was required for the delivery of NO via the INOvent machine to deliver the study drug or placebo to patients. All of these resulted in minimal interruption to delivery of the inhaled therapy while the medical air cylinder was changed, which did not have had any effect on the overall delivery of the inhaled therapy to the patient.

There was one incorrectly reported AE which occurred in a patient who had been screened, but not randomised (and hence was not an AE according to the protocol). This patient was admitted to hospital for an operation on their small bowel as a result of a hernia/volvulus before they had been randomised which was unrelated to their involvement in the study.

The events detailed in table 45 are 3 adverse reactions that could possibly be related to the study intervention. These were an episode of epistaxis and an episode of haemoptysis while on therapy and increased cough and reduced appetite during follow-up. All of these symptoms were mild and self-resolving and are not unexpected with CF in clinical practice, so it is possible they were related to the NO therapy, but could also be related to the patients clinical condition and disease process. 5 adverse events were thought either not related or unlikely to be related to the study intervention. These included epistaxis during follow-up which is not uncommon in CF and thought unrelated to the NO as it was not being administered at this stage in the study. Reduced oxygen saturations occurred in one patient overnight; however, this is very common in CF and again thought unrelated to the NO. One patient had coryzal symptoms during follow-up and there was a clinical error in Tobramycin prescribing (extra dose given) which was identified, levels and bloods were checked and were not raised and the patient received the correct dosing following this. This was unrelated to the study drug which was prescribed and administered by the research team.

The majority of AEs and all the ARs are in the NO group, but this may be due to reporting bias as the people reporting AEs were the research nurses who were not blinded to treatment allocation.

Chapter 7: Discussion and Conclusions

7.1 Discussion

7.1.1 Introduction

This proof of concept pilot study was set up to investigate the effect of inhaled NO on bacterial biofilms when given to patients with CF. It was designed following observations made in pre-clinical studies that NO, delivered via SNP (an NO donor) to *ex vivo* sputum samples, could induce dispersal of PA bacteria from biofilms. This study delivered NO gas or placebo to CF patients concurrently with IV antibiotic therapy during pulmonary exacerbation to see if antibiotic therapy could be enhanced by disruption of PA biofilms.

The results are variable. The primary outcome did not show any difference between the 2 groups. Analysis of secondary outcomes shows a possible benefit of using low-dose NO as adjunctive therapy to enhance the efficacy of antibiotics used to treat acute *P. aeruginosa* exacerbations in CF and eliminate the potential pseudomonal biofilm-enhancing effect of tobramycin. There were a number of challenges encountered through this study which limited recruitment and in turn led to small numbers being randomised, which, alongside wide variability between and within patients, resulted in a reduction in validity of the results. These factors will now be discussed in more detail including strengths and weaknesses of various aspects of the study.

7.1.2 Methodology

There were a number of factors relating to the methodology that affected recruitment and resulted in lower than planned numbers of patients being randomised in the study. This had an impact on all the results.

Sample size

Analysis of the primary outcome for this study was not simple. Detailed assessment of biofilms grown from patient sputum has not been used as an outcome measure in previous clinical trials. Being able to accurately quantify bacteria in biofilm was important in order to assess treatment effect. Small sample size (only 12 randomised participants) affected all microbiological endpoints.

Recruitment to the study was affected by the requirement for additional study procedures (over and above routine clinical care) in particular the requirement for admission to hospital to receive the treatment and the additional blood tests required. In addition, the criteria for enrolment after screening were tight to enable microbiological changes as a result of therapy to be detected; however, this did limit the pool of potential participants. Overall, 35 patients were screened and 21 enrolled, however, only 12 of these suffered pulmonary exacerbations during the study period of a year, which fell short of the 20 that had been planned.

Screening was crucial for this small proof of concept clinical trial. The primary outcome measure looked at changes in the proportion of PA bacteria detected using 3 different microbiological methods (FISH, CFUs and Q-PCR). As such, it was important that any patients who were randomised had enough detectable PA in their sputum to enable changes to be seen (if present) as a result of the treatment given. Chronic pseudomonas colonisation (>50% or more of all cultures in the last 12 months positive for PA) was selected as criteria for inclusion with a screening sputum sample to assess the amount of pseudomonas present in the patient's sample in order to see whether they met the threshold for enrolment. Although when designing the study, this was felt to be essential to obtaining useful results from this pilot clinical trial, it resulted in a highly selected sample of patients with severe disease being recruited.

There were a number of other factors that had an impact on recruitment into the study. Inclusion criteria were patient age 12 and above and PA colonisation. There were very few paediatric patients who were eligible to be approached as they did not fulfil the criteria for chronic PA colonisation (needing 50% or greater sputum cultures positive for PA in the last 12 months). This could be due to improvements in treatment of early PA colonisation of the CF lung, local cohort or chance. This limited the number of eligible patients for the study and in particular reduced the number of paediatric patients recruited. Only one participant under 18 years (aged 16) was randomised.

Secondly, due to the screening process (which was necessary for the reasons stated above), there were 13 patients who fulfilled the criteria for having chronic PA infection, but who did not grow enough PA on their screening sputum sample to be enrolled into the study. Future larger studies could include a wider range of participants, but this was not practical for this study as the purpose of this study was

to see if there was an effect on PA biofilms *in vivo* when NO was administered alongside IV antibiotics. The primary outcome was a microbiological assessment of sputum, and if there had been no PA to assess in the sputum, then it would not have been possible to see whether the NO had had any effect. Much consideration was given to the use of clinical outcomes in the design phase whilst writing the protocol, however, it was felt that for this proof of concept study, a microbiological outcome was appropriate with clinical outcomes as secondary, exploratory and safety measures.

Exclusion criteria were more extensive because the study was only planned to randomise 20 participants and it was felt that reducing other variable factors that could have a significant impact on the results was important. Therefore, colonisation with *b. cepacia* was an exclusion criteria, as were patients on NIV, patients with NTM and severely unwell patients, unlikely to survive. In addition, patient factors that were anticipated in advance of the study that would affect recruitment such as inability to tolerate nasal cannula, for example patients who have nasal polyposis and are unable to breathe through their nose were also exclusion criteria as it was felt, it would not be possible to reliably deliver the treatment in these patients.

There were other factors that impacted on recruitment that were not part of the exclusion criteria, but were important considerations for patients when approached about the study, The intensity of the trial was a factor for some patients who declined to be screened. The study required them to be admitted to hospital and receive inhaled therapy for 8 hours a night for the first 5-7 nights of their IV antibiotics. In addition there was a follow-up visit 2 weeks after finishing IV antibiotics. For some patients who fulfilled the inclusion criteria, they were regularly receiving their IV antibiotics for exacerbations at home (as part of their routine CF care) and were reluctant to enter a study that would require them to be admitted to hospital for the first 5-7 days of their treatment (required by the protocol) in order to receive the inhaled therapy and be appropriately monitored. Some patients did not want a nurse monitoring them for 8 hours a night while they received the inhaled therapy, others did not want to be attached to the machine delivering the inhaled therapy for 8 hours a night and some did not want to travel to the hospital for the follow-up visit. Some patients approached were interested until they found out that the study would involve an additional 4 blood tests over those required for routine clinical care (2 of which were vital safety measures and hence completely

unavoidable). This was enough to lead these needle phobic patients to decline to take part in the study.

Two further patients enrolled in the study, but changed their minds when they were admitted with a pulmonary exacerbation as at that point, they didn't want to stay in hospital as required by the protocol. One of these patients subsequently re-consented to the study and was randomised on his next admission to hospital with an exacerbation.

Some enrolled patients were not able to proceed with the study as there was no CF bed available for admission to and their IV antibiotics had to start at home, making them ineligible to be randomised. This was a significant factor in the delivery of the study. Bed pressures were high on the CF unit at the time of the study, as is common in the NHS, so there were occasions where patients couldn't be admitted due to a lack of bed, but could receive their IV antibiotics at home. This then led to a clinical decision being made that the patient required IV antibiotics and could not wait until a bed was available to be able to be randomised into the study, hence the exacerbation was missed as an opportunity to randomise the patient. Clinical need had to come first and although this was frustrating for the study team, it was more important that the patient was able to receive the treatment they required.

One frequently exacerbating patient who was enrolled and fully expected to be randomised at some stage during the study suffered a gastrointestinal complication of CF and was admitted to hospital for alternative reasons. He had a long stay as an inpatient and received a number of IV antibiotic courses, but was ineligible for randomisation as his primary reason for admission was not pulmonary exacerbation.

At the stage of having randomised 12 participants, the study had been running for a year and was due to stop, but the recruitment target of 20 randomised patients had not yet been met. Experienced PIs on other respiratory studies were invited by the study team to be involved in a discussion as to whether it was best to continue the study longer than had been planned and recruit a further 8 patients or stop the study with 12 randomised and complete treatment and follow-up. A decision was made to stop with 12 randomised patients and complete the follow-up and analysis of the results. It was felt that the study was originally designed to look for trends to demonstrate an effect of NO *in vivo* rather than demonstrate statistical significance

and the consensus opinion was that if trends were present, they would probably be seen with data from 12 patients. Continuing the study to achieve the full randomisation of 20 would not necessarily lead to increased significance of the results as the numbers would still be low with large variation between and within participants. So, the study was stopped after 12 patients had completed follow-up and the results analysed looking for trends in the data.

The impact of only having 12 participants in the study is seen in all the results. A huge amount of data has been collected from these 12 participants, but there is great variability between and within patients which impacts of the validity of the results. Despite this there are many interesting observations that can be made from the results and would encourage further work in this area.

Sputum quantity and quality

The variability in sputum sample quality and quantity between and within patients affected the sample quality available for analysis. This was in part because sputum samples were spontaneously expectorated, rather than induced and for some patients there were occasions where they could only expectorate a very small sample and times where no sample could be produced. Prior to commencement of the study, it had been discussed at length whether to use induced sputum to obtain samples if/when the participant was unable to produce a sample spontaneously. However, previous studies suggest that the bacterial content of induced sputum varies from that of spontaneously expectorated sputum and therefore one cannot be reliably compared to the other (Mussaffi, Fireman et al. 2008, Rogers, Skelton et al. 2010). Consideration was given to using induced sputum for all specimens in the study and visits were made to another hospital which uses induced sputum routinely in its monitoring of CF patients to consider this procedure (Leicester). The method observed was not complicated, but, it was not feasible for this small study as it required equipment that was not routinely available for this group of patients and practical skills (from physiotherapists and/or specialist nurses) to obtain the samples that were not available to the department at the time of the study (Blau, Linnane et al. 2014).

Image analysis

As discussed in chapter 3 (image analysis) the initial image analysis programme that was planned for analysis of the results was COMSTAT. This was used during

the pre-clinical studies and is widely used in biofilm assessment and reported in scientific papers. However, it is based on an assumption that the biofilm is attached to the base layer, which was not the case in the samples from this study. The difference between the sputum samples from the participants in this study and those in the pre-clinical studies, was the fact that PA biofilms appeared to be suspended in the sputum in the study samples, whereas in the pre-clinical work, the biofilms had been grown from a base layer. As a result, the planned image analysis programme was felt to not fit the study samples and an alternative was sought.

Fiji was subsequently selected for analysis of the FISH data after wide consultation with biofilm and image analysis experts from within the university and hospital microbiology departments. Fiji is widely used for image analysis of 3D objects and is well recognised amongst image analysis experts as appropriate software for this use. It has not however, as far as was possible to ascertain from the literature and expert experience, been used for biofilm analysis in sputum samples previously.

The process for image analysis using Fiji was standardised and hence easily reproducible. It was time consuming for a single operator, but was not subject to variability in set threshold values and hence was reliable in analysis of each image stack. This method (as described in chapter 3) would be completely reproducible by another operator in these samples or in future studies looking at biofilm volume.

This is the first time this method of image analysis has been used in this way. Because of the reproducibility of the method, it may be possible for an individual or team, who are experienced in computer programming, to write a programme to run the analysis without operator involvement after the image stack to be analysed has been selected. If this was achieved it would make this method of analysis more widely available and practicable for use as it would remove the need for an individual operator to spend considerable time running the analyses. This would make analysis of much larger numbers of samples possible in a shorter time frame and hence increase the potential for biofilm volume to be used as an outcome measure in future studies.

7.1.3 Discussion of results

Primary outcome measure

The primary outcome in this study was to investigate a difference in proportion of bacteria in biofilm between the groups at follow-up, approximately 2 weeks after

finishing IV antibiotics (T20-25). This study showed no difference between the groups at this time point. The primary outcome measure was an assessment of microbiological outcomes including assessment of biofilm quantity, CFUs and qPCR. The reason that 3 microbiological assessments were included in one outcome assessment was because there was felt to be the potential to develop a new biomarker for assessing biofilms in clinical trials. When the primary outcome was discussed during the study design with the statistician, it was felt that it might be possible to do combined analysis on these results to form a composite biomarker assessment. However, this was not statistically possible in the analysis of the results because of the number of missing samples and data and the huge variability in the results both within and between patients. Hence the data is presented as 3 separate results; FISH, CFUs and qPCR. None of these results show any difference in the primary outcome measure between treatment and placebo group from baseline to follow-up.

FISH analysis of the quantity of biofilm in the sputum between baseline and follow-up showed no difference between the groups. This is likely because the patients included in the study are severely affected by their CF and hence chronically colonised with PA and other bacteria. Eradication of PA (and other bacteria) from these patients' lungs is not possible and hence as soon as antibiotics are stopped the bacteria that remain within the lung start to proliferate again. In retrospect, even if NO was effective in dispersing bacteria from biofilms, in this group of patients, the effect was unlikely to be sustained and the failure to show any difference in the primary outcome measure is a reflection of the severity of the patients included in the study.

On reflection, the selection of the primary outcome measure as difference between baseline and follow-up was not ideal. The assessment of the difference between groups at other time-points were included as secondary outcome measures, but the differences seen between the groups (as discussed further below) were during the treatment period rather than sustained at follow-up. This was potentially a result of the severity of the patients included in the study. However, this group of patients were selected because of the requirement for chronic pseudomonas colonisation and growth of enough PA in their sputum to allow comparison before and after treatment. In this small pilot study this was the most pragmatic approach given the small numbers in order to assess any effect of treatment, if present.

In retrospect it could have been predicted that the effect of NO (if present) would not be sustained in this group of chronically infected CF patients. Inhaled NO, given during a single episode of pulmonary exacerbations, was unlikely to be able to fully clear PA in chronically established biofilms in these patients' airways. The primary outcome selected was inappropriate in this group of patients and the changes seen during the period of therapy were where the effect was seen.

Missing data

During the study, sputum samples were produced at all 5 time points in 11 out of 12 patients and at 4 out of 5 time points in 1 patient (R1, T 20-25). In total, 59 sputum samples were obtained, however, 4 were too small to allow FISH analysis (7%), so these 4 samples only underwent CFU and q-PCR analysis (R1, T10; R3, T20-25; R8, T 20-25; R12 T20-25). There was no difference between the groups in production of samples, with 2 patients in each group having missing data for FISH at T 20-25, and 1 in the placebo group having missing data at T10. This has had an effect on the reliability of the results and differences between groups might have achieved significance for a greater number of variables had a more complete sample set been obtainable. The patient population was selected to include patients who had chronic PA infection and who were regular sputum producers, in order to increase the chances of obtaining sputum samples at each time point for as many patients as possible. It is not possible to correlate the production of sputum with treatment success as this is highly variable between patients, with some always producing sputum and others producing sputum less consistently. However, reduction in sputum production is one outcome that is observed clinically and is indicative of improvement after IV antibiotic therapy. It is therefore not unexpected that 4 patients had a reduction in sputum production to the point of not being able to expectorate a sample after receiving IV antibiotics. The small numbers of randomised participants increased the impact of these missing samples on the results.

Secondary Outcome measures (laboratory)

Despite the lack of effect of treatment seen at follow-up (primary outcome measure), the study did show an effect of treatment during the period of inhaled therapy as seen in the laboratory secondary outcome measures.

This proof of concept clinical study has demonstrated an effect on pseudomonal biofilm load in CF patients treated with NO gas plus conventional IV antibiotic therapy compared to IV antibiotics alone during the period of treatment which is not sustained at follow-up. Generalised estimating equations analysis showed that the treatment group had a significantly greater reduction in PA in clusters over 20 cell size during the period of inhaled therapy (NO). These findings are consistent with the hypothesis that NO causes dispersal of PA bacteria from biofilms (clusters) and, thus enhances antibiotic effectiveness.

FISH

The FISH data shows a reduction in PA in biofilms in the NO group compared to placebo, during the period of treatment with NO (T5, T7), in all splits of the data (single cells, over single cell size, >10 cell clusters and >20 cell clusters). This was found to be statistically significant in the clusters over 20 cell size (representing biofilms) as demonstrated by GEE analysis. Similar trends were seen across all FISH data, but statistical significance was only achieved for the larger clusters. This result supports the hypothesis that NO causes dispersal of bacteria from biofilms through the previously described mechanism of increasing phosphodiesterase activity and thus inhibiting or degrading c-di-GMP and its ability to promote biofilm growth.

The FISH analysis is impacted by the zero values in a couple of patients. These are included in the analysis as they are true zeroes. The FISH analysis split the data into groups according to the size of the microcolonies, so the zeroes are where there is no PA in clusters over 20 cell size. These samples still contain PA (as seen in the other splits of the data e.g, single cells). Long discussions were had with the statistician about the inclusion of these values and the advice was that they are true zeroes as clusters over 20 cell size have completely disappeared from those patients' samples.

Looking at the results in individual patients, it looks like there may be responders and non-responders to NO. In such a small sample, it is not possible to draw any real conclusions in this area, but there do appear to be a couple of patients who have a dramatic response compared to the others. This would be interesting to look at in more detail in future studies.

This was a small proof of concept pilot study where indicative trends were anticipated to be more likely than statistically significant differences for many of the selected outcome parameters. Although there was no effect of NO when assessed at follow-up which was the primary outcome measure, there was an effect seen on biofilms when NO is delivered to patients during the period of treatment in this group of severely affect CF patients. These results are based on a small group of patients, who are severely affected by their CF and therefore cannot necessarily be generalised to a wider group of CF patients. However, the study was designed as a proof of concept study to assess whether the effects of NO that had been observed in pre-clinical studies, using PA grown from sputum samples from patients with CF, could be seen when inhaled NO was given directly to CF patients.

The results show an effect during treatment, and although statistical significance is only achieved for the larger clusters of cells, the trends of improvement in the PA load during treatment is seen across the FISH analysis. The trends seen would need to be looked into further in future studies to see whether they are reproducible and have any impact on clinical outcomes that would be relevant to patients (as discussed further below).

Single cell analyses were important to ensure that, following dispersal of biofilm, there was no increase in planktonic bacteria. If this had occurred, it might have caused significant problems for patients including dispersal of bacteria to other parts of the airway and worsening respiratory compromise. For this reason, it was essential that IV antibiotics were given alongside NO, in order to treat any dispersed bacteria. Previous studies have shown that antibiotics are poorly effective against biofilms, but have greater efficacy against planktonic bacteria. Release of PA bacteria from their microcolonies might have enabled a greater antibiotic effect in reducing overall bacterial load. The study showed that there was no increase in single cells in the treatment group compared to the placebo group and this was reassuring from a safety perspective. The need for concomitant IV antibiotics was important in order to treat the released bacteria and maintain safety for the participants.

CFUs

In addition to the FISH single cell analyses, CFU counts were also analysed to assess safety of release of bacteria from biofilms with NO therapy. CFUs measure planktonic bacteria and hence any increase in CFUs in the treatment group would

cause concern. Little difference was seen between the NO and placebo group in CFUs which provided reassurance from a safety perspective.

Using CFUs to monitor treatment effect is also fraught with difficulty. Problems with traditional culture methods are well recognised, however, these methods remain central to routine clinical microbiological processing and were important to include in this study for safety reasons. There is evidence in the literature of inconsistency in results from the same patient and within samples due to the heterogeneous nature of sputum. Determination of CFUs also varies between laboratories and technicians (Pye, Stockley et al. 1995, Foweraker, Laughton et al. 2005, Rogers, Skelton et al. 2010). In order to try and minimise variability in this study, CFUs were counted manually by one of two scientists within the same laboratory under the same conditions.

The inconsistency of CFUs as a measure of bacterial load is shown by the observation that some participants reduced their CFU count to zero during the study period. However, these particular patients continued to have large amounts of PA in their samples when studied using alternative microbiological methods and in particular when using FISH. This highlights the unreliable nature of CFUs as a measure of treatment effect. Van Belkum et al showed that routine culture of CF sputum often failed to identify all bacterial species (van Belkum, Renders et al. 2000) and McMenamin et al describe the same problem (McMenamin, Zacccone et al. 2000) that routine culture on agar plates does not always identify bacteria known to be present in a sample and identified using alternative methods. The results of this study support these findings. It was felt to be important to include CFUs in the outcome assessment of this study as they are widely used and accepted in the literature, however, their limitations must be considered. In addition, CFUs can only assess planktonic bacteria and this study was primarily looking at effects on biofilm which cannot be assessed using CFUs.

'Whole colony' CFUs were measured in order to assess whether low dose inhaled NO had any effect on the whole colony of bacteria as assessed using CFUs on a general growth agar. There was no significant difference between the 2 groups during the period of the study. There was a slight trend towards the NO group showing an increase in whole colony CFUs from the end of inhaled therapy to follow-up, but this was not significant and there was wide variation in results

between individuals. This effect would need to be studied further in future studies. Because of the small numbers of participants, no significance can be placed on this result, however, as other trends have been commented on, this one is important to consider. It could be postulated that by reducing the PA load within the CF lung of a chronically infected patient, proliferation of other colonies of bacteria may occur to 'fill the space' made by the reduction in PA. This is unclear at present and needs further study. There have been studies that have shown NO to be effective against other bacteria such as *e. coli* and *staph aureus* (Barraud, Storey et al. 2009, Jardeleza, Foreman et al. 2011, Sulemankhil, Ganopolsky et al. 2012) so it is possible that there might be a beneficial effect wider than just the effect on PA, but this has not yet been studied in these patients. Samples have been stored from this study to look further at whole colony analysis using sequencing techniques, but funding for this work has not yet been secured.

q-PCR

Q-PCR has been shown to be more reliable than CFUs in some studies (Nadkarni, Martin et al. 2002, Zemanick, Wagner et al. 2010). Sputum samples were treated with Propidium Monoazide (PMA), which selectively binds to dead cells and inhibits polymerase activity, thus preventing DNA amplification by PCR. The result is that only viable cells are measured (Nocker, Sossa-Fernandez et al. 2007, Rogers, Stressmann et al. 2008). Both groups showed a decline in PA q-PCR during the period of IV antibiotic therapy which is reassuring (and would be expected with antibiotic treatment). The lack of an increase in the NO group is also further reassurance that the combination of NO and IV antibiotics did not cause any worrying increase in viable/planktonic PA as bacteria were dispersed.

Q-PCR is unable to assess PA in biofilm, but was important to include as another microbiological safety measure thought to be more sensitive than CFUs. As with CFUs, there were patients in the study who had a reduction in PA seen on q-PCR but no change in PA as determined by FISH analysis, which again highlights the less reliable nature of these methods as measures of treatment effect.

Potential combined biomarker

One possible thought in the design of this study was whether the outcome measures used for microbiological assessment of PA in sputum, could be combined to form a new biomarker that would produce a good assessment of PA load for use in clinical trials. Unfortunately, this was not possible. The wide variation seen in the

results from CFUs, qPCR and FISH analysis both within and between patients, did not allow any meaningful combination of these results into a composite marker. The difficulty in assessing biofilms for measuring outcomes in studies remains, although the potential for the use of FISH with 3D image analysis is promising and requires more work to develop as a reproducible and time efficient method for wider use.

Secondary outcome measures (clinical)

The clinical endpoints in this study were used primarily to assess safety of NO therapy when given in this way to a group of chronically infected CF patients and to help inform design of future larger studies. Clinical parameters were not selected as primary outcome measures as changes in these parameters were not expected with such a small number of patients who had wide variation in baselines values. The results show there are no safety concerns, there are no significant differences between the groups for any of the clinical outcome measures, but there are some trends in favour of NO therapy as seen with many of the laboratory secondary outcome measures.

Lung function

There are no significant differences in FEV₁ or FVC between the 2 groups. However, the pattern of change in lung function between the NO and placebo group is quite striking as demonstrated in figure 40. It shows improvement in FEV₁ and FVC in the NO group during treatment. This effect, like that seen in the laboratory secondary outcome measures, is again seen during the period of treatment with NO and not sustained to follow-up (as with the microbiological outcomes).

Trend to improved lung function (in particular FEV₁) could be due to a number of different factors. Improved microbiological clearance has been seen in trials of inhaled tobramycin (Ramsey, Pepe et al. 1999) and could be the cause in this study that demonstrated reduced PA bacteria in biofilms during treatment. It could be due to NO causing vasodilatation (as is the effect seen when used to treat pulmonary hypertension in neonates and older children on intensive care units), or the effects of NO on bronchial smooth muscle could have led to improved lung function.

It is not possible to be totally conclusive about the mechanism of improved lung function in this study, however, the dose used was low (10 ppm) compared to 20 ppm that is used continuously when treating patients with pulmonary hypertension

(via an endotracheal tube). In addition, the patients were breathing the NO gas through nasal cannula, and the actual delivery dose of NO to the lung is not known, but is likely to be lower than 10 ppm which is less likely to have an effect on the pulmonary vasculature.

There have been some small scale studies in CF that have shown no effect of inhaled NO on O₂ saturations or lung function. In one study, 13 patients with CF were given inhaled NO at 100 ppb, 1ppm and 40 ppm for 5 minutes with lung function measurements before and after inhalation. The results showed no difference in oxygen saturations or lung function at any of the concentrations of NO used (Ratjen, Gartig et al. 1999). The authors concluded that inhaled NO at these concentrations had no immediate effect on bronchial muscle tone in CF patients. Another study carried out in patients with COPD looked at administration of inhaled NO for a 24 hour period in a randomised, double blind, crossover design (Ashutosh, Phadke et al. 2000). This showed no change in lung function from baseline following 24 hours delivery of 25ppm iNO in this group of patients. Therefore, given the results from these previous studies that have specifically looked for a vasodilating or bronchodilating effect of inhaled NO and not found it, it can be hypothesised that the effect on lung function seen in this study is more likely related to removal of bacteria in biofilms than direct effects on either the pulmonary vasculature or bronchial smooth muscle.

HRQOL

Health related quality of life is important as it reflects the effects that a patient might experience with the experimental treatment. The CFQ-UK was chosen as it is the only tool validated for CF QOL assessment in the UK and has versions suitable for different ages of patient. However, it takes about 15-30 minutes to fully complete the questionnaire and is time consuming for the trial participant. As a result of this and the number of other study procedures required, it was decided to only measure HRQOL at baseline (time of pulmonary exacerbation) and at follow-up. This was chosen as the hypothesis and primary outcome measure was that there would be a difference between groups at follow-up. The results showed no difference in QOL scores between groups at follow-up, which correlates with the primary outcome measure which showed no difference and other secondary outcome measures which also showed no difference at follow-up.

In retrospect, It would have been interesting to see if there was an improvement in QOL during the period of treatment, at the same time as the other results show a trend towards improvement for example in lung function and reduction in PA in biofilms. Once the blinded results had been analysed, the study allocation was unblinded and study staff then knew which treatment each group had received, the research nurses (who were the only people who had been aware of treatment allocation) commented that the participants who had received NO therapy had reported to the research nurses caring for them whilst they were receiving their inhaled therapy overnight that they felt better quicker than usual with their IV antibiotics for pulmonary exacerbation. Of course, this report is not scientifically based and open to bias, but remains an interesting observation.

Length of Antibiotics

The NO group had a shorter average length of course of antibiotics (non significant). As a result of the improvement in their clinical condition, their IV antibiotic courses were shortened by the clinical teams rather than lengthened as can be the case with CF patients not responding to IV antibiotic therapy for a pulmonary exacerbation. However, this difference was not significant with such small numbers of patients. The planned length of course of antibiotics for all participants was 10 days, but the decision regarding stopping sooner or continuing antibiotics for longer, as well as whether antibiotics needed to be changed from the standard Ceftazidime and Tobramycin, was left in the hands of the clinical team, hence the variation in length of antibiotic course. Clinicians make decisions about length of course based on a number of factors including clinical improvement and lung function and when consulting with them in the design of the study, they were keen to retain some control over this aspect of the management of their patients.

Hospitalisation and treatment of pulmonary exacerbations with IV antibiotic therapy causes a significant disruption to patients daily lives, in particular for those who are in work or education and the majority of patients would welcome shorter stays in hospital and reduced length of antibiotic courses. Hence, any patients who had improved clinically before completing the 10 days of IV antibiotics, and were felt by the clinical team to be ready to stop antibiotics, were able to do so. With larger numbers of patients in future studies, this effect could be investigated in more detail and information about why antibiotics were stopped could be gathered.

Alternatively, the length of course could be standardised and/or criteria specified to be achieved in order to stop antibiotics early.

In addition to the shorter length of antibiotic course, the NO group also had more patients who received a shorter course of inhaled therapy. The original protocol was for 7 days of inhaled therapy, however, after original ethics submission, before any patients were enrolled, a substantial amendment was submitted (and granted) to change this to 5-7 days inhaled therapy to allow patients who had received by 5 days to be discharged home to complete their IV antibiotics. This was changed because of a number of factors felt important to the clinical teams in being able to deliver the study. 5 days was felt by the study team to be the minimum required to be able to see an effect of NO at a microbiological level.

With the minimum time of 5 days therapy it allowed more patients to be discharged home to complete their IV antibiotic course. Overall, this resulted in 6 out of 12 patients in the study receiving 5 or 6 days of inhaled therapy (rather than 7) of which 4 were in the NO group. It is not possible to draw any significant conclusions from this, however, the shorter courses of inhaled therapy in the NO group could have been due to greater clinical improvement and patients being ready for discharge home sooner. There are confounding factors that impact on readiness for discharge such as other conditions related to CF (aside from pulmonary exacerbation), requiring ongoing hospital admission, for example poor nutrition and weight loss. Hence, the trend towards shorter length of therapy in the NO group can only be commented on, and as with the length of IV antibiotics, would be an area to either standardise completely in future studies or collect data on exactly why therapy was stopped and the reasons behind this in order to see whether it was due to greater clinical improvement in those receiving inhaled NO.

The clinical teams were unaware of treatment allocation as the only unblinded members of staff were the research nurses, who had no involvement with the clinical teams or any influence over decision making about antibiotics or length of stay (and inhaled therapy). Because of the potential for this to cause bias the research nurses did not have contact with the clinical teams on the wards during the day when doctors rounds were occurring and decisions regarding length of antibiotic course and potential discharge home were being made. Patients are likely to have reported how they felt to the clinical teams as would be standard for clinical practice in order to inform decision making about care, but patients were unaware

of treatment allocation, so this would be open to interpretation by the clinical teams who were also blinded to treatment. It would have been most ideal for all members of the clinical and research teams to be blinded to treatment, however, for this small pilot study, this was not feasible due to cost implications in producing a matching placebo.

Exhaled Nitric Oxide

Exhaled nitric oxide levels in this study showed no difference between groups with huge variation between and within individuals at different time points. The number of factors contributing to the level of eNO in CF makes this a complex area to understand. It had been hypothesised that the patients receiving iNO would have higher eNO levels during treatment, but this was not observed. This may be because there was a gap between stopping iNO therapy and measuring eNO levels, even though it was short (a number of minutes). This would allow exhalation of any NO (which has a short half-life) remaining in the patients lungs/respiratory tract before the eNO measurement was carried out. Discussion had taken place about the measurement of eNO while the patient was receiving the therapy, but it was felt that this would only demonstrate an increase in eNO in the NO group during treatment, as would be expected whilst breathing NO, rather than an effect of increasing eNO as a result of giving inhaled therapy. This could have led to blinded staff being aware of treatment allocation as a result of reviewing the CRFs and would have been another potential source of bias, hence was not included. It is possible that useful information could have been gained by measuring eNO during therapy to see whether giving iNO had actually increased eNO even during the treatment period, but this would have had to have been recorded separately outside of the standard CRF in order to not reveal treatment group to unblinded staff unintentionally. This is another area for further work in future studies to gain more information and understanding of how iNO works.

Estimation of NO uptake by lung

The measurement of NO metabolites in blood shows great variation due to inter-individual differences in NO processing. There is a lack of published data available on circulating levels of NO metabolites in CF patients and this study shows that low-dose inhaled NO in CF did not significantly affect circulating NO metabolites. This is different to the effects seen in infants with pulmonary hypertension, where NO metabolites in blood are raised following treatment with twice the dose used in this

study (Ibrahim, Ninnis et al. 2012). The absence of a rise in systemic NO as represented by blood nitrate and NO-heme levels is reassuring and could be due to a number of factors including accelerated break down as a result of increased oxidative stress in epithelial cells and/or consumption of NO by the bacterial biofilm or thick mucus impairing gas exchange (de Winter-de Groot and van der Ent 2005, Zetterquist, Marteus et al. 2009).

NO metabolites in blood were measured at 1 and 7 hours during the period of inhaled therapy on the first night of treatment. This was timed to coincide with the samples being taken to measure methHb (a vital safety measure) in order to prevent any further blood tests being required over those already necessary for safety monitoring. The requirement for additional tests may have limited recruitment further. Had NO metabolites been measured at baseline and through the course of the study, change from baseline and cumulative effects over the days of treatment could have been assessed. Further studies could look at a subset of patients NO metabolites in more detail to allow patients to consent separately to the additional tests. However, due to the great variability seen between patients this might be difficult to interpret and a larger sample might be needed to detect any differences between groups.

Attempts were also made 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 Micro sensor, glass sensor NO-10). However, due to difficulties in equilibrating and calibrating the probe within CF sputum, the short half-life of NO and insufficient volumes of sputum produced by patients to carry out NO measurements alongside FISH and molecular analyses, this analysis was not carried out after trials in the first patient. It was unfortunate that this method was not practicable for use in the study as it had been hoped to give an estimation of NO levels in fresh sputum.

Safety data

As described earlier in the discussion there was no increase in CFUs or qPCR during the treatment of participants with inhaled NO which was reassuring that treatment with NO did not lead to a rise in planktonic PA.

MetHb levels were not raised in the NO group compared to the placebo group. This shows there is no significant systemic absorption of NO. Interestingly, methHb levels

were higher in all patients than <1% which is reported as 'normal'. This may be due to the chronic nature of CF and other factors that may impact on metHb levels, including medications of which antibiotics, analgesics and antipyretics are included. A study carried out in the US showed a large number of hospitalised patients with higher levels of metHb, so it is likely that this is more common than we currently recognise (Ash-Bernal, Wise et al. 2004). Symptoms and signs are not usually apparent below 5% and at the levels found in our group of patients (mean 1.4%) there would be no significant effects. For this study it was important to show that delivering NO in this way to a group of CF patients did not result in an increase in metHb levels. Future studies on iNO may not require such detailed monitoring of metHb as this study has shown it to be safe when given this way.

Patients were monitored closely during the period of inhaled therapy including continuous ECG and pulse oximetry and these clinical parameters showed no difference between the 2 groups. There was a theoretical possibility of seeing signs of sepsis or systemic response to bacterial release in patients treated with NO due to release of bacteria from biofilms and the subsequent inflammatory response, but this was not observed.

One current licensed use of inhaled NO is to treat pulmonary hypertension in neonates and paediatric patients on the intensive care unit. Importantly, NO therapy is never withdrawn suddenly because of the risk of causing rebound pulmonary hypertension. In this study a clear protocol for weaning NO during the last half an hour of therapy was used to monitor the patient for changes in clinical parameters that might suggest signs of pulmonary hypertension. The results showed that there was no effect on clinical parameters when weaning the patients and no difference between the 2 groups (placebo patients had 'sham' reductions in their therapy in order to keep the participants blinded to therapy). Future studies may not need to monitor so closely during weaning as this study has shown it to be safe in this group of patients when weaning from a low dose of inhaled NO (10ppm) given via nasal cannula.

From a safety perspective, the length of course of antibiotics was important to ensure that there was not an increase in length of antibiotic course as a result of dispersed bacteria being released with the NO therapy. The results were reassuring and showed that there were no significant differences between groups, but there

was a trend towards a shorter course for those receiving inhaled NO, perhaps due to a greater and faster clinical improvement.

Adverse events

There were only a few minor adverse events or adverse reactions in the study. A number of these were related to supply of medical gases (air and oxygen) resulting in non-significant interruptions to therapy. Other minor events occurred mainly in the NO group (6 out of 8 events) and the most commonly reported was nose bleeds. This may be as a result of the flow of NO gas through the nasal passages causing some irritation and increased tendency to nose bleeds. It could potentially be due to the vasodilating effects of NO on the fragile nasal blood vessels. Delivery via nasal cannula is not the usual method of delivery of NO to patients on intensive care units who are ventilated. Most commonly, NO is delivered via an endo-tracheal tube directly into the lungs and nose bleeds have not been reported. All episodes of epistaxis were mild, resolved spontaneously and did not require further intervention. Epistaxis is common in CF and many of these events occurred during the follow-up period, and so are less likely to be related to the study intervention because NO has such a short half life, more likely being related to the patient's underlying condition.

One patient experienced an episode of haemoptysis which could have been related to the NO therapy; however, a number of CF patients experience haemoptysis, especially when they are having a pulmonary exacerbation so there is a reasonable chance that this was related to the underlying condition rather than the study treatment. There was one reduction in oxygen saturations to 78% during the night which recovered spontaneously and quickly and was thought to be unrelated to the study intervention. It is common for oxygen saturations to drop overnight in CF patients admitted to hospital for pulmonary exacerbations. The increased reporting of AEs in the NO group could be due to events occurring as a result of the NO therapy, but could also be a result of reporting bias as all AEs were reported by the research nurses who were not blinded to therapy and hence may have unintentionally shown reporting bias to events occurring in the treatment group. This could be investigated in further studies with full blinding.

There were no SAEs reported in this study. In many studies, longer than expected stay in hospital would result in an SAE, however, for this group of severely affected patients, the length of stay in hospital when admitted for a pulmonary exacerbation is hugely variable and was not felt to have been adversely impacted in any

participants. There was no expected length of stay set at the start of the study because it was felt to be too unpredictable in this group of patients.

7.1.4 Potential sources of bias

This study has a number of potential sources of bias. Attempts were made to minimise these during the design of the study, but due to the limitations with regards to number of participants and funding for the study, this was not always completely possible and these areas could have impacted on the results of the study.

The research nurses were unblinded to treatment in this study. This is perhaps the greatest source of potential bias. As with any trial, unblinding can lead to reporting bias and influence of participants in a non-intentional or even intentional way to encourage greater effort with tests such as lung function or reporting of outcomes. There may well be a reporting bias in this study with the adverse events (which were reported by the research nurses) as most were from within the NO group. Every effort was made to reduce the impact of this source of bias by keeping all other staff blinded including clinical staff, investigators, and laboratory staff. In particular the study team members who were assessing the microbiological outcomes (including the primary outcome) were blinded to the treatment allocation. This was very important, because, although it was not possible for the study to be fully blinded, the main outcomes were microbiological and so maintaining blinding of all study team members involved in the assessment of these outcomes reduced this as a source of bias.

Variation in the additional treatment of participants outside of the IMP is a potential source of performance bias. The protocol allowed for changes to antibiotic therapy to be made by the clinical teams. This resulted in more patients in the NO group receiving a shorter course of antibiotics than in the placebo group. This then had an impact on the length of treatment course with the IMP. Variation was allowed for practicable reasons in order to be able to deliver the study, however, more patients in the NO group received a shorter course of inhaled therapy. This potential bias was minimised by blinding of all staff aside from the research nurses, so the clinicians involved in making these decisions were unaware of treatment group. The groups in this study did receive different treatment in terms of length of antibiotics and length of inhaled therapy (non-significant), however, it is not possible to say

whether this is a result of bias or because these patients improved faster. Further details about why decisions were made to stop antibiotics and discharge patients was not collected, but might have helped provide some more information and possibly reassurance that these decisions were based on clinical parameters and not as a result of bias.

The criteria for inclusion in this study was mainly around chronic pseudomonas colonisation. This selected out a specific group of patients who were severely affected by their CF and had advanced lung pathology. This is a potential source of bias in selecting a group of patients that may be more likely to respond to NO. This needs to be taken into account when considering the generalisability of the results. This study shows some possible effects of NO, albeit mainly trends rather than significant results, in this group of patients. Further work would be needed before this could be generalised to a wider group of CF patients. This would involve future studies as described below.

Selection bias may have affected the results. There was a detailed exclusion criteria, but in addition to the specified exclusion criteria participants 'self selected' into the study. This is a key part of informed consent that patients can decline to take part. There were some who declined due to needle phobia. Others who declined due to the need to be admitted to hospital for their IV antibiotics and to take part in the study, when they usually received their antibiotics at home. This selected out a group of severely affected patients who were those that would usually require admission for their exacerbations due to the severity of their disease. Selection bias is increased by the small number of participants. This could have resulted in 2 very different groups of participants at baseline. This bias was minimised by the randomisation of participants using allocation sequence concealment in order that staff would not be able to predict which group the next participant would be randomised into.

There were no patients who withdrew from the study during the period of treatment, so this did not cause bias. However, there were some who could not produce sputum samples either at all, or of an adequate size for assessment of the full range of microbiological outcome measures. This is a potential source of bias as it leads to missing data, which with a very small number of participants included, has a greater impact on results. The samples that were not produced were spread across the 2 groups therefore the effect is balanced and therefore, this is a less significant

source of bias, but could have had a greater impact if more patients in one group than the other had not been able to produce a sputum sample at certain time points. Use of induced sputum for all samples could have helped reduce this potential source of bias.

Reporting bias is also important in this study. As discussed above, the primary outcome selected was possibly not the most appropriate for this group of patients and did not show any significant difference between the groups. The differences seen were in the secondary outcomes and were mainly trends in the data and therefore may all be due to chance. This report has covered all aspects of the data collected in the study including all the negative findings. In a study of this size, it was not expected that significant differences would be seen in the data and the purpose of the study was to detect positive trends that would further support the findings of the pre-clinical work. Larger studies are required to investigate whether any of the effects seen are reproducible and or statistically significant. In addition to see whether there are significant impacts on clinically relevant outcomes before treatment using NO in CF could be taken any further into clinical practice.

7.1.5 Future work

Further studies are needed to look into the effect of NO on biofilms further and to explore other potential targeted anti-biofilm strategies. There are number of factors that are important to consider in planning these studies.

This study required patients to be chronically infected with PA, and demonstrated an effect of NO during therapy which was not sustained to follow-up. It would be interesting in further studies to look at patients who are younger and earlier in their clinical course to see what effect NO might have on disease progression and PA colonisation. A further hypothesis might include whether NO could prevent chronic colonisation, but detailed studies would need to investigate this question. However, microbiological outcomes will be incredibly difficult to assess as this group of patients are much less likely to be producing large amounts of sputum and therefore PA may not even be identifiable in the sputum pre-treatment, raising the question as to how treatment effect could be assessed.

With larger groups of patients and a longer period of follow-up, clinical outcomes could be investigated in more detail and this would also be required before a new

therapy could be introduced into routine clinical care. These clinical factors are vitally important for assessing a patient's progress, and any improvements are recognised as being important to patients and the progression of their disease. Lung function, rate of exacerbations/time to next exacerbation and IV antibiotic use are linked very closely with quality of life for CF patients and ultimately improvement in any of these factors may lead to an improvement in survival. QOL is a key consideration for patients because it reflects their everyday life with the condition. When designing studies, engagement with patients (patient and public involvement – PPI) is key to understanding which outcomes matter to patients and what factors they feel are important to be assessed through studies as well as what their views are on study procedures and how the study might work.

In order to recruit more patients, alternative methods of delivering NO need to be considered. Inhaled NO is time consuming and can currently only be given in a hospital setting with appropriate monitoring, limiting its use. It was selected for this study as the cleanest way to deliver NO to patients using an already licenced drug for a new indication. However, there are drugs in development that might be able to deliver NO in a more feasible way to the CF lung. For example, a nebulised antibiotic that has an NO molecule attached that would release on contact with the lung (Barraud, Kardak et al. 2012) might enable more effective prevention/removal of biofilm from patients at an earlier stage in the disease process and needs to be investigated further. This would need early phase clinical trials to demonstrate safety of this potential treatment before further randomised controlled trials could be considered. Nebulised antibiotics are a routine part of daily treatment schedules in CF care, so incorporating a treatment such as this into everyday regimes might not be too burdensome and could be more acceptable to CF patients. Other possibilities such as tablets that cause NO release could again be used on a more long term basis and are taken easily by patients.

Ideally, future studies should be double blind so that all study personnel as well as patients are blinded to treatment. Complete double blinding was not feasible in this small study so we minimised bias by only allowing the research nurses to be aware of treatment allocation, but to eliminate this form of bias totally, all staff should be blinded to treatment allocation.

This study allowed some changes to be made in length of therapy (5-7 days) and length of course of antibiotics in order to include patients that would otherwise have

been ineligible or declined to take part, but this did increase variability in the results. This could be standardised further in future studies to remove any confounding differences between groups. Another strategy could be to record fully any deviation from the ideal length of therapy and IV antibiotics, so that any differences between the groups could be analysed further on a case by case basis to assess the reasons for reduction in length of treatment.

This study used a new method of image analysis for PA biofilm number and volume from FISH images (low throughput microscopic biofilm assay in which PA are fluorescently-labelled via in-situ hybridisation (FISH) and assessed microscopically for planktonic or biofilm status). However, the process was laborious and time consuming and would not be practicable for larger studies. Work is ongoing with the aim of increasing throughput of FISH DNA-hybridisation analysis (previously 2 samples/day) by partial automation and use of fluorescence array scanner (target 20 – 50 samples/day). This would reduce the time taken for analysis of the results and allow this type of analysis to be used for larger numbers of patients and more widely for assessment of treatment on biofilms in clinical studies.

Although the NO metabolites were helpful in demonstrating that there was no systemic absorption of NO, and reassuring from a safety perspective, it was not possible in this study to demonstrate how much NO was delivered to the CF lung. It could be helpful to be able to investigate this in more detail prior to further studies, perhaps with lung diffusion models to be able to quantify the dose of NO that actually reaches the lung.

This study has provided a huge amount of data and information about this small group of chronically infected patients and there is a lot of learning that can be applied in the development of future studies. Depending on outcome measures selected for studies, there may be data from this study that could be used in sample size calculation. For example, if FEV₁ were to be used, the data from this study may be able to inform a power calculation. However, it would need to be taken into account that this study was of a group of severely affected patients and the large changes seen may not be so prominent in a group of patients less severely affected and with much better FEV₁ at baseline. Data on bacteria in biofilms and the changes seen could be used to inform power calculations if these microbiological outcomes are used in future studies.

7.2 Conclusion

Chronic PA biofilm infection plays a significant role in the progression of CF lung disease and currently there are no clinically recognised approaches for eradicating this. New treatments and strategies to remove biofilms are a key area for development. This study (alongside the preceding laboratory work) shows that inhaled NO when given alongside standard IV antibiotic therapy can disrupt PA bacteria from biofilms and is the first description of this novel approach for treating biofilms in CF lung disease. This approach is a clinical application of the discovery that low-dose NO mediates dispersal of biofilms by increasing bacterial phosphodiesterase activity leading to a decrease in c-di-GMP levels (where high c-di-GMP levels promote the biofilm phenotype). Strategies to induce the disruption of biofilms have the potential to overcome biofilm associated antibiotic resistance in CF and other biofilm-related diseases.

This study was designed as a proof of concept pilot study with no clinical data to inform study design. It was an exploratory study hoping to identify changes in microbiological end points of relevance to the treatment of pulmonary exacerbations in CF patients with chronic PA infection. The small sample size impacts on the validity of all the results and makes it difficult to draw robust conclusions. There was no effect seen in the primary outcome measure (proportion of bacteria in biofilm between baseline and follow-up). But, the results show a significant between group difference in the amount of PA bacteria in clusters over 20 cell size (biofilms) during the period of NO treatment, taking into account the use of repeated measurements in individuals over time. The results of this clinical trial are consistent with the beneficial effects of NO as described in the preliminary studies using *ex vivo* sputum samples treated in the laboratory. This an encouraging step forward in the development of targeted anti-biofilm strategies.

The study also provides evidence for the usefulness of FISH and 3D image analysis as a microbiological endpoint for assessing the efficacy of novel anti-microbial therapies in clinical trials.

CFU and qPCR analyses showed no increase in planktonic or viable PA as a result of biofilm dispersal. These analyses provided reassurances as to the overall effects of the treatment intervention on the microbial status of the PA community within the

lung. There were no safety concerns in relation to the use of NO as described in this study.

This study recruited older patients with longstanding chronic PA infection. This has influenced the results seen in this study and given the severe nature of the disease in these patients, raises the question of how NO might affect CF patients at other stages of their disease process.

There are a number of trends in the clinical parameters that show a positive effect of NO during the period of therapy; lung function shows a greater improvement in the treatment group and the length of antibiotic course is shorter. However, none of these changes reach statistical significance due to the small numbers randomised in this study. However, they support the conclusion that NO therapy is safe when given in this way to CF patients alongside IV antibiotics during pulmonary exacerbation. HRQOL shows no difference between the 2 groups, but was not measured during the period of inhaled therapy, which is when the other trends towards improvement were seen. This area should be included in more detail in future studies in order to assess these important clinical effects in greater detail and draw any conclusions about effect of treatment on parameters that are important to patients.

The safety data collected during this study is also reassuring. NO metabolites show that there was no systemic absorption of NO of which there are no previous descriptions in the literature. MetHb levels and clinical monitoring during therapy confirm that this treatment is safe when given to CF patients at the low dose of 10ppm for 8 hours a night for 5-7 nights. The adverse events and reactions were all minor and it is possible they may have been reported preferentially in the NO group as the nurses who reported them were not blinded to treatment

Anti-biofilm strategies are crucial to the further development of CF care and prevention and treatment of CF lung infection. This study has identified a potential new strategy for targeting biofilms in this group of patients which requires further studies to explore in more detail.

Further studies into the effects of NO on the CF lung or alternative agents that are in development that can deliver NO to the CF lung in a more practicable manner are needed to look into the potential of using NO as an adjunctive treatment in CF.

The difficulty in conducting such a study, using the outcome measures described, would be the ability to obtain sputum samples consistently throughout and in particular at the end of treatment interventions, when patients are often non-productive. This problem could be addressed by using induced sputum for all samples in the study, or in a larger scale study, using clinical parameters as primary outcome measures such as lung function or time to next exacerbation. These would require a larger sample size and longer period of follow-up for any differences between groups to be statistically significant. Future studies will need to include larger numbers of participants and focus on clinical outcomes with prolonged periods of follow-up in order to assess whether this therapy could be introduced into routine clinical care. In addition, more practicable ways of delivering NO to patients need to be developed that are more feasible to be used on a wider scale, are acceptable to patients and can be incorporated into CF daily treatment regimes.

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Appendix 1

Pre-clinical study protocol and regulatory approvals



National Research Ethics Service

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RESEARCH ETHICS COMMITTEE (A)

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10 December 2008

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Dear Dr Connett

Full title of study: Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory diseases.

REC reference number: 08/H0502/126

Thank you for your letter of 13 November 2008, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

The Committee noted the study endpoint 3¹/₂ years after the start.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at <http://www.rdforum.nhs.uk>.

This Research Ethics Committee is an advisory committee to South Central Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

<i>Document</i>	<i>Version</i>	<i>Date</i>
Participant Information Sheet: Parent - Bronchoscopy	1.1	01 September 2008
Participant Information Sheet: Parent - Naso-pharyngeal aspirate	1.1	01 September 2008
Participant Information Sheet: Parent - Sputum	1.1	01 September 2008
Participant Information Sheet: Young Person - Sputum	1	01 September 2008
Participant Information Sheet: Adult Patient - Sputum	1.1	01 September 2008
Summary/Synopsis	1	01 September 2008
Covering Letter		01 September 2008
Application		01 September 2008
Participant Information Sheet: Parent - Research Bronchoscopy	1.1	01 September 2008
Participant Information Sheet: Young Person - Research Bronchoscopy	1	01 September 2008
Participant Information Sheet: Young Person - Bronchoscopy	1	01 September 2008
Letter from Department of Health		20 March 2008
Advertisement	1	01 September 2008
Compensation Arrangements		01 August 2007
Peer Review		22 August 2008
Letter from Sponsor		22 August 2008
Protocol	1.1	01 September 2008
Investigator CV		01 September 2008
Response to Request for Further Information		13 November 2008
Participant Consent Form: Parent/Child - Routine Samples	1.2	01 September 2008
Participant Information Sheet: For Children	1.2	01 September 2008
Participant Consent Form: Adult - Routine Samples	1.2	01 September 2008
Participant Consent Form: Parent/Child - Bronchoscopy	1.1	01 September 2008
Participant Consent Form: Adult - Bronchoscopy	1.1	01 September 2008
Participant Information Sheet: Adult - Bronchoscopy	1.2	01 September 2008

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

This Research Ethics Committee is an advisory committee to South Central Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

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21 May 2009

Dear Dr Connett

ID: RHM MED0837 Investigations of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease.

EudraCT:

Thank you for submitting all the required documentation for Trust R&D approval. I write to inform you that your study has full SUHT R&D approval. Please find attached the Conditions of Trust R&D approval which you are obliged to adhere to.

You are required to keep copies of all your essential documents relating to this study. Please download a copy of the relevant Investigator Site File template from the R&D website: <http://tinyurl.com/p8vuek>.

Your project is subject to R&D monitoring and you will be contacted by our office to arrange this.

Please note: A condition of approval is that any changes need to be timeously notified to the R&D office. This includes providing copies of:

- . All NRES substantial amendments and favourable opinions;
- . All Serious Adverse Events (SAEs);
- . NRES Annual Progress Reports;
- . Annual MHRA Safety Reports;
- . NRES End of Study Declaration;
- . Notifications of significant breaches of GCP or protocol

Please quote the above RHM No. on any correspondence with our office.

Should you, or any of your team, require training in any of the policies and procedures required to ensure compliance with the conditions of approval, please refer to the R&D Training website <http://tinyurl.com/prkd65> for an up-to-date calendar of training events.

Yours sincerely



Dr Claudia Fellmer

Research Governance & Quality Assurance Manager

Copy to: Dr Katrina Cathie,



National Research Ethics Service
SOUTHAMPTON & SOUTH WEST HAMPSHIRE
RESEARCH ETHICS COMMITTEE (A)

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17 September 2009
 (Amended letter dated 30 October 2009)

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Dear Dr Connett

Study title: Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory diseases.
REC reference: 08/H0502/126
Amendment number: April 2009 Amendment 1.2
Amendment date: April 2009

Thank you for submitting the above amendment, which was received on 09 September 2009. It is noted that this is a modification of an amendment previously rejected by the Committee (our letter of 15 June 2009 refers).

The modified amendment has been considered on behalf of the Committee by the Chair.

Ethical opinion

I am pleased to confirm that the Committee has given a favourable ethical opinion of the modified amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved are:

Document	Version	Date
Participant Information Sheet: Adult Patient	1 revised April 2009	April 2009
Modified Amendment	April 2009 Amendment 1.2	April 2009
Protocol	Version 1	01 September 2008
Participant Information Sheet: Information sheet for children	Version 1	01 April 2009
Participant Information Sheet: Young person information sheet	Version 1	01 April 2009
Participant Consent Form: Parent/Child consent form	Version 1	01 April 2009
Participant Consent Form: Adult Patient consent form	Version 1	01 April 2009
Questionnaire for healthy volunteers	Version 1	01 April 2009

→ Sibb Anderson Sibb Anderson
 file

This Research Ethics Committee is an advisory committee to South Central Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

Protocol version: 1.3: 07-11-12

Sponsor: University Hospital Southampton NHS Foundation Trust

Sponsor Code: RHM MED0837

NHS REC No: 08/H0502/126

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Study site: UHS Department of Child Health/Respiratory medicine

Other departments involved in the study: Southampton Wellcome Trust Clinical Research Facility
NIHR Southampton Respiratory Biomedical Research Unit
University of Southampton School of Medicine

University of Southampton School of Biological Sciences
Collaborating Universities specified for particular investigation

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1. Hypothesis

This study is designed to investigate methods of infection and inflammation in a wide variety of respiratory diseases that affect children and adults. The processes involved in these diseases are often similar, and knowledge that emerges about one disease can sometimes be applicable to others. Generating new laboratory data will allow the development of new therapies that will be tested in separate clinical trials.

2. Background

2.1 Introduction

Detailed scientific analysis and investigation has been ongoing in Southampton since 2000 as part of a clinical research collaboration, carried out under LREC 306/00/w, looking at the role of inflammatory mediators and surfactant phospholipids and proteins in respiratory disease. The National Institute For Health Research (NIHR) has recently (April 2008) awarded Southampton a Respiratory Biomedical Research Unit (BRU) grant to investigate childhood and adult respiratory disease at all stages of life. This grant was awarded following open competition and international peer review based on 5 programmed themes of research. Overall the BRU has a specific role to undertake translational clinical research in priority areas of disease, ill health and clinical need that are currently under-represented in the NHS. This protocol is designed to replace that previously covered by LREC 306/00/w and to include new work funded from within the BRU. These studies will potentially benefit large numbers of children and adults who suffer from chronic respiratory illness and disease.

This protocol describes in detail a project designed to investigate inflammatory and infective processes in the lung. Children and adults may be entered into the BRU study if they are known to suffer from, or are being investigated for, a range of respiratory illness, such as (but not limited to) cystic fibrosis (CF), primary ciliary dyskinesia (PCD), bronchiectasis and asthma.

2.2 Inflammation and surfactant

The pathological basis of many chronic respiratory conditions involves an inflammatory process driven by both innate and adaptive immune mechanisms. A major focus of this component of the project is the analysis of the innate response involved in orchestrating the characteristic infiltration of activated inflammatory cells into pulmonary air spaces and the subsequent release of pro-inflammatory cytokines. The severity of this inflammation is determined by the balance between pro-inflammatory and anti-inflammatory regulatory processes. This cellular inflammatory response in the airways takes place within an alveolar and airway lining fluid environment that is rich in pulmonary surfactant. Components of pulmonary surfactant, especially specific phospholipids and surfactant proteins A and D (SP-A,

SP-D), are important regulators of the innate immune system and are in turn subject to hydrolysis and inactivation by reactive oxygen species, proteases and phospholipases.

We have characterised changes in surfactant phospholipid compositions in asthma, CF and acute respiratory distress syndrome (ARDS) and have demonstrated that inflammation associated with infection, for instance in chronic *Pseudomonas aeruginosa* infection in CF, leads to proteolytic inactivation of SP-A and SP-D. In addition, polymorphisms in SP-A and SP-D have been linked to increased susceptibility to respiratory infection.

The central hypothesis of this component of the project is that dysregulated innate immunity in respiratory infection may in part be due to abnormalities in the composition and function of lung surfactant phospholipids and surfactant related proteins (specifically surfactant proteins A and D).¹

An improved understanding of the cellular and immunological mechanisms that are common to respiratory disease in children as well as the genetic basis of dysregulated innate immunity might help us formulate recombinant therapy. We are currently developing a bioactive recombinant fraction of SP-D and intend to undertake phase II/III studies to investigate its potential for the enhancement of innate immunity.

2.3 Infection

Bacteria are now known to exist *in vivo* as single cells that float or swim independently in a liquid medium (the planktonic phase) or as microcolonies. These microcolonies, known as biofilms, are structured bacterial communities encased in a polymeric matrix adherent to a biological or inert surface. Bacteria in biofilms are relatively resistant to antibiotics. This resistance profile can be explained in 3 ways:²

1. As a consequence of a protective barrier effect of the polymeric matrix.
2. Bacteria in the biofilm may be metabolically quiescent thus reducing the activity of antibiotics that rely upon disrupting energetic processes to be maximally effective.
3. By facilitating the survival of resistant bacterial phenotypes.

A bacterial population in a biofilm may have a degree of bacterial resistance up to 1000 times as great as an identical population that is planktonic.³ Although aggressive early antibiotic treatment can eradicate planktonic bacterial infection in CF, the formation of biofilms and the antibiotic resistance that they infer are becoming increasingly recognized as risk factors for chronic infection in diseases such as CF.^{4,5}

This is particularly important in chronic purulent lung diseases (CF and non-CF bronchiectasis and PCD). Nitric oxide (NO) is an endogenously produced neurotransmitter with vasodilatory properties. It is known to down regulate biofilm formation *in vitro* and exhaled NO levels are reduced in CF^{6,7} and PCD.⁸ This could therefore predispose to biofilm formation *in vivo*.⁹ NO also contributes to the regulation of bronchial tone, and a single inhalation of nebulised L-arginine (which stimulates NO production in epithelial cells) has been shown to improve FEV₁ in patients with CF.¹⁰ We have shown that biofilm formation by *Pseudomonas aeruginosa* is reversed *in vitro* by nanomolar, non-toxic concentrations of NO.¹¹ In this model the proportion

of *P.aeruginosa* in the planktonic (vulnerable) phase is increased and a reduction in antibiotic tolerance was observed. Exposure of biofilms to NO also greatly enhanced ability of antibiotics such as Tobramycin (a typical antibiotic used in CF) to remove the bacteria/biofilms from a glass surface.¹¹ In this study we will test the hypothesis that the administration of NO will enhance the bactericidal efficacy of antibiotic therapy in an *in vitro* model of CF and non-CF bronchiectasis.

The lungs of patients with CF and other chronic suppurative lung diseases are colonized with bacteria. The intense host inflammatory response to these bacteria results in damage to the lung and the progressive loss of lung function.¹² This is the main cause of mortality in CF patients.¹²⁻¹⁵ Although appropriate antibiotic therapy is used against bacterial species identified by traditional culture methods, patients continue to deteriorate. Microbiological culture media used routinely are only capable of supporting the growth of a small fraction of the diverse bacterial community present¹⁶ and only a limited number of important species are assayed (including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Burkholderia cepacia*).¹⁷ This produces results that are unrepresentative of the actual populations of bacteria present within the lungs.

Techniques have been developed to further study the bacterial diversity within specimens provided from patients with CF. Terminal restriction fragment length polymorphism (T-RFLP) is a molecular biology technique for profiling microbial communities based on the position of a restriction site closest to the labeled end of an amplified gene. It has been shown to be an important means of analyzing the composition and diversity of the bacterial community in samples from patients with cystic fibrosis.¹⁸ Studies have shown that species not previously reported in CF lung infection have been identified using TRFLP.¹⁹

Multilocus sequence typing (MLST) is another technique in molecular biology for characterizing isolates of bacterial and fungal species using the DNA sequences of internal fragments of multiple housekeeping genes. MLST helps increase our knowledge of the genetic variation that occurs within a species. This can provide data for epidemiological surveillance and be used to inform public health policies and develop new treatments and vaccines.²⁰

We have discovered that tobramycin binds copper and as well as being an effective antibiotic, the tobramycin-copper complex has anti-inflammatory properties and positive effects on the function of the CF protein that is abnormal in this condition. We aim to investigate whether tobramycin is converted into copper-tobramycin in patients. We hope to find out if copper-tobramycin is a safe and effective therapy, and superior to tobramycin.

3. Aims and objectives

3.1 Main hypothesis

Our hypothesis is that more detailed understanding of the interactions between inflammatory and infective processes in paediatric and adult lung disease will lead directly to novel and improved therapies to prevent disease, disability and death.

3.2 Primary aims

To investigate the pathophysiology of inflammation and infection in children and adults with a variety of respiratory diseases as detailed below:

3.2.1 Inflammation

1. To quantify the number of inflammatory cells and their degree of activation within pulmonary air spaces.
2. To define the roles of lung surfactant phospholipids and surfactant proteins in the regulation of the pulmonary innate immune response.
3. To investigate the relationship between the protease:antiprotease balance and pulmonary inflammation.
4. To investigate the oxidative mechanisms leading to pulmonary damage.
5. To define the relationship between genotype and phenotype (with particular regard to polymorphic diversity in genes encoding SP-A and SP-D) in children and adults with inflammatory pulmonary disease.

3.2.2 Infection

1. To quantify and describe *in vitro* the effect of Nitric oxide (NO) on bacteria in biofilms.
2. To quantify *in vitro* the ability of NO to enhance the efficacy of antibiotics conventionally used to treat infective exacerbations in CF and non-CF patients with bronchiectasis.
3. To quantify *in vitro* the ability of L-Arginine (which stimulates NO production in biofilms) and NO itself to disrupt biofilms.
4. To quantify *in vivo* the distribution of nitric oxide through the airway, in health and disease.
5. To inform design of a phase 2 trial to test the hypothesis that introducing low dose nitric oxide into chronically infected airways (such as *pseudomonas* in cystic fibrosis) will induce biofilm dispersal and make the organisms more susceptible to antibiotic treatment.

6. To more precisely characterise bacterial communities within the airways of diseased lungs using molecular microbiological techniques for species identification (e.g. TRFLP and MLST).
7. To determine whether Tobramycin-copper complexes are present in urine samples of children receiving IV or nebulised tobramycin treatment.

4. Study design

This study will use aliquots of samples collected as part of the routine clinical management of adults and children attending the paediatric and adult respiratory service with a variety of respiratory diseases including CF. Samples will include:

1. Sputum
2. Cough swabs
3. Naso-pharyngeal aspirates
4. Broncho-alveolar lavage fluid (BAL)
5. Urine

Consent to obtain blood samples at the time of a clinically indicated cannulation or blood test will also be sought.

We will also use lung tissue, removed during surgery from Southampton and other thoracic centres, from an ethical approved source with appropriate consent.

It is important to establish whether sputum or BAL fluid is a direct marker of infection and inflammation on the actual surface of the lung and to do this it is necessary to collect a number of endobronchial and brush biopsy specimens. Some of these may be collected from patients undergoing routine clinical bronchoscopy where consent will be requested to obtain additional endobronchial and brush biopsy specimens. However, this may not provide the number (up to 10) or type of specimens needed (particularly from patients with CF) within a 1 year time span preceding planned phase 2 studies, and therefore it is necessary to have the option of inviting a small number of older children and adults to give informed consent for a research bronchoscopy (i.e. not for clinical indications).

Separate more detailed information sheets (see appendix 3) will be used for individuals meeting inclusion criteria for this additional procedure solely for research purposes.

We intend to collect the samples described above from patients recruited prospectively into this study. We intend to analyse these samples together with samples collected from a previous ethics committee approved study ('Analysis of immune and inflammatory mechanism in paediatric respiratory disease': LREC 306/00/w). Patients who had participated in LREC 306/00/w had agreed to provide linked anonymised samples for storage and have consented for those specimens to be used in future studies (providing ethical approval is granted) (see Appendix 7 for original consent form referring to study LREC 306/00/w).

Recruited patients will therefore fall into 3 groups:

1. Children and adults attending the respiratory service who would normally provide samples of respiratory secretions as part of their routine clinical care (sputum and cough swabs in children and adults, naso-pharyngeal aspirates in young children). These patients will be asked to consent to aliquots of their samples being used for research purposes. They will not undergo any additional procedures as a result of taking part in this research.
2. Children and adults undergoing clinically indicated bronchoscopies who will be asked to consent to aliquots of BAL fluid and blood being used for research purposes and to consider additional endobronchial and/or brush biopsy.
3. Adults and older children who will be asked to consent to research bronchoscopy which is not part of their routine clinical care in order to obtain the necessary specimens (namely endobronchial and brush biopsies) to carry out laboratory investigations.

We will measure nitric oxide (NO) levels from children and adults attending the respiratory service to obtain normative data for different respiratory conditions. We will obtain repeat measurements during subsequent clinic visits, and will take measurements if attending with respiratory exacerbations or infections. Additionally we will recruit healthy volunteers from members of staff, University students and family members accompanying volunteers from clinic. Exhaled NO will be measured at four respiratory flow rates to calculate fractional NO from small and large airways. Nasal NO will also be measured. Measurements will be made using a NIOX Flex machine (Aerocrine) following standard protocols.

4.1 Subject selection

Potential participants will be identified in 3 ways:

1. Patients with known or suspected chronic inflammatory or infective pulmonary disease admitted to a ward in Southampton University Hospitals NHS Trust (SUHT) for treatment.
2. Patients with chronic inflammatory or infective respiratory disease attending an out patient clinic at SUHT.
3. Patients attending the SUHT based Wellcome Trust Clinical Research Facility (WTCRF) for clinically indicated bronchoscopy or patients undergoing bronchoscopy for clinical indications in other areas within SUHT.

4.1.1 Inclusion criteria

1. Any patient with a known respiratory diagnosis (including the following conditions) who is attending the ward or outpatient department for routine follow up:
 - a. Cystic fibrosis
 - b. Primary ciliary dyskinesia syndrome
 - c. Bronchiectasis
 - d. Immune deficiency with susceptibility to infection
 - e. Asthma

2. Any child scheduled for a routine bronchoscopy for the investigation of suspected pulmonary disease.
3. Adults and children over the age of 12 years under routine follow up by the respiratory consultants whose disease is stable and who are judged by objective criteria to be fit to undergo bronchoscopy/brushings/biopsy safely. The criteria that will be used are as follows:
 - a. Clinically stable and free from acute infection.
 - b. Able to voluntarily give consent (for children over 12 years they must be deemed to be Gillick competent).
 - c. Normal oxygen saturations.
 - d. No complications from previous bronchoscopies.
 - e. Not currently involved in a trial of interventional medicinal products.

4.1.2 Exclusion criteria

1. Patients who do not give consent.
2. Patients where the treating clinical consultant does not consider it appropriate to offer participation in this study.
3. Patients whose disease state excludes them from being fit for research bronchoscopy.
4. Patients involved in trials of interventional medicinal products will be excluded from research bronchoscopy (as this represents an additional intervention), but will still be able to provide specimens as part of their routine clinical care.

4.1.3 Subject recruitment

Children and adults attending the respiratory service (clinic or ward) who would normally provide samples of respiratory secretions as part of their routine clinical care.

Patients attending the respiratory service who fit the inclusion criteria will be sent (before their appointment) or given (on the ward or in clinic) an information sheet about the study. All participants and/or their parents and legal guardians will have time to read the information sheets and discuss any concerns or questions with a doctor. They will be asked to consent to aliquots of their specimens (sputum and cough swabs in children and adults; naso-pharyngeal aspirates (NPA) in young children) being used for research purposes. Consent will be obtained from either the patient (>18 years) or from a parent/adult with legal responsibility for the child. We will also ask children over 12 years who are able to understand for their verbal assent. (See appendix 1 for information sheets and appendix 4 for consent forms).

Patients with chronic respiratory disease will be asked to consent for the repeated collection of specimens on different occasions e.g. visits to clinic, inpatient episodes. Some patients will

therefore be able to provide a number of specimens over time. All patients will be free to withdraw their consent at any time, and consent to continue in the study will be confirmed verbally before each new collection of non-invasive specimens (cough swab, sputum, NPA) or in writing prior to bronchoscopy. (See appendix 4 for consent forms).

Children undergoing routine clinical bronchoscopy

Patients attending the WTCRF for bronchoscopy for clinical reasons will be approached as part of the work up for their day case bronchoscopy (they will be sent the information sheet as part of the appointment package along with the procedural leaflets). Patients attending for routine bronchoscopy in other SUHT areas will be given information sheets ideally 24 hours before the procedure. However, due to the nature of the clinical procedures, information sheets may be given to the families on the day of the procedure but this will be avoided wherever possible. All participants and/or their parents and legal guardians will have time to read the information sheets and discuss any concerns or questions with a doctor.

They will be asked to consent to aliquots of broncho-alveolar lavage fluid taken during the procedure to be used for research purposes. They will also be asked to consent to an additional blood test at the time of cannulation. Endobronchial and brush biopsies are routinely carried out as part of the evaluation of patients with respiratory problems (including CF) in some centres,²¹ but this is not currently routine practice in Southampton and therefore informed consent will also be sought for this additional specimen collection.

If they agree to take part the parents will be asked to sign a consent form. They will be given the option to decline/consent to some or all parts of the study (for example they could consent to broncho-alveolar lavage but not biopsy). Consent for the research project will be sought at the same time as the standard NHS procedural consent in the case of patients undergoing elective bronchoscopy. Consent will be obtained from a parent/adult with legal responsibility for the child. We will also ask children over 12 years, who are able to understand, for their verbal assent. (See appendix 2 for information sheets and appendix 4 for consent forms).

Adults and older children who will be asked to consent to research bronchoscopy which is not part of their routine clinical care.

This will be a carefully selected group of patients (who fit the criteria set out above in section 4.1.1), known to the respiratory service, who will be invited to consider a bronchoscopy for research purposes. As this will result in an additional procedure solely for research, separate more detailed information sheets will be used (see appendix 3 for information sheets and appendix 4 for consent forms). Parents will not be asked to enroll younger children in this part of the study and all children >12 years, who will be approached, must be considered by their responsible consultant to be Gillick competent to consent to this part of the study.

Public information

A patient information poster will be displayed in the Wellcome Trust Clinical Research Facility at the new patient information point (see Appendix 6). The aim of the poster is not to recruit patients, but to provide information about the project. Patients will only be recruited if their responsible consultant considers it appropriate and they fit the objective inclusion criteria.

4.1.4 Controls

Most paediatric bronchoscopies are currently being conducted within the Wellcome Trust Clinical Research Facility (WTCRF) in order to ensure that, where consent is obtained, specimens are appropriately handled to sub-aliquot for research purposes (LREC 306/00/w). This allows us to generate data on infection and inflammatory markers in children who subsequently have been shown not to have any lower airway disease, to provide the closest one can ever get to normal controls without asking normal children to undertake invasive procedures. No completely healthy children will undergo bronchoscopy to act as a control group.

Exhaled and nasal NO measurements will be made in healthy volunteers to obtain normative data.

4.1.5 Withdrawal of subjects

Patients are free to withdraw their consent at any time during the study. This will not affect their clinical care in any way.

4.2 Sample size

No formal power calculations have been performed as the intention is to collect phenotypic data and clinical specimens from all eligible patients enrolled to inform future translational studies. This is standard practice in descriptive or “clinical cohort definition” studies. Bronchoscopy carried out purely for research purposes will be limited to the minimum number of patients necessary to test the hypothesis that sputum or BAL fluid is a direct marker of infection and inflammation on the actual surface of the lung. It is not feasible to determine exactly how many additional biopsies will be required until the first biopsies have been analysed in the laboratory but it is not anticipated that this will be more than 10 patients. Formal statistical support will be provided as part of the NIHR Respiratory Biomedical Research Unit with the support of the Southampton Research and Development Support Unit (RDSU, Director Mr. Mark Mullee).

4.3 Sample collection and testing

4.3.1 Collection of specimens

Specimens will be collected from all eligible patients who consent to participation in this trial from three clinical areas: the ward for investigation or treatment of their condition, from those attending the respiratory outpatient clinic and from patients undergoing bronchoscopy in the WTCRF or other clinical areas. The specimens are described below:

1. **Sputum** – patients will be asked to expectorate sputum into a sterile universal container.
2. **Cough swabs** – patients will be asked to cough when two small sterile swabs are placed together in the oro-pharynx (i.e. clinical and research swabs collected together).
3. **Nasopharyngeal aspirates (NPA)** – these specimens will be collected by passing a soft suction catheter up the nose and collecting the material that is aspirated in a sterile container.
4. **Broncho-alveolar lavage fluid (BAL)** – these specimens will be collected at bronchoscopy. Aliquots of sterile saline (1ml/kg up to a maximum of 20ml) will be washed into a section of the lung and then suctioned back through the bronchoscope into a sterile container. It is normal practice to collect 2 sequential aliquots from 2 separate areas of the lung.
5. **Endobronchial/brush biopsy** – these specimens will be collected at bronchoscopy. Endo bronchial biopsy specimens will be taken using cupped forceps under direct vision from a large airway. Brush biopsy specimens will be taken using a soft nylon sampling brush rubbed gently across the wall of a central airway. The risks involved in this procedure are minimal and in some centres these investigations are part of routine practice at the time of bronchoscopy.
6. **Lung tissue** – We will also use lung tissue, removed during surgery, from Southampton and other centres, from an ethical approved source with appropriate consent.
7. **Blood** – up to a teaspoonful of blood (no more than 5ml) will only be collected from patients who need to be cannulated for clinical reasons prior to bronchoscopy or treatment on the ward, or who are undergoing a blood test for clinical reasons. Blood will be collected into 2 bottles (2.5ml in each) – EDTA and plain (serum).
8. **Urine** – Up to 20ml of mid stream urine will be collected from in-patients receiving intravenous antibiotics and out-patients on nebulised antibiotics when attending routine out-patient clinic visits. These samples will be stored at -80°C prior to analysis.

4.3.2 Transportation and preparation of samples

All samples will be labeled with a unique study number before preparation begins. Preparation of specimens will take place in the Wellcome Trust Clinical Research Facility.

Respiratory secretions will be split into 3 aliquots:

1. For clinical testing as appropriate (for example routine NHS microbiological testing).
2. Fresh sample transfer to the appropriate research laboratory.
3. Immediate freezing of an aliquot at -80°C for further tests to be carried out at a later date and to enable testing to be conducted in batch format.

Blood will be spun, separated and frozen at -80°C for testing at a later stage and to enable testing to be conducted in batch format.

Biopsy specimens will be prepared and transferred immediately to the appropriate research laboratory.

Lung tissue, removed during a surgery, from Southampton or other centres, will be prepared and transferred promptly in appropriate media to the appropriate research laboratory.

The samples will be stored in a locked -80°C freezer in the Division of Infection, Inflammation & repair. The key will be kept secure in a locked key cupboard with access restricted to authorized staff. Authorization of staff for such access will be determined by the management committee for the project.

4.3.3 Laboratory experiments

4.3.3.1 Inflammation

This component of the project will provide diagnostic clinical information as part of the research protocol. The differential leukocyte count of bronchoalveolar lavage (BAL) fluid is becoming an increasingly important diagnostic method.^{22 23} The University Department of Child Health has expertise in this technique and is now routinely processing samples for clinical diagnostic purposes. Consequently, aliquots of BAL samples taken at bronchoscopy will be used for microbiological culture and detailed differential cell count.

Surfactant phospholipid compositions will be determined by electrospray ionisation mass spectrometry (ESI-MS) analysis of lipid extracts of BAL fluid. Concentrations of Surfactant proteins A and D (SP-A and SP-D) will be determined by a combination of enzyme-linked immunoSorbent assay (ELISA) and immunoblotting, which will differentiate between native active proteins and inactive proteolytic fragments.

The inflammatory immune response will be quantified by measuring levels of expressed cytokines in BAL fluid (Interleukin-6 (IL-6), interleukin-8 (IL-8), tissue necrosis factor alpha (TNF α), protease and antiproteases, neutrophil elastase, matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinases (TIMPS) and phospholipase A₂).

Where sample volumes permit, membrane phospholipid compositions and activation responses will be determined in both freshly isolated and cultured (24 h) inflammatory cells from BAL in the presence and absence of surfactant components.

Blood samples (5ml) will allow DNA extraction and an analysis of the relationship between genotype and phenotype at gene loci involved in the pulmonary inflammatory response, with particular regard to polymorphic diversity in genes encoding SP-A and SP-D, Toll like receptor 4 and macrophage migration inhibitory factor.

4.3.3.2 Infection

To test whether increased *in vitro* susceptibility of *Pseudomonas aeruginosa* biofilms to antibiotics after treatment with Nitric Oxide (NO)²⁴ can be extended to biofilms obtained from individuals with chronic suppurative pulmonary disease, samples (sputum, cough swabs and biopsy specimens) will be subjected to three treatments:

1. Nitric Oxide treatment (micromolar concentrations) – the most effective delivery of NO will be evaluated using three different compounds:
 - a. sodium nitroprusside
 - b. L-arginine
 - c. gas phase NO
2. Nitric oxide treatments (as above) combined with antibiotic treatment (including ciprofloxacin and tobramycin).
3. Control which will be left untreated.

To analyse the efficacy of each treatment a number of methods will be used:

1. **Viable colony enumeration** – Samples will be treated as above, diluted and plated onto solid medium to determine the number of colony forming units (CFUs).
2. **Microscopy** – Direct imaging of biofilm structures collected from samples will be performed using an inverted light microscope with long working distance objectives, with samples held within 6-well tissue culture plates. Samples will be subjected to treatments as above and then stained using Baclight Live/Dead Imaging to highlight viable and non-viable cells.
3. **Continuous culture** – Samples will be cultured on solid *Pseudomonas aeruginosa* selective media and single colonies sub-cultured, species ID confirmed by PCR, and stored at –80 degrees. Individual *P. aeruginosa* clinical isolates will be grown in monospecies continuous culture biofilm flow cells and allowed to develop into structured biofilms. These biofilms will then be subjected to treatments as above. Cells and biofilm structure can be visualised on an upright light microscope with oil immersion x100 objective and stained with Live/Dead kit. General biofilm phenotype can also be compared to that of wild type laboratory strains currently studied within our laboratories.

Other markers of inflammation and infection will be measured to increase our understanding of the pathophysiology of lung disease.

Specimens will be further characterised using the following:

Terminal restriction fragment length polymorphism (TRFLP)

Clinical samples for molecular biological assays will be snap frozen in liquid nitrogen, prior to longer term storage at -80°C prior to use. Following sample transfer, bacterial nucleic acids and proteins will be extracted directly from the samples. Specific target molecules in these extracts will be studied by gel electrophoresis approaches with other target molecules e.g. bacterial ribosomal sequences studied after polymerase chain reaction (PCR) amplification. PCR products will be characterised either through direct sequence analysis or by a profiling strategy such as Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis, with the data generated on the bacteria present in the samples interpreted statistically. TRFLP generates fragments of different lengths due to the variation in position of the first specific restriction endonuclease site in ribosomal sequences. These fragments will be fluorescently labeled to allow detection on automated DNA sequencing machines. This allows complex bacterial communities to be sequenced rapidly.¹⁸ Separate samples will be processed by routine culture-based microbiological analysis, with these samples stored at 4°C prior to use. These samples will also be used for a range of microscopic based analysis systems such as fluorescent in situ hybridization. This will include the analysis of ribosomal region clone libraries derived from culture-independent analysis of the clinical samples obtained. In detail, regions of ribosomal RNA genes will be amplified from the nucleic acids extracted from the clinical samples. These ribosomal RNA genes will then be cloned into the appropriate vectors for subsequent sequence analysis. Once cloned, sequencing will be performed, that will in turn provide the data to identify unambiguously the species present in individual cloned constructs through comparison with ever increasing databases of ribosomal sequences. It is envisaged that this work will be shared between King's College London (KCL) and the Sanger Institute.

Multilocus sequence typing (MLST)

DNA extracted from organisms that have been isolated from the patient will be analysed using molecular methods (such as PCR and multi locus sequence typing) to determine the strain type to which they belong. Such information about certain organisms (for example *pneumococcus*) can inform on whether it is of a vaccine, non vaccine, or vaccine related serotype and give further information via the MLST type assigned.

High-performance liquid chromatography (HPLC)

Urine will be tested for the presence of tobramycin and copper-tobramycin using HPLC.

5. Study sponsorship

The sponsor is Southampton Universities Hospitals NHS Trust (SUHT).

6. Data

6.1 Data collection

The following data will be collected from all patients at enrolment (see Appendix 5 for data collection sheet):

- Age
- Sex
- Diagnosis
- Medications

In the case of patients providing multiple specimens over time, further data will be collected at the time of each specimen collection (see Appendix 5):

- Specimen type (e.g. sputum/cough swab)
- Current problems (e.g. well/acute exacerbation or illness)

The data will be collected (with consent) by the doctor enrolling the patient in the study or the doctor who collects the specimen from the patient. Data will be obtained from the patients' medical records and by direct questioning.

6.2 Data handling and record keeping

Data will be collected on paper and then entered into a secure electronic database. This data will be stored on a non-networked computer and will be password protected.

All data will be anonymised. Patients will be allocated a study number and all specimens and data will be labeled with the date of collection and this number only. The same study number will be applied to data and specimens from the same patient thus allowing laboratory results to be anonymously linked with patient data. The chief investigator will keep a book with details of patients enrolled and study numbers assigned. Only senior investigators will have access to this information.

Data will be collected and retained in accordance with the Data Protection Act 1998 and standard SUHT procedures.

7. Research governance, monitoring and Ethics and R&D approval

7.1 Monitoring

The study will be monitored and audited in accordance with SUHT procedures. All study related documents will be made available on request for monitoring and audit by SUHT, the relevant REC or other regulatory bodies.

7.2 Adverse Event reporting

Many of the study participants are recruited whilst in hospital and receiving treatment for an infective exacerbation of their lung problem. Due to their illness, prolonged hospitalisation and re-admissions to hospital will be commonly occurring events during routine clinical care. Similarly, study participants with severe underlying lung diseases are at risk of dying during the course of the study as a result of the natural history of their disease.

In agreement with the sponsor of this study all Adverse Events fulfilling the reporting criteria should be recorded on the CRF and submitted to the Sponsor within the defined timelines as detailed below.

If a participant experiences an Adverse Event after entry into the study (informed consent has been received) but the only protocol procedures the participant has undertaken are one or more of:

1. Informed consent
2. Sputum sample
3. Exhaled nitric oxide measurement
4. Nasal nitric oxide measurement
5. Cough swab
6. Blood sample at the same time as a clinically indicated blood test or cannula (no more than 5 ml – 1 teaspoon)
7. Explanted lung tissue removed at surgery that would otherwise be discarded
8. Urine

Then the event will not be reported unless the investigator feels that the event may have been caused by a protocol procedure. This is because these protocol procedures involve extremely minimal risk to the participant and events that would otherwise be classified as SAEs (hospital admissions etc.) are common in the clinical course of the respiratory diseases that these participants suffer from (e.g. CF, bronchiectasis, PCD etc.)

Adverse events will be reported for the following protocol procedures in the following time period after the procedure has been carried out:

1. Nasopharyngeal aspirate – any adverse events occurring up to 24 hours following the procedure will be reported. Any events occurring after this time will not be reported unless the investigator feels that the event may have been caused by the protocol procedure.
2. Bronchoalveolar lavage at (clinically indicated) bronchoscopy - any adverse events occurring up to 48 hours following the procedure will be reported. Any events occurring after this time will not be reported unless the investigator feels that the event may have been caused by the protocol procedure.

3. Endobronchial or brush biopsy at (clinically indicated) bronchoscopy - any adverse events occurring up to 7 days following the procedure will be reported. Any events occurring after this time will not be reported unless the investigator feels that the event may have been caused by the protocol procedure.
4. Research bronchoscopy with BAL +/- endobronchial or brush biopsy - any adverse events occurring up to 7 days following the procedure will be reported. Any events occurring after this time will not be reported unless the investigator feels that the event may have been caused by the protocol procedure.

Any adverse events occurring within the above timeframes that are classified as serious and assessed as being related to study procedures will be reported to the sponsor and hosting R+D departments and reported in the annual reports. Adverse Events will be reported in accordance with the UHS Research Related Adverse Event Reporting Policy. Depending on the nature of the event the reporting procedures listed in the sections below should be followed. Any events occurring outside the timeframes listed above will not be reported or included in annual safety reports unless they are deemed to be related to the study procedures by the investigator.

7.2.1 Adverse Reactions/Adverse Events

All such events, whether expected or not, should be recorded on an Adverse Event form which should be submitted to the sponsor within 7 days. They will also be recorded in the CRF.

7.2.2 Serious ARs/AEs/SUSARs

SARs, SAEs and SUSARs should be reported within 24 hours of the investigator becoming aware of the event. The SAE form asks for the nature of the event, date of onset, severity, corrective therapies given, outcome and causality. The responsible investigator should assign the causality of the event. Additional information should be sent within 5 days if the reaction has not resolved at the time of reporting. The sponsor will notify the REC of all 'fatal' or life-threatening' SUSARs occurring during the study within 7 days after the sponsor became aware of the event and within 15 days where the SUSAR was not assessed as 'life-threatening' or 'fatal'. The sponsor will report any additional relevant information to the REC within 8 days of the last report.

7.3 Ethical considerations

7.3.1 Research involving children

This research involves patients who are children. This is essential for research into lung diseases that start in childhood, because there may be windows of opportunity in which intervention with novel therapeutic options may reduce or prevent progression to chronic lung infection or inflammation that might otherwise occur. The research however, does not involve

healthy subjects of any age. Most children who are being invited to take part will not undergo any additional invasive procedures as part of the research.

This protocol aims to reduce and simplify the process from a child and family perspective by using a combined protocol and a clear and concise consent form. The previous study (LREC 306/00/w) has given us great experience in taking consent for bronchoscopy from children and their parents. From this experience, we understand that families do not require a large amount of clinical detail and the standard information sheet and consent form are based on this previous work. This will not be the case for the limited number of patients (children over 12 years and adults) invited to undergo bronchoscopy as part of the project where it is not clinically indicated and these patients will receive different information sheets with more detail to allow them to make an informed choice about participating.

7.3.2 Confidentiality

The study requires trained researchers not directly involved with the patients' medical care to have access to confidential information. It is necessary to gain consent from the patient and/or their parents (if they are <18 years) to link anonymised clinical information with the laboratory data generated. This will be done by using study numbers to identify specimens and clinical data.

7.3.3 Risk to participants

Children and adults attending the respiratory service (clinic or ward) who would normally provide samples of respiratory secretions as part of their routine clinical care.

These patients will be asked to consent to aliquots of their samples (sputum, cough swabs and naso-pharyngeal aspirates) being used for research purposes. They will not undergo any additional procedures as a result of taking part in this research and are therefore at no increased risk.

Children undergoing routine clinical bronchoscopy.

These patients will be asked to provide bronchoalveolar lavage specimens as part of their routine clinical care, which will result in no additional risk. They will also be asked to consent to a blood test (5ml) at the time of cannulation for the bronchoscopy. They will not undergo any additional blood sampling to obtain this sample and therefore this also presents no additional risk to the patient. They will be asked to consider additional endobronchial and brush biopsy at the time of bronchoscopy. Brush biopsies are safe and involve a soft brush being rubbed against the surface of the airway to collect some epithelial cells. Considering the risks of endobronchial biopsy, a report in September 2005 investigating the safety of bronchoscopy in children with difficult asthma showed that bronchoscopy with BAL and endobronchial biopsy can be performed safely in children, and that the procedure is acceptable to parents and children.²⁵ In a review of 278 endobronchial biopsies in children (8 months – 19

years) there was only trivial and superficial bleeding lasting less than 2 minutes and no other complications were seen.²⁶ It is our opinion that carrying out endobronchial biopsy at the time of bronchoscopy does not represent additional risk to the patient. This view is supported by most tertiary respiratory paediatric centres, and in some places endobronchial biopsy is now carried out routinely as part of bronchoscopies performed for clinical indications.

Adults and older children who will be asked to consent to research bronchoscopy, which is not part of their routine clinical care.

For this small group of patients who will be asked to consent to bronchoscopy in addition to their routine clinical care, there will be some additional risk. Those risks are that of bronchoscopy – transient hypoxia (low oxygen saturations), which will be corrected by withdrawing the bronchoscope and administering oxygen as soon as it occurs. There is a small chance of a nosebleed, which will be addressed by applying pressure. There are theoretical risks of pneumothorax and infection but these would be extremely low in the carefully selected study population. Over 1,000 bronchoscopies have been performed in Southampton over the last 12 years. Only 1 pneumothorax has occurred in a child with cystic fibrosis undergoing bronchoscopy in Southampton in the last 12 years. Patients undergoing bronchoscopy may also experience a raised temperature during the following 48 hours, which can be treated with paracetamol. We have considered the risks of endobronchial and brush biopsy (see above for further details) and it is our opinion that carrying out endobronchial biopsy at the time of bronchoscopy does not represent additional risk to the patient over and above that of the actual bronchoscopy.

Bronchoscopy will only be carried out by experienced trained doctors to minimize the risk of the procedure. It will be done under sedation to minimize any discomfort the patient may experience.

We have spent some time discussing whether this research question could be answered without additional bronchoscopy and have designed the protocol specifically to minimise the number of additional bronchoscopies that will be required. However, it is important for the research that biopsy specimens are collected to be able to establish whether sputum and bronchoalveolar lavage specimens are a direct marker of infection and inflammation on the surface of the lung, and it is not feasible to determine exactly how many additional biopsies will be required until the first biopsies have been analysed in the laboratory.

7.4 Ethics and R+D approval

The study will be performed subject to any provisions of favorable opinion from the Research Ethics Committee (REC), including Site Specific Assessment (SSA), and local Research and Development (R&D) approval.

7.5 Research governance

This study will be conducted in accordance with the Research Governance Framework for Health and Social Care (2005) and Good Clinical Practice.

7.6 Indemnity

This is an NHS-sponsored research study. For NHS sponsored research HSG(96)48: *NHS indemnity – arrangements for handling clinical negligence claims against NHS staff* applies. If there is negligent harm during the clinical trial when the NHS body owes a duty of care to the person harmed, NHS Indemnity covers NHS staff, medical academic staff with honorary contracts, and those conducting the trial. NHS Indemnity does not offer no-fault compensation and is unable to agree in advance to pay compensation for non-negligent harm. Ex-gratia payments may be considered in the case of a claim. Regardless of this, if a participant has concerns about any aspect of the way they have been approached or treated during the course of this study they may wish to contact the hospital's Patient Advice and Liaison Service (PALS) on 023 8079 8498, email PALS@suht.swest.nhs.uk or write to PALS, C Level, Centre Block, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD.

8. Finance

The study is funded by the National Institute for Health Research as part of the Respiratory Biomedical Research Unit.

Patients will not receive any payment for their involvement in this research.

9. Reporting and dissemination

Results from this study may be published in peer-reviewed journals and presented at national and international scientific and medical meetings. No patients will be identifiable in any such publications and presentations.

Poster presentations in language understandable to lay people will be made available for display in the Southampton Wellcome Trust Clinical Research Facility.

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Appendix 1 Information sheets for children and adults attending the respiratory service who would normally provide respiratory secretions as part of their routine clinical care

Adult patient information sheet

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

Adult Patient information sheet – sputum

We are conducting research into new ways of treating lung infection in cystic fibrosis and other chronic respiratory conditions. You are being invited to take part in a research study because you have either been diagnosed with one of these conditions or are being cared for by the respiratory service. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

People who suffer with cystic fibrosis and other respiratory diseases often produce lots of sputum. Sputum and cough swabs can be used to see if there is infection in the lungs that needs antibiotic treatment.

The research we are doing looks at new ways of growing bacteria from these samples and possible ways of enhancing antibiotic treatment of infection. By investigating these things we hope to improve our understanding of how infections cause lung disease. Finding out about the genes that cause respiratory diseases, or make them worse, is important so we can better understand the processes involved in lung infection and inflammation and hopefully devise new treatments. If you agree we would also like to use the samples for this genetic research.

It is up to you to decide whether or not to take part. If you decide to take part you will be asked to sign a consent form. Even after you have decided to take part you are still free to withdraw from the study at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen if I take part?

If you agree to take part we will ask you to provide us with a sputum sample or cough swab in the same way as usual when you attend the clinic. It is only the processing of the sample once it gets to the laboratory that will change. Part of the specimen will be sent for routine processing (as clinically indicated) and the rest will be used for research purposes. Some of the sample will be frozen and stored for analysis at a later date. All samples will be given a study number which cannot be directly traced

back to you. A doctor involved in the study will check your medical notes to record certain details of symptoms, history and treatment which will help in the analysis of these samples. A research study number will also be given to any documentation so that it cannot be traced back to you. Section B on the consent form asks you to give us permission to store the sample for use in future studies. The official name for such permission is linked anonymised, whereby results of future studies can be linked with your medical details but will not have access to your identity.

What are the side effects of taking part?

None.

What are the possible disadvantages and risks of taking part?

None. The extra laboratory tests will not affect you in any way.

What are the possible benefits of taking part?

The information we get from this study may help us to treat people with respiratory diseases better. It will not directly benefit you now but may help you or people with similar problems in the future.

Will my taking part in this study be kept confidential?

Yes. Any information about you that leaves the hospital will have your details removed so that you cannot be identified.

What will happen to the results of the research?

The results may be published in a scientific or medical journal. They may also be presented at a scientific conference. Your confidentiality will not be broken by publication, as the information presented will not be related to named individuals.

Contact for further information:

If you have any questions relating to this research you will have the opportunity to discuss them with a doctor either on the ward or when you come to clinic. Otherwise you can contact **Dr Mary Carroll**, Consultant Respiratory Physician, Infectious Diseases Unit, West wing, C level, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, Tel: 02380 796777 or **Dr Gary Connett**, Paediatric Respiratory Physician, Department of Child Health, Mailpoint 228, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, Tel: 02380 798973.

General information about participating in research in the NHS is available on the National Patient Safety Agency website at

<http://www.nres.npsa.nhs.uk/public/index.htm>. **Thank you for reading this**

Young person information sheet

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

Young person information sheet – sputum

You are being invited to take part in a research project. This sheet will tell you a bit about the research and why we are doing it. If you don't understand or want more information then please ask us.

Why are we doing this research?

You have been chosen to take part in this study because you have a long-term lung disease. We are looking at new ways of finding and treating infection in samples we collect from patients. The samples we need are sputum and cough swabs. We want to look at these samples under a microscope in the lab to see if we can understand more about lung disease and hopefully find new treatments.

Do I have to take part?

It is up to you and your parents to decide whether you wish to take part. You or your parents can ask for you to stop taking part at any time.

What will happen if I take part?

We will ask you to cough some sputum into a pot or to cough while a nurse holds a swab in your mouth. These samples will then be used for some extra tests as part of the research.

Are there any side effects or risks of taking part?

No.

What are the possible benefits of taking part?

We may be able to help children with illnesses like yours in the future.

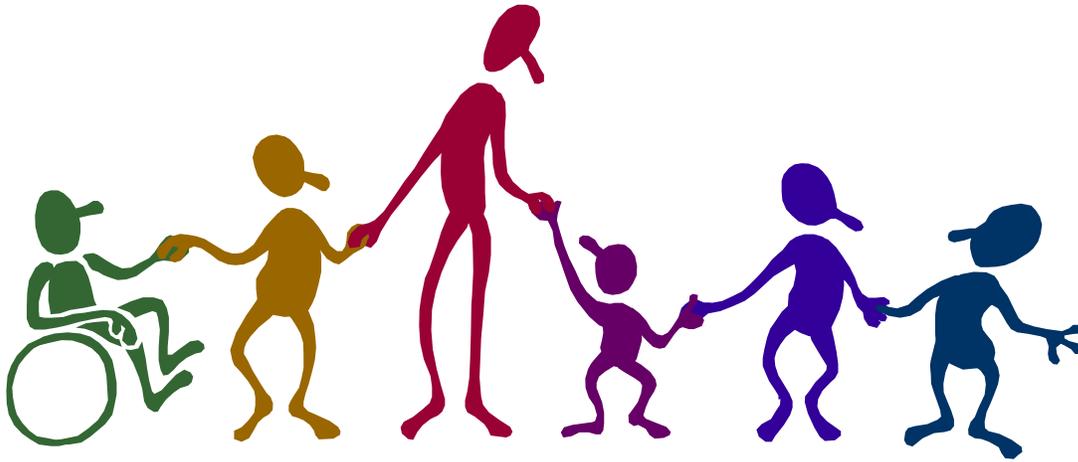
Will my taking part in this study be kept confidential?

Yes. The samples we take will be labeled with a number only, not with your personal details.

How can I get more information?

We know that children and young people want different amounts of information depending on how they are feeling, how old they are and many other reasons, so if you want to know more then please ask your parents or your doctor or ask your parents to contact Dr Connett.

Thank you very much for reading this



Parent information sheet – sputum/cough swab

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

Parent information sheet – sputum

We are conducting some research into new ways of treating lung infection in chronic respiratory conditions. Your child is being invited to take part in a research study because they have either been diagnosed with one of these conditions or are being cared for by the respiratory service. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish your child to take part.

Children with cystic fibrosis and other respiratory diseases can produce lots of sputum. Sputum and cough swabs can be used to see if there is infection in the lungs that needs antibiotic treatment.

The research we are doing looks at new ways of growing bacteria from the samples and possible ways of enhancing antibiotic treatment of infection. By investigating these things we hope to improve our understanding of how infections cause lung disease. Finding out about the genes that cause respiratory diseases, or make them worse, is important so we can better understand the processes involved in lung infection and inflammation and hopefully devise new treatments. If you agree we would also like to use the samples for this genetic research.

It is up to you to decide whether or not your child takes part. If you decide they can take part you will be asked to sign a consent form. Even after you decide to participate you are still free to withdraw your child from the study at any time and without giving a reason. This will not affect the standard of care they receive.

What will happen if my child takes part?

If you agree for your child to take part we will ask them to provide us with a sputum sample (by coughing and spitting the phlegm into a pot) or cough swab (by coughing while a nurse holds a swab in their mouth) when you attend the clinic (many children will be used to giving these samples). It is only the processing of the sample once it gets to the laboratory that will change. Part of the specimen will be sent for routine processing (as clinically indicated) and the rest will be used for research purposes. Some of the sample will be frozen and stored for analysis at a later date. All samples will be given study numbers, which cannot be directly traced back to your child. A doctor involved in the study will also check your child's notes to record details of symptoms, history and treatment, which will help in the analysis of the samples. A

research study number will also be given to any documentation so that it cannot be traced back to your child. Section B on the consent form asks you to give us permission to store the sample for use in future studies. The official name for such permission is linked anonymised, whereby results of future studies can be linked with your child's medical details but the researchers will not have access to his or her identity.

What are the side effects of taking part?

None.

What are the possible disadvantages and risks of taking part?

None. The extra laboratory tests will not affect your child in any way.

What are the possible benefits of taking part?

The information we get from this study may help us to treat children with respiratory diseases like cystic fibrosis better. It will not directly benefit your child now but may help them or children with similar problems in the future.

Will my taking part in this study be kept confidential?

Yes. Any information about your child that leaves the hospital will have their details removed so that they cannot be identified.

What will happen to the results of the research?

The results may be published in a scientific or medical journal. They may also be presented at a scientific conference. Confidentiality will not be broken by publication, as the information presented will not be related to named children.

Contact for further information:

If you have any questions relating to this research you will have the opportunity to discuss them with a children's doctor either on the ward or when you come to clinic. Otherwise you can contact Dr Connett, a children's respiratory doctor, Department of Child Health, Mailpoint 228, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, Tel: 02380 798973.

General information about participating in research in the NHS is available on the National Patient Safety Agency website <http://www.nres.npsa.nhs.uk/public/index.htm>.

Thank you for reading this

Parent information sheet – naso-pharyngeal aspirate

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

Parent information sheet – naso-pharyngeal aspirate

We are conducting some research into new ways of treating lung infection in children. Your child is being invited to take part in a research study because they have been admitted with respiratory problems. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish your child to take part.

Children with lung problems may suffer with increased infections. Naso-pharyngeal aspirates (samples of phlegm from the area between the roof of the mouth and behind the nose) can help up to identify viruses and bacteria that cause these infections.

The research we are doing looks at new ways of growing bacteria and viruses from the samples and possible ways of enhancing treatment of infection. By investigating these things we hope to improve our understanding of how infections cause lung disease in children. Finding out about the genes that cause respiratory diseases, or make them worse, is important so we can better understand the processes involved in lung infection and inflammation and hopefully devise new treatments. If you agree we would also like to use the samples for this genetic research.

It is up to you to decide whether or not your child takes part. If you decide they can take part you will be asked to sign a consent form. Even after you have decided you are still free to withdraw your child from the study at any time and without giving a reason. This will not affect the standard of care they receive.

What will happen if my child takes part?

If you agree for your child to take part we will need to take a naso-pharyngeal aspirate by sucking out some phlegm from the back of their nose with a soft tube. The tube is small and flexible and it will be passed though one nostril until it reaches the nasopharynx where the sample will be taken. It only takes a minute or two to take the sample. Part of the specimen will be sent for routine processing (as clinically indicated) and the rest will be used for research purposes. All samples will be given a study number, which cannot be directly traced back to your child. A doctor involved in the study will also check your child's notes to record certain details of symptoms, history and treatment, which will help in the analysis of these samples. A research study number will also be given to any documentation so that it cannot be traced back to your child. Section B on the consent form asks you to give us permission to store

the sample for use in future studies. The official name for such permission is linked anonymised, whereby results of future studies can be linked with your child's medical details but will not have access to his or her identity.

What are the side effects of taking part?

Although the tube used to take the sample is small and flexible, sometimes children can feel some discomfort. We will not continue with taking the sample if your child experiences excessive discomfort.

What are the possible disadvantages and risks of taking part?

None. The extra laboratory tests will not affect your child in any way.

What are the possible benefits of taking part?

The information we get from this study may help us to treat children with lung diseases better. It will not directly benefit your child now but may help children with similar problems in the future.

Will my taking part in this study be kept confidential?

Yes. Any information about your child that leaves the hospital will have their details removed so that they cannot be identified.

What will happen to the results of the research?

The results may be published in a scientific or medical journal. They may also be presented at a scientific conference. Confidentiality will not be broken by publication, as the information presented will not be related to named children.

Contact for further information:

If you have any questions relating to this research you will have the opportunity to discuss them with a children's doctor either on the ward or when you come to clinic. Otherwise you can contact Dr Connett, a children's respiratory doctor, Department of Child Health, Mailpoint 228, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, Tel: 02380 798973.

General information about participating in research in the NHS is available on the National Patient Safety Agency website at <http://www.nres.npsa.nhs.uk/public/index.htm>.

Thank you for reading this

Appendix 2 Information sheets for children undergoing routine clinical bronchoscopy

Parent information sheet

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

Parent information sheet – bronchoscopy

Your child is due to have a bronchoscopy to investigate his or her chest symptoms. You are being invited to take part in a research study in addition to the bronchoscopy. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Your child has been chosen to take part in this study because he or she is due to have a bronchoscopy. During the bronchoscopy, while the child is sedated, it is normal practice to take a washing of part of the lung in order to look for infections. This is called bronchoalveolar lavage or BAL. We are also able to look at the immune cells in the washing fluid, which can help us to make the diagnosis.

We would like to look at some other aspects of the composition of the BAL fluid. In particular the way bacteria grow and a substance called surfactant, which is found naturally in the lung and allows it to function normally. By investigating these things we hope to be able to better understand how some diseases work. Blood samples can also give us useful information about inflammation and we would like to take an extra teaspoonful (5ml) of blood from your child when they have the cannula sited for the procedure. We are also interested in looking at the lining of the airways and would like to take a very small sample of this (called a biopsy). Finding out about the genes that cause respiratory diseases, or make them worse, is important so we can better understand the processes involved in lung infection and inflammation and hopefully devise new treatments. If you agree we would also like to use the samples for this genetic research.

It is up to you to decide whether or not you wish your child to take part. They can take part in some or all of the study. If you decide to take part you will be asked to sign a consent form on the day of the bronchoscopy. Even after you have decided to take part you are still free to withdraw your child at any time and without giving a reason. This will not affect the standard of care they receive.

What will happen if my child takes part?

Very little will change if you consent to your child taking part. They will have the bronchoscopy as planned. The blood sample will be taken when the cannula is put in (this is for the medicine to make your child sleepy for the bronchoscopy), so we will not use any extra needles. The BAL will be taken during the bronchoscopy if the doctor feels it is needed to help diagnose your child's problems. Then if you have consented, some of that sample will be used for research after the other tests have been done. If you consent to a small biopsy then this will be taken during the bronchoscopy.

All samples will be given a study number, which cannot be directly traced back to your child. A doctor involved in the study will also check your child's notes to record certain details of symptoms, history and treatment which will help in the analysis of these samples. A research study number will also be given to any documentation so that it can not be directly traced back to your child. Section B on the consent form asks you to give us permission store the sample for use in future studies. The official name for such permission is linked anonymised, whereby results of future studies can be linked with your child's medical details but will not have access to his or her identity.

What are the side effects of taking part?

The lung washing is only performed if it is needed to help diagnose your child's symptoms. The doctor performing the bronchoscopy will decide if this is necessary during the procedure. The lung washing can cause a raised temperature in the following 48 hours, this can be treated with paracetamol.

What are the possible disadvantages and risks of taking part?

The biopsy has been shown to be safe and there are no risks above that of the bronchoscopy which your child is having for clinical reasons. The blood sample will only be taken when a cannula is being placed for clinical reasons (for sedation for the procedure). The extra laboratory tests that will be carried out on the BAL fluid, blood and biopsy will not affect your child in any way.

What are the possible benefits of taking part?

The information we get from this study may help us to treat children with respiratory illnesses better. It will not directly benefit your child but may help children with similar problems in the future.

Will my taking part in this study be kept confidential?

Yes. Any information about you that leaves the hospital will have your child's details removed so that they cannot be identified.

What will happen to the results of the research?

The results may be published in a scientific or medical journal. They may also be presented at a scientific conference. Confidentiality will not be broken by publication as the information presented will not be related to named children.

Contact for further information:

If you have any questions relating to this research you will have the opportunity to discuss them with a children's doctor on the day of the bronchoscopy. Otherwise you can contact Dr Connett, a children's respiratory doctor, Department of Child Health, Mailpoint 228, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, Tel: 02380 798973.

General information about participating in research in the NHS is available on the National Patient Safety Agency website at <http://www.nres.npsa.nhs.uk/public/index.htm>.

Thank you for reading this

Young person information sheet

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

Young person information sheet – bronchoscopy

You are being invited to take part in a research project. This sheet will tell you a bit about the research and why we are doing it. If you don't understand or want more information then please ask us.

Why are we doing this research?

You have been chosen to take part in this study because you are going to have a bronchoscopy to investigate your lung problems. Bronchoscopy is where a small tube with a camera on the end is passed down your nose into your lungs to look at the airways and take samples. You will be given some medicine to make you sleepy during the procedure (through a cannula). We are looking at new ways of finding and treating infection in samples we collect from patients during bronchoscopy. The samples we need are lung washing fluid and small pieces of the lining of the airway. We also need blood samples, which can be taken when you have a cannula put in for the medicine to make you sleepy for the bronchoscopy. We would like you to think about whether some of the samples that will be taken during your bronchoscopy can also be used for research.

We want to look at these samples under a microscope in the lab to see if we can understand more about lung disease and hopefully find new treatments.

Do I have to take part?

It is up to you and your parents to decide whether you wish to take part. You or your parents can ask for you to stop taking part at any time.

What will happen if I take part?

You will come into hospital for your bronchoscopy. When you arrive you will be seen by a doctor who will examine you and will discuss the procedure with you and your parents. The doctor will ask your parents to sign a consent form (because you are under 18 years) for the bronchoscopy and for the samples to be taken and they will check that you agree. The blood sample will be taken when the cannula is put in (this is for the medicine to make you sleepy for the bronchoscopy). The lung washing (BAL) will be taken during the bronchoscopy by washing some sterile salt water into your lung and sucking it back up the tube into a container. The small biopsy will be taken at the same time.

What are the side effects of taking part?

The lung washing can cause a raised temperature in the next 2 days; this can be treated with paracetamol.

What are the possible disadvantages and risks of taking part?

The doctor will discuss this with your parents. Bronchoscopy is a common procedure and we rarely see any problems.

What are the possible benefits of taking part?

We may be able to help children with illnesses like yours in the future.

Will my taking part in this study be kept confidential?

Yes. The samples we take will be labeled with a number only, not with your personal details.

How can I get more information?

We know that children and young people want different amounts of information depending on how they are feeling, how old they are and many other reasons, so if you want to know more then please ask your parents or your doctor or ask your parents to contact Dr Connatt.

Thank you very much for reading this



Children's information sheet

Q. Is there anything else I need to know?

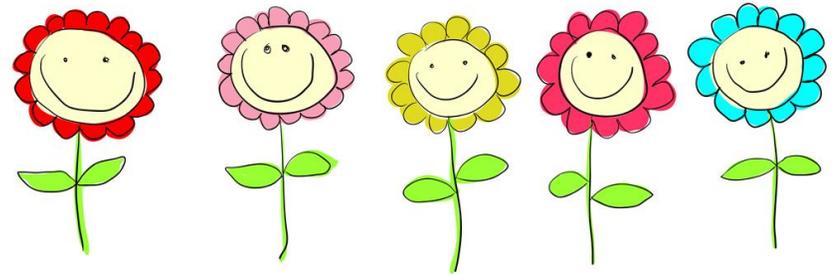
We are doing a study looking at how to improve treatment for children with breathing problems like yours. We would like you and your parents to allow us to use some of the samples we will take when you have your bronchoscopy for this research. It will not make things any different for you when you have your bronchoscopy. We have given your parents more information about the research and we would be happy to talk to you more about it if you would like us to.

If you have any questions make sure you ask your doctor or nurse.



Teddy can come along too!

Information about your bronchoscopy



Information sheet for children

Wellcome Trust Clinical Research Facility
Southampton General Hospital
Tel: 023 8079 4989

Version 1.2 01-09-08

NHS REC No. 08/H0502/126

Reviewed by Southampton and South West Hampshire
Research Ethics Committee A on 14/10/08

Q. Why are you coming into hospital?



You have 2 lungs in your chest which fill with air when you breathe. The doctor wants to look at your lungs and see how they are working.

Q. What do you need to do before coming in?

Your letter will tell you what time to come to the hospital. It is important that you do not have anything to eat or drink for 4 hours before you come into hospital.



Q. What will happen when you arrive?

When you arrive you will meet the nurses. They will show you to your bed. They will weigh you. They will take your temperature. They will check your oxygen levels by putting a soft clip on your finger or toe. The clip may need to stay on for a while after your bronchoscopy but it doesn't hurt at all.



You will then meet the doctor who will talk to you and your mum and dad and make sure you are well and will listen to your chest with their stethoscope.

The doctor needs to put a small tube in a vein in the back of your hand so we can give you some medicine. The nurse will put some magic cream or cold spray on the back of your hand so it numbs



the skin and you may not even feel a thing. This is quick and it will be hidden away by a bandage. The nurse will take the small tube out of your hand before you go home.

Q. What happens next?

The nurse will take you and your parent(s) through to a special room where the bronchoscopy will happen. The doctor will then give you some medicines through the small tube in your hand, this will make you feel sleepy. When you are sleepy we will wrap you warmly in a blanket and then the doctor will put a very special narrow tube into your nose. This tube has a tiny light with a camera on the end so the doctor can see into your lungs.

This is called a **bronchoscopy** (try and say bron-kos-ka-pee)

Q. How long will it take?

It takes about half an hour and you will be sleepy all that time. The nurse may need to give you some oxygen through a plastic mask for a while.

Your mum and dad will be there when you wake up. We will take you back to your comfy bed and you can have a sleep while the nurses make sure you are ok.



We will give you something to eat and drink before you go home.

Appendix 3 Information sheets for adults and older children who will be asked to consent to research bronchoscopy which is not part of their routine clinical care

Adult patient information sheet

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

Adult Patient information sheet – research bronchoscopy

You are being invited to take part in a research project. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

You have been chosen to take part in this study because you have a chronic lung disease. We are looking at new ways of identifying bacteria in specimens from patients and potential new treatments of infection. To carry out this research we need samples of lung washing and very small samples of the airway lining (called a biopsy) to look at under a microscope in the lab. These samples can only be collected during a bronchoscopy and we are asking you to consider whether you would be prepared to undergo bronchoscopy so that we can collect these samples.

Bronchoscopy is where a small tube containing a camera is passed down the nose into the lungs to look at the airways and take samples. You will be given some medicine (through a cannula) to make you sleepy during the procedure.

The lung washing samples (known as bronchoalveolar lavage – BAL fluid) allows us to look at the way bacteria grow and how a substance called surfactant, allows it to function normally. Blood samples can give us useful information about inflammation and we would like to take an extra teaspoonful (5ml) of blood when you have the cannula sited for the procedure. We are also interested in looking at the lining of the airways and would like to take a very small sample of this (called a biopsy). By investigating these things we hope to be able to better understand how some diseases work. Finding out about the genes that cause respiratory diseases, or make them worse, is important so we can better understand the processes involved in lung infection and inflammation and hopefully devise new treatments. If you agree we would also like to use the samples for this genetic research.

It is up to you to decide whether or not you wish to take part. You can take part in

some or all of the study. If you decide to take part you will be asked to sign a consent form on the day of the bronchoscopy. Even after you have decided to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen if I take part?

You will come into hospital for your bronchoscopy. When you arrive you will be seen by a doctor who will examine you and will discuss the procedure with you. The doctor will ask you to sign a consent form for the bronchoscopy and for the samples to be taken. The blood sample will be taken when the cannula is put in (this is for the medicine to make you sleepy for the bronchoscopy). The lung washing (BAL) will be taken during the bronchoscopy by washing some sterile salt water into the lung and sucking it back up the tube into a container. The small biopsy will be taken at the same time.

All samples will be given a study number, which cannot be directly traced back to you. A doctor involved in the study will also check your notes to record certain details of symptoms, history and treatment which will help in the analysis of these samples. A research study number will be given to any documentation so that it can not be directly traced back to you. Section B on the consent form asks you to give us permission store the sample for use in future studies. The official name for such permission is linked anonymised, which means that results of future studies can be linked with your medical details but will not have access to your identity.

What are the side effects of taking part?

The lung washing can cause a raised temperature in the following 48 hours, this can be treated with paracetamol.

What are the possible disadvantages and risks of taking part?

The risks associated with bronchoscopy are brief low oxygen saturations which will be corrected by removing the bronchoscope and giving oxygen as soon as it occurs. There is a small chance of a nose bleed which will be stopped by applying pressure. There are extremely low risks of pneumothorax (air escaping outside the lung – which usually resolves itself) and infection but the risks are negligible when performed in clinically stable patients. The biopsy has been shown to be safe and there are no risks above that of the bronchoscopy. The blood sample will only be taken when a cannula is being placed (for sedation for the procedure). The laboratory tests that will be carried out on the BAL fluid, blood and biopsy will not affect you in any way.

What are the possible benefits of taking part?

The information we get from this study may help us to treat patients with respiratory illnesses better. It will not directly benefit you at this time but may help you or patients

with similar problems in the future. If we discover anything of significance for you in the course of the research we will let you know and arrange for treatment as appropriate.

Will my taking part in this study be kept confidential?

Yes. Any information about you that leaves the hospital will have your details removed so that you cannot be identified.

What will happen to the results of the research?

The results may be published in a scientific or medical journal. They may also be presented at a scientific conference. Confidentiality will not be broken by publication as the information presented will not be related to named patients.

Contact for further information:

If you have any questions relating to this research please do not hesitate to contact either **Dr Mary Carroll**, Consultant Respiratory Physician, Infectious Diseases Unit, West wing, C level, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, Tel: 02380 796777 or **Dr Gary Connett**, Paediatric Respiratory Physician, Department of Child Health, Mailpoint 228, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, Tel: 02380 798973 directly. You will also be given ample opportunity to ask any questions you might have with a doctor on the day of the bronchoscopy.

General information about participating in research in the NHS is available on the National Patient Safety Agency website at <http://www.nres.npsa.nhs.uk/public/index.htm>.

Thank you for reading this

Young person information sheet

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

Young person information sheet – research bronchoscopy

You are being invited to take part in a research project. This sheet will tell you a bit about the research and why we are doing it. If you don't understand or want more information then please ask us.

Why are we doing this research?

You have been chosen to take part in this study because you have a long-term lung disease. We are looking at new ways of finding and treating infection in samples we collect from patients. The samples we need are collected during a bronchoscopy. Bronchoscopy is where a small tube with a camera on the end is passed down your nose into your lungs to look at the airways and take samples. You will be given some medicine to make you sleepy during the procedure (through a cannula). The samples we need are lung washing fluid and small pieces of the lining of the airway. We also need blood samples, which can be taken when you have a cannula put in for the medicine to make you sleepy for the bronchoscopy.

We want to look at these samples under a microscope in the lab to see if we can understand more about lung disease and hopefully find new treatments.

Do I have to take part?

It is up to you and your parents to decide whether you wish to take part. You or your parents can ask for you to stop taking part at any time.

What will happen if I take part?

You will come into hospital for your bronchoscopy. When you arrive you will be seen by a doctor who will examine you and will discuss the procedure with you and your parents. The doctor will ask your parents to sign a consent form (because you are under 18 years) for the bronchoscopy and for the samples to be taken and they will check that you agree. The blood sample will be taken when the cannula is put in (this is for the medicine to make you sleepy for the bronchoscopy). The lung washing (BAL) will be taken during the bronchoscopy by washing some sterile salt water into your lung and sucking it back up the tube into a container. The small biopsy will be taken at the same time.

What are the side effects of taking part?

The lung washing can cause a raised temperature in the next 2 days; this can be treated with paracetamol.

What are the possible disadvantages and risks of taking part?

The doctor will discuss this with your parents. Bronchoscopy is a common procedure and we rarely see any problems.

What are the possible benefits of taking part?

We may be able to help children with illnesses like yours in the future.

Will my taking part in this study be kept confidential?

Yes. The samples we take will be labeled with a number only, not with your personal details.

How can I get more information?

We know that children and young people want different amounts of information depending on how they are feeling, how old they are and many other reasons, so if you want to know more then please ask your parents or your doctor or ask your parents to contact Dr Connett.

Thank you very much for reading this



Parent information sheet

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

Parent information sheet – research bronchoscopy

Your child is being invited to take part in a research project. Before you decide whether or not your child should take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Your child has been chosen to take part in this study because he or she has a chronic lung disease. We are looking at new ways of identifying bacteria in specimens from patients and potential new treatments of infection. To carry out this research we need samples of lung washing and very small samples of the airway lining (called a biopsy) to look at under a microscope in the lab. These samples can only be collected during a bronchoscopy and we are asking you to consider whether your child would be prepared to undergo bronchoscopy so that we can collect these samples.

Bronchoscopy is where a small tube containing a camera is passed down the child's nose into the lungs to look at the airways and take samples. The child is given some medicine (through a cannula) to make them sleepy during the procedure and they will not remember what happens.

The lung washing samples (known as bronchoalveolar lavage – BAL fluid) allows us to look at the way bacteria grow and a substance called surfactant, which is found naturally in the lung and helps it to function normally. Blood samples can also give us useful information about inflammation and we would like to take an extra teaspoonful (5ml) of blood from your child when they have the cannula sited for the procedure. We are also interested in looking at the lining of the airways and would like to take a very small sample of this (called a biopsy). By investigating these things we hope to be able to better understand how some diseases work. Finding out about the genes that cause respiratory diseases, or make them worse, is important so we can better understand the processes involved in lung infection and inflammation and hopefully devise new treatments. If you agree we would also like to use the samples for this genetic research.

It is up to you to decide whether or not you wish your child to take part. They can take part in some or all of the study. If you decide to take part you will be asked to sign a consent form on the day of the bronchoscopy. Even after you have decided to take part you are still free to withdraw your child at any time and without giving a reason. This will not affect the standard of care they receive.

What will happen if my child takes part?

They will come into hospital for their bronchoscopy. When they arrive they will be seen by a doctor who will examine them and will discuss with you the procedure. The doctor will ask you to sign a consent form for the bronchoscopy and for the samples to be taken. The blood sample will be taken when the cannula is put in (this is for the medicine to make your child sleepy for the bronchoscopy). The lung washing (BAL) will be taken during the bronchoscopy by washing some sterile salt water into the lung and sucking it back up the tube into a container. The small biopsy will be taken at the same time.

All samples will be given a study number, which cannot be directly traced back to your child. A doctor involved in the study will also check your child's notes to record certain details of symptoms, history and treatment, which will help in the analysis of these samples. A research study number will be given to any documentation so that it cannot be directly traced back to your child. Section B on the consent form asks you to give us permission store the sample for use in future studies. The official name for such permission is linked anonymised, which is where results of future studies can be linked with your child's medical details but will not have access to his or her identity.

What are the side effects of taking part?

The lung washing can cause a raised temperature in the following 48 hours; this can be treated with paracetamol.

What are the possible disadvantages and risks of taking part?

The risks associated with bronchoscopy are brief low oxygen saturations, which will be corrected by removing the bronchoscope and giving oxygen as soon as it occurs. There is a small chance of a nosebleed, which will be stopped by applying pressure. There are extremely low risks of pneumothorax (air escaping outside the lung – which usually resolves itself) and infection but these risks are negligible when performed in clinically stable patients. The biopsy has been shown to be safe and there are no risks above that of the bronchoscopy. The blood sample will only be taken when a cannula is being placed (for sedation for the procedure). The laboratory tests that will be carried out on the BAL fluid, blood and biopsy will not affect your child in any way.

What are the possible benefits of taking part?

The information we get from this study may help us to treat children with respiratory illnesses better. It will not directly benefit your child at this time but may help them or children with similar problems in the future. If we discover anything of significance for your child in the course of the research we will let you know and arrange for treatment as appropriate.

Will my taking part in this study be kept confidential?

Yes. Any information about you that leaves the hospital will have your child's details removed so that they cannot be identified.

What will happen to the results of the research?

The results may be published in a scientific or medical journal. They may also be presented at a scientific conference. Confidentiality will not be broken by publication, as the information presented will not be related to named children.

Contact for further information:

If you have any questions relating to this research you will have the opportunity to discuss them with a children's doctor on the day of the bronchoscopy. Otherwise you can contact Dr Connett, a children's respiratory doctor, Department of Child Health, Mailpoint 228, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, Tel: 02380 798973.

General information about participating in research in the NHS is available on the National Patient Safety Agency website at <http://www.nres.npsa.nhs.uk/public/index.htm>.

Thank you for reading this

Appendix 4 Consent forms

Adult patient staged consent form – Research bronchoscopy

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

ADULT PATIENT CONSENT FORM (Staged) – Research bronchoscopy Version 1.1

Subject Identification Number for this trial:

Name of Researcher: Dr Gary Connett

PART A Consent for the main study

PLEASE **INITIAL** THE BOXES FOR EACH SECTION YOU WISH TO TAKE PART IN (please put a cross through any you do not wish to consent to):

1. I confirm that I have read the information sheet for the above study. I have had the opportunity to ask questions, understand why the research is being done and any possible risks have been explained to me.
2. I understand that my participation is voluntary and that I am free to withdraw at any time.
3. I give permission for sections of my medical notes to be looked at by research doctors involved in this study.
4. I agree to take part in the above study and provide a sputum specimens/cough swabs.
5. I agree to take part in the above study to collect a sample of lung washing fluid at bronchoscopy.
6. I agree to take part in the above study to collect a small biopsy from my lung at bronchoscopy.
7. I agree to take part in the above study to collect a blood sample at the time of cannulation.
8. I agree that the samples I provide can be used for genetic studies.

SAMPLES GIFTED FOR STORAGE AND USE IN FUTURE STUDIES

PART B **Linked Anonymised Samples**

9. I indicate my consent for the samples to be stored (potentially for many years) for the following types of studies (provided they have gained ethical approval):

a. I give permission for the samples to be used for treatments/investigations lung diseases in children and adults.

b. I give permission for the samples to be stored for use in other unrelated research studies, including genetic studies, the precise nature of which will depend upon future scientific advances.

c. In the case of linked anonymised samples, I give permission for a member of the research team to look at my medical records.

d. I understand that the samples may be used for Commercial development, without financial or other benefit to myself in keeping with the gift nature of my sample.

Name of Patient

Date

Signature

Name of Person taking consent Date
(if different from researcher)

Signature

Researcher

Date

Signature

1 for patient, 1 for researcher, 1 to be kept with hospital notes.

Parent/child staged consent form – Research bronchoscopy

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

PARENT/CHILD CONSENT FORM (Staged) – Research bronchoscopy Version 1.1

Child Identification Number for this trial:

Name of Researcher: Dr Gary Connett

PART A Consent for the main study

PLEASE **INITIAL** THE BOXES FOR EACH SECTION YOU WISH YOUR CHILD TO TAKE PART IN (please put a cross through any you do not wish to consent to):

1. I have read the information sheet for the above study. I have had the opportunity to ask questions, I understand why the research is being done and any possible risks have been explained to me.
2. I understand that my child's participation is voluntary and that we can withdraw at any time.
3. I give permission for sections of my child's notes to be looked at by research doctors involved in this study.
4. I agree to take part in the above study and for my child to provide sputum specimens/cough swabs.
5. I agree to take part in the above study and for my child to provide a naso-pharyngeal aspirate.
6. I agree to my child taking part in the above study to collect a sample of lung washing fluid at bronchoscopy.
7. I agree to my child taking part in the above study to collect a small biopsy from my child's lung at bronchoscopy.
8. I agree to my child taking part in the above study to collect a blood sample during cannulation for bronchoscopy.
9. I agree that the samples my child provides can be used for genetic studies.

SAMPLES GIFTED FOR STORAGE AND USE IN FUTURE STUDIES

PART B **Linked Anonymised Samples**

10. I indicate my consent for the samples to be stored (potentially for many years) for the following types of studies (provided they have gained ethical approval):

- a. I give permission for the samples to be used for treatments/investigations of lung diseases in children and adults.
- b. I give permission for the samples to be stored for use in other unrelated research studies, including genetic studies, the precise nature of which will depend upon future scientific advances.
- c. In the case of linked anonymised samples, I give permission for a member of the research team to look at my child's medical records.
- d. I understand that the samples may be used for Commercial development, without financial or other benefit to myself in keeping with the gift nature of the sample.

Name of Parent

Date

Signature

Name of Person taking consent
(if different from researcher)

Date

Signature

Researcher

Date

Signature

For children >12 years:

I agree to take part in this study

Name

Date

1 for patient, 1 for researcher, 1 to be kept with hospital notes.

Adult patient staged consent form – Routine samples

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

ADULT PATIENT CONSENT FORM (Staged) – Routine Samples Version 1.2

Subject Identification Number for this trial:
Name of Researcher: Dr Gary Connett

PART A Consent for the main study

PLEASE **INITIAL** THE BOXES FOR EACH SECTION YOU WISH TO TAKE PART IN (please put a cross through any you do not wish to consent to):

1. I confirm that I have read the information sheet for the above study. I have had the opportunity to ask questions, understand why the research is being done and any possible risks have been explained to me.
2. I understand that my participation is voluntary and that I am free to withdraw at any time.
3. I give permission for sections of my medical notes to be looked at by research doctors involved in this study.
4. I agree to take part in the above study and provide a sputum specimens/cough swabs.
5. I agree that the samples I provide can be used for genetic studies.

Parent/child staged consent form – Routine samples

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

PARENT/CHILD CONSENT FORM 1 – Routine samples

Version 1.2

Child Identification Number for this trial:

Name of Researcher: Dr Gary Connett

PART A Consent for the main study

PLEASE **INITIAL** THE BOXES FOR EACH SECTION YOU WISH YOUR CHILD TO TAKE PART IN (please put a cross through any you do not wish to consent to):

1. I have read the information sheet for the above study. I have had the opportunity to ask questions, I understand why the research is being done and any possible risks have been explained to me.
2. I understand that my child's participation is voluntary and that we can withdraw at any time.
3. I give permission for sections of my child's notes to be looked at by research doctors involved in this study.
4. I agree to take part in the above study and for my child to provide sputum specimens/cough swabs.
5. I agree to take part in the above study and for my child to provide a naso-pharyngeal aspirate.
6. I agree that the samples my child provides can be used for genetic studies.

Appendix 5 Data collection sheets

Data collection sheet – enrolment

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

Data collection sheet Enrolment data

Patient study number	
Date of enrolment	
Age	
Sex	
Diagnosis	
Medications	

Data collection sheet – specimen collection

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

Data collection sheet Specimen collection

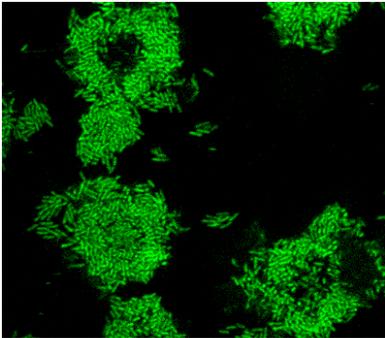
Patient study number	
Specimen type	
Date of specimen collection	
Age	
Sex	
Current problems <input type="checkbox"/> Well <input type="checkbox"/> Acute exacerbation	
Diagnosis	
Medications	

Appendix 6 Patient information poster for display

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

Do you or your child have a respiratory illness?

If so, we may ask you to consider taking part in some research we are doing



Bacteria in biofilms seen under a microscope

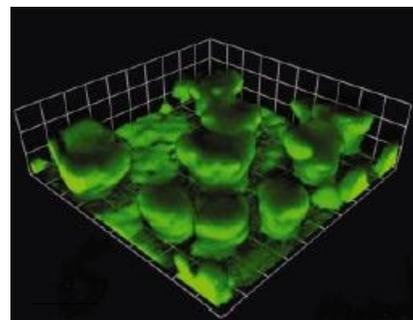
We are looking at inflammation in the airways and new ways of identifying bacteria in specimens from patients. From a better understanding of the processes involved in lung infection and inflammation we hope to eventually devise new treatments.

To carry out this research we need samples from patients with lung diseases such as cystic fibrosis, primary ciliary dyskinesia, asthma and many others.

The samples we need are blood, sputum, cough swabs, naso-pharyngeal aspirates, lung washing (taken at bronchoscopy) and very small samples of the airway lining (called a biopsy – also taken at bronchoscopy).

You or your child may be asked to allow us to use some of their samples for research. These are extra tests that will be carried out on samples that have been collected for clinical reasons.

If you would like more information about this study then please contact Dr Connett (a children's respiratory doctor) or ask the nurse or doctor who is looking after you.



Bacteria growing in biofilms

Appendix 7 Old consent form (LREC306/00/w)

Study Number: 306/00

Subject Identification Number for this trial:

PARENT'S CONSENT FORM (Staged)

PART A *Consent for the main study*

Title of Project: Analysis of immune and inflammatory mechanisms in paediatric respiratory disease

Name of Researcher: Professor J.O. Warner

PLEASE **INITIAL** THE BOXES IF YOU AGREE WITH EACH SECTION:

8. I confirm that I have read the information sheet dated January 2002 (version 3) for the above study, have had the opportunity to ask questions, understand why the research is being done and any possible risks which have been explained to me.

9. I understand that my child's participation is voluntary and that he/she is free to withdraw at any time by contacting Professor Warner without giving any reason and without his/her medical care or legal rights being affected. If he/she withdraws, I understand that any unused donated tissue will be disposed of, in the case of linked anonymised samples.

10. I understand that sections of any of my child's medical notes may be looked at by research doctors from the Department of Child Health where it is relevant to taking part in this study. I give permission for these individuals to have access to these records.

11. I understand that (my Doctor and/or I, as appropriate) will be informed if any of the results of the medical tests done as part of the research are important for my child's health.

12. I agree to my child taking part in the above study to collect a sample of lung washing fluid to study lung inflammation in children.

SAMPLES GIFTED FOR STORAGE AND USE IN FUTURE STUDIES

PART B Linked Anonymised Samples:

13. Provided that specific study protocols have been reviewed and approved by the Local Research Ethics Committee, I indicate my consent for the sample and its derived cells to be stored (potentially for many years) for the following types of studies. I understand that these studies are not for the purpose of directly benefiting my health:

a. I give permission for the sample to be used for treatments/investigations of medical conditions relating to lung diseases of children.

b. I give permission for the sample to be stored for use in other unrelated research studies the precise nature of which will depend upon future scientific advances, but excluding genetic studies and germ-line research.

c. In the case of linked anonymised samples, I give permission for a member of the research team to look at my child's medical records, to obtain information on his/her history of lung infection and/or inflammation. I understand that all information will be kept confidential

14. I understand that the sample may be used for Commercial development, without financial or other benefit to myself, for the investigation and treatment of medical conditions, potentially leading to new preventative measures against such conditions in keeping with the gift nature of my sample.

Name of Patient

Date

Signature

Name of Person taking consent
(if different from researcher)

Date

Signature

Researcher

Date

Signature

1 for patient, 1 for researcher, 1 to be kept with hospital notes.

Appendix 8 Information sheets and consent forms for urine samples

Adult patient information sheet - urine

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

Adult Patient information sheet – Urine

We are conducting research into new ways of treating lung infection in cystic fibrosis and other chronic respiratory conditions. You are being invited to take part in a research study because you have cystic fibrosis. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

The research we are doing looks at new ways in which antibiotics work in treating infection. By investigating these things we hope to improve our understanding of how infections cause lung disease and how they can best be treated.

It is up to you to decide whether or not to take part. If you decide to take part you will be asked to sign a consent form. Even after you have decided to take part you are still free to withdraw from the study at any time and without giving a reason. This will not affect the standard of care you receive.

What will happen if I take part?

If you agree to take part we will ask you to provide us with a urine sample. It is only the processing of the sample once it gets to the laboratory that will change. This sample will be used for research purposes. All samples will be given a study number, which cannot be directly traced back to you. A doctor involved in the study will check your medical notes to record certain details of symptoms, history and treatment which will help in the analysis of these samples. A research study number will also be given to any documentation so that it cannot be traced back to you. Section B on the consent form asks you to give us permission to store the sample for use in future studies. The official name for such permission is linked anonymised, whereby results of future studies can be linked with your medical details but will not have access to your identity.

What are the side effects of taking part?

None.

What are the possible disadvantages and risks of taking part?

None. The extra laboratory tests will not affect you in any way.

What are the possible benefits of taking part?

The information we get from this study may help us to treat people with respiratory diseases better. It will not directly benefit you now but may help you or people with similar problems in the future.

Will my taking part in this study be kept confidential?

Yes. Any information about you that leaves the hospital will have your details removed so that you cannot be identified.

What will happen to the results of the research?

The results may be published in a scientific or medical journal. They may also be presented at a scientific conference. Your confidentiality will not be broken by publication as the information presented will not be related to named individuals.

Contact for further information:

If you have any questions relating to this research you will have the opportunity to discuss them with a doctor either on the ward or when you come to clinic. Otherwise you can contact **Dr Mary Carroll**, Consultant Respiratory Physician, Infectious Diseases Unit, West wing, C level, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, Tel: 02380 796777 or **Dr Gary Connett**, Paediatric Respiratory Physician, Department of Child Health, Mailpoint 228, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, Tel: 02380 798973.

General information about participating in research in the NHS is available on the National Patient Safety Agency website at <http://www.nres.npsa.nhs.uk/public/index.htm>.

Thank you for reading this

Parent information sheet - urine

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

Parent information sheet – Urine

We are conducting some research into new ways of treating lung infection in chronic respiratory conditions. Your child is being invited to take part in a research study because they have either been diagnosed with one of these conditions or are being cared for by the respiratory service. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish your child to take part.

The research we are doing looks at new ways in which antibiotics work in treating infection. By investigating these things we hope to improve our understanding of how infections cause lung disease and how they can best be treated.

It is up to you to decide whether or not your child takes part. If you decide they can take part you will be asked to sign a consent form. Even after you decide to participate you are still free to withdraw your child from the study at any time and without giving a reason. This will not affect the standard of care they receive.

What will happen if my child takes part?

If you agree for your child to take part we will ask them to provide us with a urine sample. This sample will be used for research purposes. All samples will be given study numbers, which cannot be directly traced back to your child. A doctor involved in the study will also check your child's notes to record details of symptoms, history and treatment which will help in the analysis of the samples. A research study number will also be given to any documentation so that it cannot be traced back to your child. Section B on the consent form asks you to give us permission to store the sample for use in future studies. The official name for such permission is linked anonymised, whereby results of future studies can be linked with your child's medical details but the researchers will not have access to his or her identity.

What are the side effects of taking part?

None.

What are the possible disadvantages and risks of taking part?

None. The research tests will not affect your child in any way.

What are the possible benefits of taking part?

The information we get from this study may help us to treat children with respiratory diseases like cystic fibrosis better. It will not directly benefit your child now but may help them or children with similar problems in the future.

Will my taking part in this study be kept confidential?

Yes. Any information about your child that leaves the hospital will have their details removed so that they cannot be identified.

What will happen to the results of the research?

The results may be published in a scientific or medical journal. They may also be presented at a scientific conference. Confidentiality will not be broken by publication as the information presented will not be related to named children.

Contact for further information:

If you have any questions relating to this research you will have the opportunity to discuss them with a children's doctor either on the ward or when you come to clinic. Otherwise you can contact Dr Connett, a children's respiratory doctor, Department of Child Health, Mailpoint 228, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, Tel: 02380 798973.

General information about participating in research in the NHS is available on the National Patient Safety Agency website
<http://www.nres.npsa.nhs.uk/public/index.htm>.

Thank you for reading this

Young person information sheet - urine

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

Young person information sheet – Urine

You are being invited to take part in a research project. This sheet will tell you a bit about the research and why we are doing it. If you don't understand or want more information then please ask us.

Why are we doing this research?

You have been chosen to take part in this study because you have cystic fibrosis. We are looking at new ways of treating infection. The samples we need to do this include urine samples from people who are receiving antibiotics.. We want to look at these samples and see if we can understand more about how antibiotics might work better to treat infections.

Do I have to take part?

It is up to you and your parents to decide whether you wish to take part. You or your parents can ask for you to stop taking part at any time.

What will happen if I take part?

We will ask you to provide a urine sample in a pot. The sample will then be used for some extra tests as part of the research.

Are there any side effects or risks of taking part?

No.

What are the possible benefits of taking part?

We may be able to help children with illnesses like yours in the future.

Will my taking part in this study be kept confidential?

Yes. The samples we take will be labeled with a number only, not with your personal details.

How can I get more information?

We know that children and young people want different amounts of information depending on how they are feeling, how old they are and many other reasons, so if you want to know more then please ask your parents or your doctor or ask your parents to contact Dr Connett.

Thank you very much for reading this



Adult staged consent form - urine

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

**ADULT PATIENT CONSENT FORM (Staged) – Urine Samples
Version 1**

Subject Identification Number for this trial:

Name of Researcher: *Dr Gary Connett*

PART A Consent for the main study

PLEASE **INITIAL** THE BOXES FOR EACH SECTION YOU WISH TO TAKE PART IN
(please put a cross through any you do not wish to consent to):

7. *I confirm that I have read the information sheet for the above study. I have had the opportunity to ask questions, understand why the research is being done and any possible risks have been explained to me.*

8. *I understand that my participation is voluntary and that I am free to withdraw at any time.*

9. *I give permission for sections of my medical notes to be looked at by research doctors involved in this study.*

10. *I agree to take part in the above study and provide a urine sample.*

Parent staged consent form - urine

Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease

PARENT/CHILD CONSENT FORM 1 – Urine samples
Version 1

Child Identification Number for this trial:

Name of Researcher: *Dr Gary Connett*

PART A Consent for the main study

PLEASE **INITIAL** THE BOXES FOR EACH SECTION YOU WISH YOUR CHILD TO TAKE PART IN (please put a cross through any you do not wish to consent to):

12. *I have read the information sheet for the above study. I have had the opportunity to ask questions, I understand why the research is being done and any possible risks have been explained to me.*

13. *I understand that my child's participation is voluntary and that we can withdraw at any time.*

14. *I give permission for sections of my child's notes to be looked at by research doctors involved in this study.*

15. *I agree to take part in the above study and for my child to provide a urine sample.*

Appendix 2

Letter to potential participants

The RATNO Study

Reducing Antibiotic Tolerance using low dose Nitric Oxide in cystic fibrosis
A phase 2 pilot study

Dear

The new Respiratory National Institute for Health Research Biomedical Research Unit in Southampton has provided NHS clinical research resources for the CF clinical teams to work with University colleagues to investigate new treatments that might be of benefit to people with cystic fibrosis. We are writing to you as part of a group of researchers who are doing a study that might be of interest to you.

We are writing to you because you have Cystic Fibrosis and you fit the criteria for taking part in this study. If you want to know more, please read the enclosed information sheet which will give you lots of information about the study, what is involved if you decide to take part and what to do if you are interested. The research team would like to hear from you if this study is of interest to you or if you have further questions. Please contact them on 023 8079 4989. Alternatively we can explain a bit more about the study to you and ask the research team to speak to you if you are interested.

Thank you for taking the time to read this letter and we hope you will read the information enclosed about the study and consider taking part.

Yours Faithfully

Dr Gary Connett (on behalf of the Paediatric CF team)/Dr Thomas Daniels (on behalf of the adult CF team)

Research team contact details (RATNO study team)

Southampton Centre for Biomedical Research
Southampton University Hospitals NHS Trust
Mailpoint 218
Tremona Road
Southampton
SO16 6YD

Tel: 023 8079 4989

Appendix 3

Participant, Parent and Young person Information Sheets

The RATNO Study

Reducing Antibiotic Tolerance using low dose Nitric Oxide in cystic fibrosis
A phase 2 pilot study

Principal Investigator: Dr Gary Connett

Study Site: Southampton University Hospitals NHS Trust, Tremona Road, Southampton, SO16 6YD

INFORMATION SHEET FOR PARTICIPANTS Version 1.1

We would like to invite you to take part in a research study which aims to find out if a potential treatment for pseudomonal lung infection can benefit patients with Cystic Fibrosis. Before you decide we would like you to understand why the research is being done and what it would involve. One of our team will talk through the information sheet with you and answer any questions you have.

- Part 1 of this information sheet tells you the purpose of the study and what will happen if you take part.
- Part 2 gives you more detailed information about how the study will be organized.

Please take your time to read the information carefully and discuss it with your family or GP if you wish. If anything is not clear or if you would like more information, please ask us or telephone the study team on 023 8079 4989.

Part 1

What is the purpose of the study?

One of the main bacteria causing lung infection in CF is a bug called *Pseudomonas*. This causes a chronic (long term) lung infection. Whilst intravenous antibiotics can be used to treat this bug they are not completely effective and the infection is often a recurring problem. We have been researching new ways to treat *Pseudomonas* infection. To do this, we are studying a medicine called nitric oxide. Nitric oxide is currently used to treat blood vessel contraction in premature babies and in some lung diseases in adult and children's intensive care, but we have found in the laboratory that very low doses of nitric oxide can help kill *Pseudomonas* from patients with CF. Nitric oxide is given by inhalation and works by bringing the bacteria out of the mucous in which they are embedded so that they are easier for the antibiotics to kill. This small study has been designed to find out whether nitric oxide can help make our usual intravenous antibiotics (given during an episode of acute infection or exacerbation) more effective in killing *Pseudomonas* and improve the health of CF patients.

Why have you been chosen?

We have asked you to take part because you have CF and you also have *Pseudomonas* infection. We are asking adults and children aged 12 or above to take part. You may be well at the moment, but if you agree, when you have an exacerbation we will give you an additional inhaled treatment with your usual IV antibiotics.

Research study design

Sometimes we don't know which way of treating patients is best. To find out, we need to compare different treatments. We put people into groups and give each group a different treatment. The results are compared to see if one is better. To try to make sure the groups are the same to start with, each patient is put into a group by chance (randomly).

In this study half the patients will receive the study therapy (nitric oxide) and the other half will receive air (this is called a placebo because it is made to look the same as for the patients being given the study therapy). The study medicine is tasteless and it will not be possible for you to know what you will be receiving, although you will find out at the end of the study. Both groups will receive intravenous antibiotics as usual for an exacerbation and all other standard treatment.

During the trial period, if you become unwell with symptoms of a respiratory infection (exacerbation) and need intravenous antibiotics you will be randomly allocated to one of the 2 groups. Neither you nor the doctors or study team will be able to choose which group you will be in.

What is the drug that is being tested?

The drug that we will be studying is called "nitric oxide". This will be given by inhalation through nasal cannula (similar to giving oxygen). Although this is the first time that nitric oxide has been used specifically to treat infection, it has been widely used in other ways to treat illnesses in both preterm babies and children and adults with breathing difficulties on intensive care units.

What are the alternatives for diagnosis or treatment?

All standard treatment will be given to you at all times. This study is testing nitric oxide as an extra medicine to be given alongside all the standard treatments.

What are the possible disadvantages and risks of taking part?

When you are admitted with an exacerbation you will receive the inhaled therapy overnight for the first 7 nights of your course of antibiotics. You will be monitored carefully by research or ward nurses who will keep a very close eye on you. If you would usually give your own antibiotics at home you will need to stay in hospital for the first 7 nights of your course of antibiotics to receive the inhaled therapy. During the day you will be on the ward or allowed home as is your usual practice.

You will have some blood tests taken as part of the study, but these should be done at the same time as the ones they would have taken anyway for your exacerbation.

Although we do not anticipate any major risk to you, we are monitoring to detect any unexpected serious safety issues with the use of nitric oxide in people with CF (see "side effects" paragraph below).

What are the possible benefits of taking part?

We hope that this potential treatment will help clear infection from the lungs better than intravenous antibiotics given alone, so there is a small chance you may benefit from this. However, only one course of this treatment will be given and so the actual benefit from this study is likely to be small. The information we get from this study will help us find out whether this potential treatment could be used for patients with CF in the future and reduce the amount they have to come into hospital.

What are the side effects of nitric oxide treatment?

Although side effects from nitric oxide at the low dose we are using are rare, they are possible. You will be monitored very closely while you are on the treatment to look out for any side effects. The most common are caused if the nitric oxide therapy is stopped too quickly, which can cause headaches and/or a reduction in blood pressure and oxygen levels in the blood. To avoid this we will slowly reduce the levels before stopping the therapy each morning and you will be monitored very closely for any of these potential problems. If troublesome side effects occur then you will be reviewed by a nurse or doctor involved in the study. We will record carefully anything we think may be related to the study treatment. You have the option of withdrawing from the study at any time without affecting your standard treatment. There is also a special study committee who will monitor the effects and if it is felt the treatment is causing problems the study will be stopped.

It is not known whether nitric oxide is harmful to unborn babies, therefore all female participants will have a pregnancy test before being allocated to a treatment group. If this test is positive, they will not be able to take part.

What will happen to me if I take part?

Initial assessment:

1. We will examine you (like you would have done at clinic) and take some details about the medications you are on.
2. We will ask you to cough up some sputum into a pot for us to test in the lab.

Exacerbations/acute respiratory infection:

We will ask you and your CF doctor to let us know when you have an exacerbation (increased cough/sputum/breathlessness/fever). In addition to the usual tests you would have if you came in for IV antibiotics (lung function, bloods, height and weight) you will be asked to do the following:

1. Give an extra sample of sputum and blood (taken at the same time as the routine samples).
2. Fill out a questionnaire about how your CF has been affecting you over the last 2 weeks.
3. If/when you have an exacerbation you will be allocated to one of 2 groups:
 - a. Nitric oxide
 - b. Placebo (air or air+oxygen – depending on your needs)

The treatment is breathed by the patient via nasal cannula. So if you take part you will have to wear nasal cannula overnight for the first 7 nights of your admission.

You will receive all the other standard care that you would usually have when you have an exacerbation including IV antibiotics, physiotherapy and monitoring on the ward.

Follow-up:

We would like to review all patients approximately 2 weeks after they finish their IV antibiotics. At this appointment we will:

1. Examine you (like you would have done at clinic) and take some details about the medications you are on.
2. Ask you to cough up some sputum into a pot for us to test in the lab.

3. Ask you to fill in a questionnaire about how your CF has been affecting you since you finished your intravenous antibiotics.
4. Do some lung function tests like you would usually do in clinic.

Not all patients who agree to take part will have an exacerbation while we are doing the study. If this is the case for you, we will write to you to let you know that the study has ended and you will continue to receive standard clinical care as before.

Additional optional procedures:

These procedures are optional and you do not have to consent to these to take part in the trial:

1. Nasal examination – We will suggest a nasal examination if you have a history or symptoms of nasal blockage or if you have had previous sinonasal surgery. This will allow us to exclude significant blockage which may affect delivery of the inhaled treatment to the lungs.
2. Nasal brushings – This is a quick procedure involving a small brush of the inside of the nose. It is slightly uncomfortable, but only lasts a few seconds. It allows us to look at the cells that help clear particles from the nose.

Do I have to take part?

It is up to you to decide whether or not you take part in this research. It is entirely voluntary. If you do not want to take part please be assured the care you receive will not be affected in any way. If you agree to take part in this study then you will be asked to sign a consent form. You are free to withdraw your consent at any time and without giving a reason. This will not affect the standard of care you receive.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

Who should I contact if I have any questions or concerns or if I want more information?

Please contact a member of the study team on 023 8079 4989, or you can discuss your concerns with your CF doctor.

If you want more general information about taking part in research the following websites may be helpful: <http://www.nres.npsa.nhs.uk/aboutus/protecting-participant-safety/> and <http://www.peopleinresearch.org/>

This completes part 1.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

Part 2

Will my taking part be kept confidential?

Yes, all information about you will be kept confidential. We will tell your GP that you have taken part in this study. All information about you will be coded with a unique study number. This allows us to link your results with your medical information, but keep the details confidential. Data collected during the study may be shared with our research colleagues in other hospitals (possibly abroad); however they will not know who the information belongs to, as your name and address will only be kept at Southampton University Hospitals NHS Trust. Only the research team will have access to your personal details. The sponsor of the study (Southampton University Hospitals NHS Trust) or regulatory authorities may also wish to access the records as part of their monitoring of ongoing research, but no identifiable information with leave Southampton University Hospitals NHS Trust. You will not be individually identified in any reports or publications resulting from the study.

What will happen to any samples I give?

The sputum samples will be analysed for the amount of *Pseudomonas* bacteria. The blood samples will look at markers of infection. Some of the sputum and blood samples will be stored securely at Southampton University Hospitals NHS Trust or the University of Southampton for later tests to be done in connection with this study or other CF or lung infection studies. It may be that researchers from this hospital or other groups in the UK or abroad ask us for small amounts of the stored samples to use in additional research on children and adults with cystic fibrosis. The samples will not be labelled with your name or address so that other researchers analysing them will not know who the sample belongs to. All future studies will be approved by a research ethics committee before being able to use the samples.

What if relevant new information becomes available?

Sometimes we get new information about the treatment being studied. If this happens, the doctors will tell you and discuss whether you should continue in the study. If you or your doctors decide you will not carry on, the standard clinical care will not be affected in any way. If the study is stopped for any other reason, we will tell you.

What will happen if I don't want to carry on with the study?

You can decide to withdraw from the study at any time and are not obliged to give any reasons. Whatever you decide, the standard of clinical care you receive will not be affected in any way. If you withdraw from treatment you can keep in contact with us to let us know about your progress. Information collected before withdrawal will still be used. Any stored blood and sputum will be destroyed if you wish.

What if there is a problem?

If you have a concern about any part of this study you should ask to speak to a member of the research team who will do their best to answer any of your questions (Tel 023 8079 4989). You can also discuss any concerns you have with your CF doctor.

If you still have questions or concerns you can contact Research and Development at Southampton University Hospitals NHS Trust. In the very unlikely event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence

then you may have grounds for legal action for compensation against Southampton University Hospital NHS Trust but you may have to pay your legal costs.

Regardless of this, if you have concerns about any aspect of the way you have been approached or treated during the course of this study you may wish to contact the hospital's Patient Advice and Liaison Service (PALS) on 023 8079 8498, email PALS@suht.swest.nhs.uk or write to PALS, C Level, Centre Block, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD).

What will happen to the results of the research study?

All the results of the study will be made available by publication in medical journals. You will not be identified in any report/publication to do with this trial.

What happens when the research study stops?

If this study finds that nitric oxide has potentially beneficial effects for patients with cystic fibrosis, a much larger and definitive trial will be designed to determine whether this treatment should become standard for all patients with cystic fibrosis.

Who is organising and funding the research?

This study has been designed by a group of researchers from the National Institute of Health Research Respiratory Biomedical Research Unit at Southampton University Hospitals NHS Trust and the University of Southampton. All the researchers have been involved in trying to determine better treatments for patients with cystic fibrosis for many years. None of the doctors, nurses or researchers are being paid to include patients in this study. This project is being paid for by a grant from the National Institute for Health Research, the body that funds clinical research in the NHS.

Expenses and payments

Because of the additional inconvenience for a number of adult patients of staying in hospital overnight for the first 7 nights of their course of IV antibiotics, you will be given a gift of £150 (at the final visit) if you have an exacerbation during the study period and receive the inhaled therapy.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by Research Ethics Committee. Prior to submission to the ethics committee, the study was reviewed by doctors and scientists independent from our team. The study was also reviewed as part of the application to the National Institute of Health Research (NIHR) for the Southampton Respiratory Biomedical Research Unit.

How long do I have to decide whether I should take part?

Your decision to participate in this study is entirely voluntary. You should take as much time as you need.

Thank you for taking time to read this information sheet.

You will be given a copy of this information sheet to keep, together with a copy of the signed consent form if you decide that you can take part.

The RATNO Study

Reducing Antibiotic Tolerance using low dose Nitric Oxide in cystic fibrosis
A phase 2 pilot study

Principal Investigator: Dr Gary Connett

Study Site: Southampton University Hospitals NHS Trust, Tremona Road, Southampton, SO16 6YD

INFORMATION SHEET FOR PARENTS Version 1.1

We would like to invite your son/daughter to take part in a research study which aims to find out if a potential treatment for pseudomonal lung infection can benefit patients with Cystic Fibrosis. Before you decide we would like you to understand why the research is being done and what it would involve. One of our team will talk through the information sheet with you and answer any questions you have.

- Part 1 of this information sheet tells you the purpose of the study and what will happen if your son/daughter takes part.
- Part 2 gives you more detailed information about how the study will be organized.

Please take your time to read the information carefully and discuss it with your family or GP if you wish. If anything is not clear or if you would like more information, please ask us or telephone the study team on 023 8079 4989.

Part 1

What is the purpose of the study?

One of the main bacteria causing lung infection in CF is a bug called *Pseudomonas*. This causes a chronic (long term) lung infection. Whilst intravenous antibiotics can be used to treat this bug they are not completely effective and the infection is often a recurring problem. We have been researching new ways to treat *Pseudomonas* infection. To do this, we are studying a medicine called nitric oxide. Nitric oxide is currently used to treat blood vessel contraction in premature babies and in some lung diseases in adult and children's intensive care, but we have found in the laboratory that very low doses of nitric oxide can help kill *Pseudomonas* from patients with CF. Nitric oxide is given by inhalation and works by bringing the bacteria out of the mucous in which they are embedded so that they are easier for the antibiotics to kill. This small study has been designed to find out whether nitric oxide can help make our usual intravenous antibiotics (given during an episode of acute infection or exacerbation) more effective in killing *Pseudomonas* and improve the health of CF patients.

Why has your son/daughter been chosen?

We have asked your son/daughter to take part because they have CF and they also have *Pseudomonas* infection. We are only asking children aged 12 or above to take part. This is because we want them to be able to understand about the study and agree to take part. Your son/daughter may be well at the moment, but if you agree, when they have an exacerbation we will give them an additional inhaled treatment with their usual IV antibiotics.

Research study design

Sometimes we don't know which way of treating patients is best. To find out, we need to compare different treatments. We put people into groups and give each group a different treatment. The results are compared to see if one is better. To try to make sure the groups are the same to start with, each patient is put into a group by chance (randomly).

In this study half the patients will receive the study therapy (nitric oxide) and the other half will receive air (this is called a placebo because it is made to look the same as for the patients being given the study therapy). The inhaled medicine is tasteless and it will not be possible for you or your son/daughter to know what they will be receiving, although you will find out at the end of the study. Both groups will receive intravenous antibiotics as usual for an exacerbation and all other standard treatment.

During the trial period, if your son/daughter becomes unwell with symptoms of a respiratory infection (exacerbation) and needs intravenous antibiotics he/she will be randomly allocated to one of the 2 groups. Neither you nor the doctors or study team will be able to choose which group they will be in.

What is the drug that is being tested?

The drug that we will be studying is called "nitric oxide". This will be given by inhalation through nasal cannula (similar to giving oxygen). Although this is the first time that nitric oxide has been used specifically to treat infection, it has been widely used in other ways to treat illnesses in both preterm babies and children and adults with breathing difficulties on intensive care units.

What are the alternatives for diagnosis or treatment?

All standard treatment will be given to your son/daughter at all times. This study is testing nitric oxide as an extra medicine to be given alongside all the standard treatments.

What are the possible disadvantages and risks of taking part?

When your son/daughter is admitted with an exacerbation they will receive the inhaled therapy overnight for the first 7 nights of your course of antibiotics, and they may have to sleep overnight in a special area in the hospital dedicated to research. They will be monitored carefully by research or ward nurses who will keep a very close eye on them. If you would usually stay with them you will still be able to do this. During the day they will be on the ward or allowed home as is your usual practice.

Your son/daughter will have some blood tests taken as part of the study, but these should be done at the same time as the ones they would have taken anyway for their exacerbation.

Although we do not anticipate any major risk to your son/daughter, we are monitoring to detect any unexpected serious safety issues with the use of nitric oxide in people with CF (see "side effects" paragraph below).

What are the possible benefits of taking part?

We hope that this potential treatment will help clear infection from the lungs better than intravenous antibiotics given alone, so there is a small chance your son/daughter may benefit from this. However, only one course of this treatment will be given and so the actual benefit from this study is likely to be small. The information we get from this study will help us find out whether this treatment could be used for patients with CF in the future and reduce the amount they have to come into hospital.

What are the side effects of nitric oxide treatment?

Although side effects from nitric oxide at the low dose we are using are rare, they are possible. Your son/daughter will be monitored very closely while they are on the treatment to look out for any side effects. The most common are caused if the nitric oxide therapy is stopped too quickly, which can cause headaches and/or a reduction in blood pressure and oxygen levels in the blood. To avoid this we will slowly reduce the levels before stopping the therapy each morning and your son/daughter will be monitored very closely for any of these potential problems. If troublesome side effects occur then your son/daughter will be reviewed by a nurse or doctor involved in the study. We will record carefully anything we think may be related to the study treatment. You and your son/daughter have the option of withdrawing from the study at any time without affecting your standard treatment. There is also a special study committee who will monitor the effects and if it is felt the study treatment is causing problems the study will be stopped.

It is not known whether nitric oxide is harmful to unborn babies, therefore all female participants will have a pregnancy test before being allocated to a treatment group. If this test is positive, they will not be able to take part.

What will happen to my son/daughter if they take part?

Initial assessment:

1. We will examine your son/daughter (like they would have done at clinic) and take some details about the medications they are on.
2. We will ask them to cough up some sputum into a pot for us to test in the lab.

Exacerbations/acute respiratory infection:

We will ask you and your son/daughter's CF doctor to let us know when your son/daughter has an exacerbation (increased cough/sputum/breathlessness/fever). In addition to the usual tests they would have if they came in for IV antibiotics (lung function, bloods, height and weight) they will be asked to do the following:

1. Give an extra sample of sputum and blood (taken at the same time as the routine samples).
2. Fill out a questionnaire about how their CF has been affecting them over the last 2 weeks.
3. If/when your son/daughter has an exacerbation they will be allocated to one of 2 groups:
 - a. Nitric oxide
 - b. Placebo (air or air+oxygen – depending on their needs)

The treatment is breathed by the patient via nasal canula. So if your son/daughter takes part they will have to wear nasal canula overnight for the first 7 nights of their admission. Your son/daughter will receive all the other standard care that they would usually have when they have an exacerbation including IV antibiotics, physiotherapy and monitoring on the ward.

Follow-up:

We would like to review all patients approximately 2 weeks after they finish their IV antibiotics. At this appointment we will:

1. Examine your son/daughter (like they would have done at clinic) and take some details about the medications they are on.
2. Ask them to cough up some sputum into a pot for us to test in the lab.
3. Ask them to fill in a questionnaire about how their CF has been affecting them since they finished their intravenous antibiotics.
4. Do some lung function tests like they would usually do in clinic.

Not all children who agree to take part will have an exacerbation while we are doing the study. If this is the case for your son/daughter, we will write to you to let you know that the study has ended and they will continue to receive standard clinical care as before.

Additional optional procedures:

These procedures are optional and you do not have to consent to these to take part in the trial:

1. Nasal examination – We will suggest a nasal examination if your son/daughter has a history or symptoms of nasal blockage or if they have had previous sinonasal surgery. This will allow us to exclude significant blockage which may affect delivery of the inhaled treatment to the lungs.
2. Nasal brushings – This is a quick procedure involving a small brush of the inside of the nose. It is slightly uncomfortable, but only lasts a few seconds. It allows us to look at the cells that help clear particles from the nose.

Does my son/daughter have to take part?

It is up to you and your son/daughter to decide whether or not they take part in this research. It is entirely voluntary. If you do not want them to take part please be assured the care they receive will not be affected in any way. If you agree to your son/daughter taking part in this study then you will be asked to sign a consent form. They will also be asked to sign to say they agree. You and your son/daughter are free to withdraw your consent at any time and without giving a reason. This will not affect the standard of care they receive.

What if there is a problem?

Any complaint about the way your son/daughter has been dealt with during the study or any possible harm they might suffer will be addressed. The detailed information on this is given in Part 2.

Will my son/daughter taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

Who should I contact if I have any questions or concerns or if I want more information?

Please contact a member of the study team on 023 8079 4989, or you can discuss your concerns with your son/daughter's CF doctor.

If you want more general information about taking part in research the following websites may be helpful: <http://www.nres.npsa.nhs.uk/aboutus/protecting-participant-safety/> and <http://www.peopleinresearch.org/>

This completes part 1.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

Part 2

Will my son/daughter taking part be kept confidential?

Yes, all information about your son/daughter will be kept confidential. We will tell their GP that they have taken part in this study. All information about your son/daughter will be coded with a unique study number. This allows us to link their results with their medical information, but keep the details confidential. Data collected during the study may be shared with our research colleagues in other hospitals (possibly abroad); however they will not know who the information belongs to, as your son/daughter's name and address will only be kept at Southampton University Hospitals NHS Trust. Only the research team will have access to your son/daughter's personal details. The sponsor of the study (Southampton University Hospitals NHS Trust) or regulatory authorities may also wish to access the records as part of their monitoring of ongoing research, but no identifiable information will leave Southampton University Hospitals NHS Trust. Your son/daughter will not be individually identified in any reports or publications resulting from the study.

What will happen to any samples given by my son/daughter?

The sputum samples will be analysed for the amount of *Pseudomonas* bacteria. The blood samples will look at markers of infection. Some of the sputum and blood samples will be stored securely at Southampton University Hospitals NHS Trust or the University of Southampton for later tests to be done in connection with this study or other CF or lung infection studies. It may be that researchers from this hospital or other groups in the UK or abroad ask us for small amounts of the stored samples to use in additional research on children with cystic fibrosis. The samples will not be labelled with your son/daughter's name or address so that other researchers analysing them will not know who the sample belongs to. All future studies will be approved by a research ethics committee before being able to use the samples.

What if relevant new information becomes available?

Sometimes we get new information about the treatment being studied. If this happens, the doctors will tell you and discuss whether your son/daughter should continue in the study. If you or your doctors decide your son/daughter will not carry on, the standard clinical care will not be affected in any way. If the study is stopped for any other reason, we will tell you.

What will happen if I don't want my son/daughter to carry on with the study?

You can decide to withdraw your son/daughter from the study at any time and are not obliged to give any reasons. Whatever you decide, the standard of clinical care they receive will not be affected in any way. If your son/daughter withdraws from treatment you can keep in contact with us to let us know about their progress. Information collected before withdrawal will still be used. Any stored blood and sputum will be destroyed if you wish.

What if there is a problem?

If you have a concern about any part of this study you should ask to speak to a member of the research team who will do their best to answer any of your questions (Tel 023 8079 4989). You can also discuss any concerns you have with your son/daughter's CF doctor.

If you still have questions or concerns you can contact Research and Development at Southampton University Hospitals NHS Trust. In the very unlikely event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence

then you may have grounds for legal action for compensation against Southampton University Hospital NHS Trust but you may have to pay your legal costs.

Regardless of this, if you have concerns about any aspect of the way your son/daughter has been approached or treated during the course of this study you may wish to contact the hospital's Patient Advice and Liaison Service (PALS) on 023 8079 8498, email PALS@suht.swest.nhs.uk or write to PALS, C Level, Centre Block, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD).

What will happen to the results of the research study?

All the results of the study will be made available by publication in medical journals. Your son/daughter will not be identified in any report/publication to do with this trial.

What happens when the research study stops?

If this study finds that nitric oxide has potentially beneficial effects for patients with cystic fibrosis, a much larger and definitive trial will be designed to determine whether this treatment should become standard for all patients with cystic fibrosis.

Who is organising and funding the research?

This study has been designed by a group of researchers from the National Institute of Health Research Respiratory Biomedical Research Unit at Southampton University Hospitals NHS Trust and the University of Southampton. All the researchers have been involved in trying to determine better treatments for patients with cystic fibrosis for many years. None of the doctors, nurses or researchers are being paid to include patients in this study. This project is being paid for by a grant from the National Institute for Health Research, the body that funds clinical research in the NHS.

Expenses and payments

No extra expense payments will be made. According to European and UK Law, children are not allowed to receive any payment for taking part in research.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by Research Ethics Committee. Prior to submission to the ethics committee, the study was reviewed by doctors and scientists independent from our team. The study was also reviewed as part of the application to the National Institute of Health Research (NIHR) for the Southampton Respiratory Biomedical Research Unit.

How long do I have to decide whether my son/daughter should take part?

Your decision to participate in this study is entirely voluntary. You should take as much time as you need.

Thank you for taking time to read this information sheet.

You will be given a copy of this information sheet to keep, together with a copy of the signed consent form if you decide that your son/daughter can take part.

The RATNO Study

Reducing Antibiotic Tolerance using low dose Nitric Oxide in cystic fibrosis
A phase 2 pilot study

Principal Investigator: Dr Gary Connett

Study Site: Southampton University Hospitals NHS Trust, Tremona Road, Southampton, SO16 6YD

SIMPLIFIED INFORMATION SHEET FOR YOUNG PERSON AGED 12-18 YEARS Version 1.1

Why are we doing this research?

We are trying to find ways to help children and young people with cystic fibrosis (CF) like you. Many children and young people with CF get problems with their lungs. When your lungs are not working properly you may find it hard to breathe. This stops you being able to do everything you want to and you may have to come to hospital.

We are doing a study to see if giving you an extra treatment when you come into hospital for antibiotics will reduce the chance of you getting problems with your lungs.

What will happen if I take part?

If you and your parents agree to take part in the study, we will ask you to write your name on a form called a consent form, and if you are under 18 your parents or guardian will also sign this form. This is to say you understand the study and what will happen. You and your parents will keep a copy of the consent form and you can keep this information sheet.

What will happen to me during the study?

A lot of what we are doing in the study is exactly the same as what would usually happen to you when you come into hospital for IV antibiotics.

Doctors will examine you and ask you to cough up sputum into a pot a few times during your stay. When you have your blood tests done we will take an extra teaspoon of blood for the study. When you come into hospital for intravenous (IV) antibiotics you might stay overnight in a special area of the hospital where we do research. It is very much like the ward you usually stay on. Your mum or dad can stay there with you if you want. In the day you will be on your normal ward and everything else will be the same (including your treatment and physio). Overnight you will need to wear nasal cannula to get the extra treatment. Half of the patients in the study will get the study treatment and half will get just air/oxygen (called a placebo). You will not know what you are getting. This is important so we can compare the study treatment fairly.

We would like you to fill out a questionnaire about how your CF has been affecting you as part of the study. We would also like you to come in for a follow-up visit 2 weeks after your IV antibiotics so we can see how you are getting on.

Does I have to take part?

No, it is totally up to you and your parents whether you take part or not.

Will taking the medicine upset me?

We don't expect the study medication to upset you, but the nurses will keep a close eye on you to make sure you are ok. If you think you feel unwell or funny then you must tell the nurse.

Why is this form not as detailed as the one for my parents?

We know that young people want different amounts of information depending on how they are feeling, how old they are, what they have done so far in school and for many other reasons. We need to be able to make sure that you get the information you want, and that you can understand any information we give you.

You and your parents have also been given a more detailed information sheet that explains the study in more detail,

The doctors and nurses will be pleased to spend any time you want explaining the treatment or the study to you in more detail if you would like this.

Thank you for reading this – please let your doctor or nurse know if you would like more information.

Appendix 4

Consent forms, participant, parent

RATNO - Reducing Antibiotic Tolerance using low dose Nitric Oxide in cystic fibrosis – a phase 2 pilot study

Participant Consent Form Version 1.0

Ethics Study Number:

Participant Identification Number for this trial:

1. I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw this participation at any time without giving any reason, without my medical care or legal rights being affected.

3. a. I agree to my anonymised data relevant to this trial being collected on case report forms and stored on electronic databases in accordance with the Data Protection Act 1998.

- b. I agree to this anonymised information being shared with other researchers in the UK or abroad.

4. a. I agree to my blood and sputum samples being stored anonymously for later testing relevant to cystic fibrosis and infection in the UK or abroad. I understand that I may not be given the results of these tests.

- b. I agree to anonymised samples being shared with other research groups in the UK or abroad.

5. I give permission for relevant sections of my medical notes and data collected during the study to be looked at by individuals from the study sponsor (Southampton University Hospital NHS Trust) and from regulatory authorities where it is relevant to my taking part in this research.

6. I agree to the optional additional procedures:
 - a. Nasal examination
 - b. Nasal brushings

7. I agree to my GP being informed of my participation in the study.

8. I agree to take part in the RATNO study.

Name of Participant

Date

Signature

Name of Researcher

Date

Signature

When completed, 1 for parent/guardian; 1 for researcher site file; 1 (original) to be kept in medical notes.

RATNO - Reducing Antibiotic Tolerance using low dose Nitric Oxide in cystic fibrosis – a phase 2 pilot study

Consent Form Version 1.0

Ethics Study Number:

Participant Identification Number for this trial:

1. I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.
2. I understand that my son/daughter's participation is voluntary and that I am free to withdraw this participation at any time without giving any reason, without my son/daughter's medical care or legal rights being affected.
3. a. I agree to my son/daughter's anonymised data relevant to this trial being collected on case report forms and stored on electronic databases in accordance with the Data Protection Act 1998.
b. I agree to this anonymised information being shared with other researchers in the UK or abroad.
4. a. I agree to blood and sputum samples taken from my son/daughter being stored anonymously for later testing relevant to cystic fibrosis and infection in the UK or abroad. I understand that I may not be given the results of these tests.
b. I agree to anonymised samples being shared with other research groups in the UK or abroad.
5. I give permission for relevant sections of my son/daughter's medical notes and data collected during the study to be looked at by individuals from the study sponsor (Southampton University Hospital NHS Trust) and from regulatory authorities where it is relevant to my son/daughter taking part in this research.
6. I agree to the optional additional procedures:
 - a. Nasal examination
 - b. Nasal brushings
7. I agree to my GP being informed of my son/daughter's participation in the study.
8. I agree to my son/daughter taking part in the RATNO study.

Name of Parent/Guardian Date Signature

Name of Researcher taking consent Date Signature

I understand the information I have been given and I agree to taking part in this study:

Name of Patient Date Signature

When completed, 1 for parent/guardian; 1 for researcher site file; 1 (original) to be kept in medical notes.

Appendix 5

Study guidance: weaning of NO

RATNO (Reducing Antibiotic Tolerance using NO)

Reducing antibiotic tolerance using low dose nitric oxide in
cystic fibrosis – a phase 2 pilot study

Study guidance: Weaning of nitric oxide

Section 1: Standard weaning guidance

The manufacturers guidelines are that the dose is reduced to 1 ppm for 30 mins and then stopped if there has been no significant change in HR, oxygen saturations and BP.¹ To allow monitoring specifically for this, the dose of NO will be reduced to 5ppm (halved) for 10 mins, then 1ppm for 20 mins before stopping. During this 30 minute period, HR and O2 saturations will be continuously monitored and BP will be measured every 10 minutes. If there is a significant change in HR, BP or drop in O2 saturations then the NO dose will be put back to 10ppm and then will be reduced more gradually (see section 2).

Section 2: Slow weaning guidance

For use with patients who have had a significant change in HR, BP or sats with standard weaning guidance (see section 1).

1. Starting dose of 10ppm
2. Reduce dose to 7ppm
 - a. Monitor HR and sats continuously and BP every 10 mins
 - b. If remains stable after 10 mins move on to step 3
3. Reduce dose to 4ppm
 - a. Monitor HR and sats continuously and BP every 10 mins
 - b. If remains stable after 10 mins move on to step 4
4. Reduce dose to 2ppm
 - a. Monitor HR and sats continuously and BP every 10 mins
 - b. If remains stable after 10 mins move on to step 5
5. Reduce dose to 1ppm
 - a. Monitor HR and sats continuously and BP every 10 mins
 - b. If remains stable after 20 mins move on to step 5
6. Stop
 - a. Monitor HR and sats continuously and BP every 10 mins
 - b. If remains stable after 10 mins stop monitoring

1. INOtherapeutics. Summary of Product Characteristics - Nitric Oxide, 2006.

Appendix 6

Laboratory methods

Proportion of *Pseudomonas Aeruginosa* (PA) in biofilm

The primary outcome measure was the difference in proportion and viability of PA in biofilms between T0 and T20-25, as determined by direct microscopic visualisation of biofilms in combination with bacterial live/dead (BacLight Live/Dead stain, Invitrogen) staining, *in-situ* molecular detection of PA, and quantitative computer-based image analysis. This was also measured at T5, T7 and T10.

Colony forming units of PA

This was a microbiological endpoint and was analysed by laboratory researchers using standardised procedures (as detailed below). CFUs were measured at randomisation (before treatment), 5 days, 7 days, 10 days and follow-up (T0, T5, T7, T10 and T20-25).

Microbial community CFU counts on non-selective agar

Sputum samples were analysed in the laboratory for microbial community CFU counts on non-selective agar. This was in addition to the PA CFUs. Although the majority of the background data regarding the use of nitric oxide to disrupt bacteria from biofilms is based on PA isolates, there is debate as to whether the effect is similar on the whole population of bacteria within the CF lung. Whole colony CFU counts were measured before the start of treatment (randomisation), at 5 days, 7 days, 10 days and at follow-up (T0, T5, T7, T10 and T20-25).

Nitric Oxide levels in sputum

Nitric Oxide levels in sputum were planned to be measured using a Unisense Nitric Oxide micro sensor. This was done in fresh sputum close to the bedside in order to investigate whether there was an increase in NO in the sputum with treatment. This was planned for T0 and T7 and one additional sample between T1-5, however, due to technical issues (discussed in the main thesis) this was not carried out during the study after trials in the first participant.

Sputum

Sample processing was performed in a Microbiological Safety Cabinet (MSC), according to the relevant risk assessment that was carried out prior to commencement of the study.

All laboratory specimens were processed and analysed by Dr Rob Howlin and team.

Figure 1 shows the outline of the sputum sample processing.

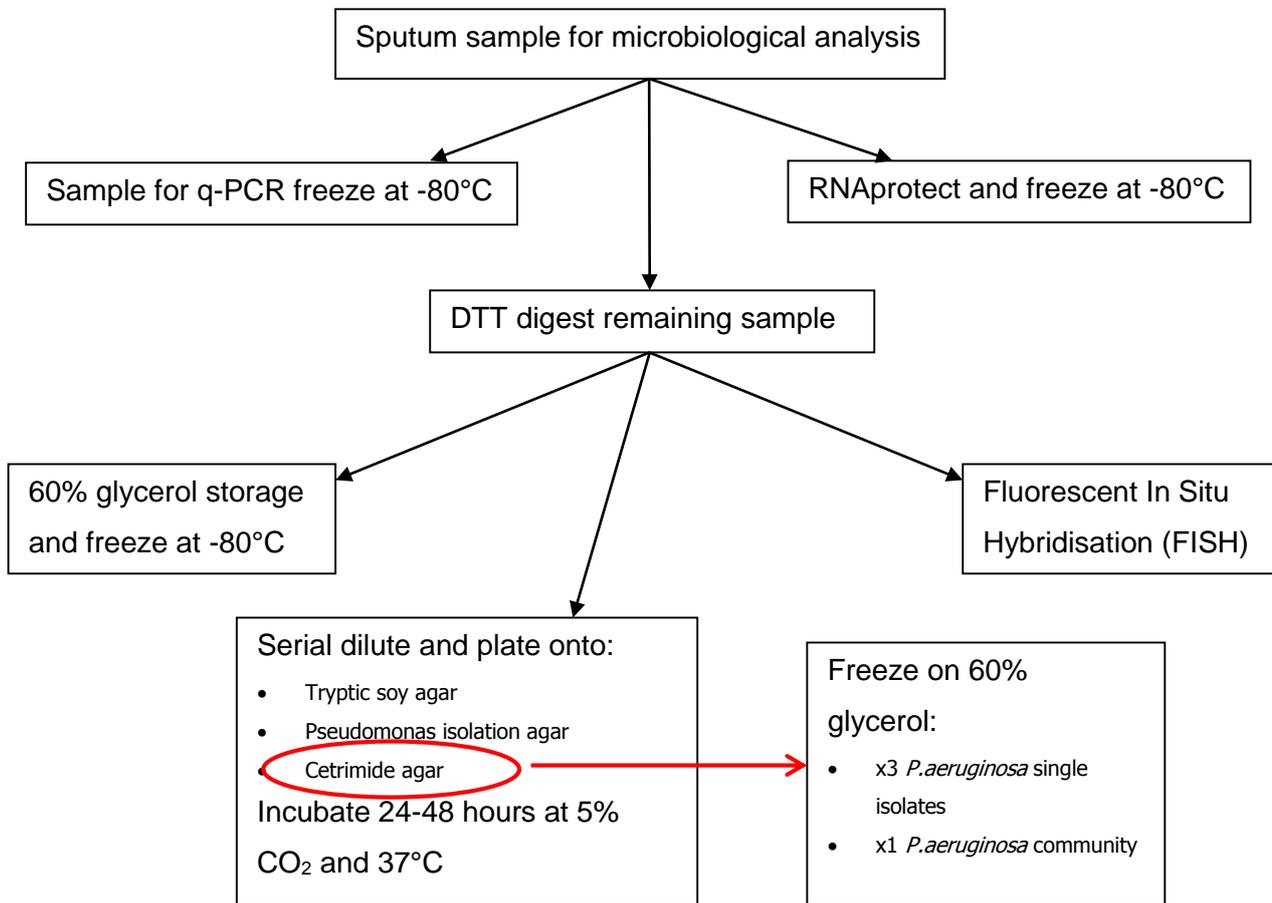


Figure 1 Flow diagram of sputum sample processing

Storage of sample for Quantitative Polymerase Chain Reaction (qPCR)

Samples were prepared and stored to allow batch testing for qPCR which was carried out at Kings College Hospital, London by Dr Geraint Rogers.

- 1 ml of the sputum sample was removed into a sterile cryovial (in situations where removal of 1 ml of sample did not leave sufficient sample for the following experiments, user discretion was used as to the volume of sample to be stored. A note of this was made in the storage log sheet).
- The 1ml sample in the cryovial was then submerged in 100% ethanol (that had been stored at -80°C) to rapidly freeze it.
- A label was attached documenting 'sputum', sample date, sample identifier (e.g. T0/T5/T7/T10/T20), sample type (e.g. q-PCR/Glycerol/RNAprotect) and the patient's unique study number.

- The sample was stored in the -80°C freezer and documented on the storage log sheet.

Storage of sample in RNAprotect

Carried out for storage of samples for future tests that may be required.

- 1ml of RNAprotect (Qiagen) was placed into a sterile cryovial.
- 1ml of sputum from the original sample was added to the cryovial (again user discretion was required if original sample volume was low).
- Sample was labelled (as in section 4.2.1.) and placed in the fridge for 12 hours.
- It was then stored at -80°C and documented on the storage log sheet.

Digestion of sputum with Dithiothreitol (DTT)

The sputum remaining (after 2ml removed for qPCR and storage in RNA protect) was added to an equal volume of 0.2% (w/v) DTT (pre-made and sterilised) and placed on ice for 30 minutes, or until the sputum bolus was liquefied (this varied according the initial sample viscosity). This was in preparation for analysis of the primary outcome.

0.2 % (w/v) Dithiothreitol DTT solution was made using the following method:
0.2 g of DTT (Sigma cat. #: 43815) added to 100ml ultrapure water (Sigma cat.#: W4502). Autoclaved for 15 minutes at 121°C to sterilise. Allowed to cool before use.

Storage of sample in 60 % glycerol

Carried out for storage of samples for future tests that may be required.

- 330µl of 60% glycerol was placed into a cryovial.
- To this, 1ml of the DTT-digested sputum was added.
- The sample was vortexed to mix.
- Sample was labelled as in section 4.2.1.

60 % glycerol was made using the following method:

60ml of glycerol (Sigma cat. #: G5516) was pipetted into a clean 100 ml beaker. 40ml of ultrapure water (Sigma cat.#: W4502) was added. Autoclaved for 15 minutes at 121°C to sterilise. Allowed to cool before use.

Serial dilution and colony forming unit counts of sputum sample

- 500µl of DTT-digested sputum was placed into a sterile eppendorf.
- This eppendorf was labelled 'sample'.

- A further 8 eppendorfs were filled with 1ml Hanks Balanced Salt Solution (HBSS).
- The eppendorfs were labelled: 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} & 10^{-8} .
- A 10-fold serial dilution was performed on the sample taking 100 μ l from the eppendorf labeled 'sample' with a sterile pipette and pipetting it into the eppendorf labeled ' 10^{-1} '. The sample was vortexed to mix.
- This process was repeated down the dilution range until the final dilution where 100 μ l of 10^{-7} had been pipetted into the 10^{-8} eppendorf and vortexed to mix.
- At this stage the 10^{-8} eppendorf contained 1100 μ l. 100 μ l was removed and discarded in 2% Virkon.
- 3 plates of each: Tryptic soy agar (TSA), Pseudomonas isolation agar (PIA) and Cetrimide agar (Cet) were identified and each plate was divided into 3 sections, labeling the sections: 'sample', ' 10^{-1} ', ' 10^{-2} ' up to ' 10^{-8} '.
- Each eppendorf was vortexed briefly before pipetting to resuspend any pelleted cells.
- Into the corresponding labeled segments of each agar plate, five 10 μ l drops of each serial diluted sample were pipetted, ensuring the drops did not overlap. The drops were allowed to dry before transferring the plates to the incubator.
- This was repeated for each agar type (TSA, PIA and Cet).
- All plates were incubated for 24 hours at 37°C and 5 % CO₂, contained within a non-air tight storage box.
- After 24 hours the plates were removed from the incubator.
- At each dilution where enumeration was possible the number of colonies per 10 μ l drop were counted.
- The data was recorded on a CFU log data sheet both manually and electronically.
- The handwritten copy was filed in the laboratory site file and the electronic copy was stored on a password protected computer.

Freezing of PA isolates

- Single colonies were carefully picked taking a sterile loop and resuspended in a cryovial (if the colonies were small and difficult to isolate after 24 hours the plates were returned to the 37°C incubator and this step was performed after a further 24 hours).
- This step was repeated 3 times to get 3 single isolates.
- Each tube was vortexed and inverted repeatedly.

- As much media as possible was pipetted off from within each cryovial and discarded as waste or into Virkon.
- The samples were labeled as per section 4.2.1 with 'PA iso 1/2/3' as the sample type.
- The vials were stored at -80°C and documented on the storage log sheet.
- Next, at the 10⁻¹ dilution, a sterile loop was used to collect each of the five spots of growth and these were resuspended in a single cryovial.
- The tube was vortexed and inverted repeatedly.
- As much media as possible was pipetted off from within each cryovial and discarded as waste or into Virkon.
- The samples were labeled as per section 4.2.1 with 'PA Com' as the sample type.
- The vials were stored at -80°C and documented on the storage log sheet.

Fluorescent in situ hybridisation (FISH) of sputum samples (2M.FISH)

Sample fixation

- The following solutions were constructed in sterile falcon tubes:
 1. 30ml 3xPBS: 21ml of ultrapure water + 9ml 10x PBS. This was vortexed to mix and stored on ice then put through a sterile filter.
 2. 40ml of 1x PBS: 36ml of ultrapure water + 4ml of 10x PBS. This was vortexed to mix and stored on ice then put through a sterile filter.
 3. 4 % Paraformaldehyde (PFA): 10 ml of 16x paraformaldehyde was mixed with 30 ml 3x PBS made up previously. The tube was inverted to mix and stored on ice. This solution was stable for 1 week at (4-6°C) and so remaining solution was stored, clearly labelled, in the fridge for use if a further sample was expected within this time frame. All PFA waste was collected in a 50 ml falcon tube for specialised disposal.
 4. PBS-Ethanol solution: 5 ml of 1x PBS was added to 5 ml of molecular biology grade ethanol. This was vortexed to mix and stored on ice then put through a sterile filter.
- The remaining DTT-digested sputum sample was added to the pre-chilled 4% PFA solution at a ratio of 1:1 with the volume of the sample (e.g. 5ml of sputum + 5ml 4% PFA)
- This was incubated for 1 hour on ice or in the fridge.
- 2ml of this fixed sample was then aliquoted into a sterile eppendorf and centrifuged for 2 minutes at 8,000 rpm in the microcentrifuge to pellet.

- The supernatant was carefully removed into the waste receptacle for PFA, taking care to ensure the pellet was not disrupted.
- 1ml 1x PBS was added to the eppendorf and vortexed to resuspend the pellet.
- This was then centrifuged for 2 minutes at 8,000 rpm to pellet.
- The supernatant was removed into 2% Virkon and replaced with a further 1ml of 1x PBS.
- This was vortexed to resuspend the pellet and centrifuged for a further 2 minutes at 8,000rpm.
- The supernatant was once again removed into 2% Virkon and 200µl of PBS-ethanol solution was added and the pellet resuspended by vortexing.

Immobilisation

- 3x 20µl drops of the above sample were placed onto a pre-made poly-L-lysine (PLL) coated slide.
- The slide was orientated to which side the sputum spots had been placed by annotating the frosted area of the slide with the date and patient sample identification number.
- The slide was placed in a Petri dish, covered with a lid and left to dry overnight.
- The fixed sputum sample eppendorf was returned to the -21°C freezer for storage.

Hybridisation of the FISH probes

- The hybridisation oven was turned on at 46°C and the water bath at 48°C.
- In a 2 ml eppendorf the hybridisation buffer was constructed by mixing the following:
 - 360 µl 5M NaCl
 - 40 µl Tris-HCL
 - 400 µl of formamide
- The eppendorf was filled to 2 ml by adding 1, 200 µl of ultrapure water.
- 2µl 10 % SDS was added and vortexed to mix.
- The PA Cy3 probe and *Eubacterial* Cy5 probe were removed from the freezer and thawed. These are fluorescent probes and therefore were shielded from the light where possible.
- The following were added to a separate 2 ml eppendorf, and using separate pipette tips:
 - 6 µl of PA Cy3 probe

- 6 µl of *Eubacterial* Cy5 probe
- 60 µl of the hybridisation buffer
- This was mixed by repeatedly drawing through the pipette with care and shielded from the dark until used.
- A small section of tissue paper was placed into the Petri dish containing the sputum slide.
- The tissue paper was carefully moistened by adding a few drops of the waste hybridisation buffer.
- Finally, 20 µl of the probe-buffer mix was carefully placed over each of the sputum drops on the slide.
- The slide was incubated in the hybridisation oven at 46°C for 2 hours.
- During this time, the wash buffer was constructed in a 50ml falcon tube (one per slide) as follows:
 - 1000 µl of 1M Tris-HCL
 - 2150 µl of 5M NaCl
 - 500 µl 0.5 M EDTA
- It was then filled to 50ml by adding 46.35ml of ultrapure water.
- 50 µl of 10 % SDS was added.
- The wash buffer was pre-warmed by leaving it in the water bath at 48°C until used.
- After 2hours hybridisation, the slide was removed from the hybridisation oven and held over a sink.
- The hybridisation buffer-probe mix was gently rinsed off by taking 1ml of wash buffer and gently dispensing at the top of the slide so that the wash buffer ran lightly over the sputum spots, removing the probe mix into the sink.
- Carefully the slide was placed vertically into the 50ml falcon tube containing the wash buffer. This was placed back into the water bath at 48°C and incubated for 15 minutes.
- During this time, a 50 ml falcon tube was filled with ultrapure water and placed in the water bath to warm.
- After the 15 minute incubation/wash step, the slide was removed and carefully placed in the falcon tube containing the water.
- This was left for 5 seconds then removed.
- The slide was placed, sputum spots facing up, onto blue roll or other absorbent material and tapped carefully on its sides to remove excess liquid.

- A drop of Vectashield mounting medium was added to the slide and coverslip, ensuring that air bubbles were not trapped underneath the coverslip.
- The slide was then stored, in the dark, in the fridge until evaluation by microscopy was possible.

Microscopy

- Microscopy was carried out using the DMI600 SP5 Scanning Confocal Laser Microscope.
- Optimised laser settings for Cy3 and Cy5 fluorescent analysis were saved to ensure consistency of analysis.
- These saved settings were based on the use of the laser settings “Cy3correct” and “Cy5correct” with both the 561 and 633 lasers set to 25 % output.
- Images (z scan stacks performed in 0.5 step increments) were acquired for 5 fields of view across the surface of each sputum spot as depicted in Figure 2. The optimal “smart gain” setting which allowed exclusion of autofluorescence from the sputum was approximately 500, however this did require changing depending on the sample density.
- Images were taken as green for PA in the Cy3 channel and red for Eubacterial signal in the Cy5 channel.

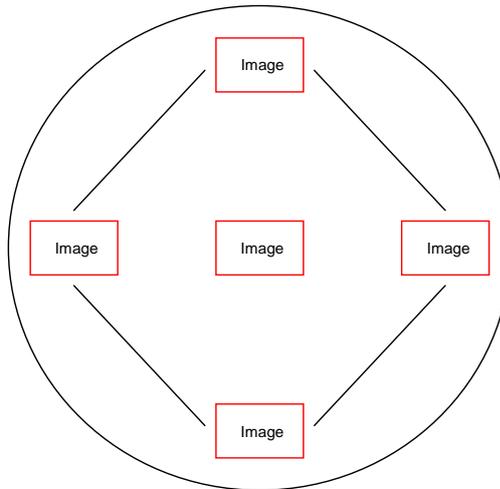


Figure 2 Diagrammatic representation of the five fields of view captured using confocal microscopy of each sputum spot.

- The image file was saved, labelled with the date, sample time (e.g. T0/T7) and patient study number.
- The file was saved in a designated location. A back-up copy of the image file was also saved to an external drive.

Quantitative Polymerase Chain Reaction

This was carried out at Kings College London by a collaborator; Dr Geraint Rogers. The method of analysis was as follows:

1. A single aliquot from each of the samples was PMA treated. DNA was then extracted from this aliquot and the PA load determined by Q-PCR in triplicate.
2. In a subset of samples (6-8, chosen on the basis of volume) multiple aliquots were PMA treated and underwent DNA extraction in parallel. This allowed observation of the degree to which intra-sample variation, or procedural variation, accounted for any differences between samples in (1).
3. DNA extracts were stored, to be available as a basis for further future assays, for example, Q-PCR based determination of total bacterial load, as required.
4. Non PMA-treated sample aliquots were retained and could be subjected to DNA extraction in the future, as required.

Blood

Sample processing was performed in a Microbiological Safety Cabinet (MSC), according to the relevant risk assessment that was carried out prior to commencement of the study. An overview of blood sample processing is shown in Table 1.

Table 1 Blood sample processing

Blood sample	Tube colour/ sample size	Time of sample	Invert	Aliquots	Special Instructions
EDTA (for NO metabolites)	Purple top (3.5ml) 	T0, T1 (x2)	5 times	1	Centrifuge at 800g (1900rpm) for 10 mins at 21 ^o C within 15 mins and pipette off plasma into 1 tube to freeze. Freeze pellet in original blood tube. Both samples to be stored in -20 ^o C freezer
PAX gene	Clear/red top (2.5ml) 	T0, T10, T20-25	10 times	Leave in blood tube	Leave at room temperature for 2-24 hours then store in tube in -20 ^o C freezer for 1-14 days then store at -80 ^o C
Plain/serum	Red top (1-2.5ml) 	T0, T10, T20-25	5 times	3	Leave to clot for 30 mins before spinning. Spin at 1200g (2300rpm) for 10 minutes at 21 ^o C and store immediately at -80 ^o C
Lith Hep (plasma)	Green top (1-2.5ml) 	T0, T10, T20-25	5 times	2	Spin at 1200g (2300rpm) for 10 minutes at 21 ^o C and store immediately at -80 ^o C
EDTA	Purple top (1-2.5ml) 	T0, T10, T20-25	5 times	2	Separate as whole blood into aliquots then store at -80 ^o C

Nitric oxide metabolites in blood

NO metabolites in blood were measured at 1-2 and 7 hours after starting inhaled therapy on day 1. These additional blood tests were carried out by local collaborators Prof Martin Feelisch and Dr Bernadette Fernandez to see whether it was possible to estimate how much nitric oxide was absorbed systemically to give an indication of the actual dose of IMP that was being absorbed by the CF lung.

- All samples were processed within 15 minutes of collection.
- The sample was placed on ice for 5 minutes while the centrifuge was turned on and adjusted to 21°C.
- The EDTA tube was spun at 1900rpm for 10 minutes at 21°C.
- The plasma was then pipetted into a fresh tube.
- The original tube (containing the pellet) and the second tube containing the plasma were labelled with sample date and time and patient identifier.
- Both were stored in the -20°C freezer and logged on freezer log and lab site file.

Other blood tests

10ml blood was taken and split between EDTA, Lithium heparin, plain and PAX gene tubes. If insufficient blood was drawn the priority was the clinical samples, then PAX gene, then plain, then lithium heparin then EDTA.

- After collection the serum bottle was left to clot for 30 mins.
- The PAX gene tube was labelled and left at room temperature for 2-24 hours.
- In the MSC the contents of the EDTA tube was separated into 2 equal aliquots as whole blood (this depended on amount of blood collected). Each aliquot was labelled with sample date, time and patient identifier.
- Once the serum tube had clotted it was spun along with the lithium heparin tube. They were spun at 21°C, 2300rpm for 10 minutes ensuring that tubes were balanced prior to spinning.
- After spinning the contents of the serum tube were separated into 3 equal aliquots and the lithium heparin tube into 2 equal aliquots. Each aliquot was labelled with date, time and patient identifier.
- All aliquots were stored in the -80°C freezer in the specified storage space study and logged on the freezer log sheet and in the lab site file.
- After 2-24 hours the PAX gene tube was put in the -20°C freezer (could be stored for 1-14 days) and recorded on the -20°C freezer storage log sheet.
- After 1-14 days the PAX gene tube was moved to the -80°C freezer and recorded on the -80°C freezer storage log sheet.

Appendix 7

CFQ-UK Questionnaire

Understanding the impact of your illness and treatments on your everyday life can help your healthcare team keep track of your health and adjust your treatments. For this reason, this questionnaire was specifically developed for people who have cystic fibrosis. Thank you for your willingness to complete this form.

Instructions: The following questions are about the current state of your health, as you perceive it. This information will allow us to better understand how you feel in your everyday life.

Please answer all the questions. There are **no** right or wrong answers! If you are not sure how to answer, choose the response that seems closest to your situation.

Section I. Demographics

Please fill-in the information or tick the box indicating your answer.

- A.** What is your date of birth?
- Date

--	--	--	--	--	--	--	--
- Day Month Year
- B.** What is your gender?
- Male Female
- C.** During the **past two weeks**, have you been on holiday or out of school or work for reasons **NOT** related to your health?
- Yes No
- D.** What is your current marital status?
- Single/never married
- Married
- Widowed
- Divorced
- Separated
- Remarried
- With a partner
- E.** Which of the following best describes your racial background?
- White - UK
- White - other
- Indian/ Pakistani
- Chinese/ Asian
- African
- Caribbean
- Other [not represented above or people whose predominant origin cannot be determined/ mixed race]
- F.** What is the highest level of education you have completed?
- Some secondary school or less
- GCSEs/ O-levels
- A/AS-levels
- Other higher education
- University degree
- Professional qualification or post-graduate study
- G.** Which of the following best describes your current work or school status?
- Attending school outside the home
- Taking educational courses at home
- Seeking work
- Working full or part time (either outside the home or at a home-based business)
- Full time homemaker
- Not attending school or working due to my health
- Not working for other reasons

Section II. Quality of Life

Please tick the box indicating your answer.

	A lot of difficulty	Some difficulty	A little difficulty	No difficulty
<i>During the past two weeks, to what extent have you had difficulty:</i>				
1. Performing vigorous activities such as running or playing sports.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
2. Walking as fast as others	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
3. Carrying or lifting heavy things such as books, shopping, or school bags.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
4. Climbing one flight of stairs.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
5. Climbing stairs as fast as others	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

	Always	Often	Sometimes	Never
<i>During the past two weeks, indicate how often:</i>				
6. You felt well	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
7. You felt worried.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
8. You felt useless.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
9. You felt tired.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
10. You felt full of energy	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
11. You felt exhausted	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
12. You felt sad.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Please circle the number indicating your answer. Please choose only one answer for each question.

Thinking about the state of your health over the last two weeks:

13. To what extent do you have difficulty walking?
 1. You can walk a long time without getting tired
 2. You can walk a long time but you get tired
 3. You cannot walk a long time because you get tired quickly
 4. You avoid walking whenever possible because it's too tiring for you

14. How do you feel about eating?
 1. Just thinking about food makes you feel sick
 2. You never enjoy eating
 3. You are sometimes able to enjoy eating
 4. You are always able to enjoy eating

15. To what extent do your treatments make your daily life more difficult?
 1. Not at all
 2. A little
 3. Moderately
 4. A lot
16. How much time do you currently spend each day on your treatments?
 1. A lot
 2. Some
 3. A little
 4. Not very much
17. How difficult is it for you to do your treatments (including medications) each day?
 1. Not at all
 2. A little
 3. Moderately
 4. Very
18. How do you think your health is now?
 1. Excellent
 2. Good
 3. Fair
 4. Poor

Please select a box indicating your answer.

*Thinking about your health during the past **two weeks**, indicate the extent to which each sentence is true or false for you.*

	Very true	Somewhat true	Somewhat false	Very false
19. I have trouble recovering after physical effort	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
20. I have to limit vigorous activities such as running or playing sports.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
21. I have to force myself to eat	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
22. I have to stay at home more than I want to	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
23. I feel comfortable discussing my illness with others	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
24. I think I am too thin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
25. I think I look different from others my age	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
26. I feel bad about my physical appearance	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
27. People are afraid that I may be contagious	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
28. I get together with my friends a lot.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

- | | | | | |
|---|--------------------------|--------------------------|--------------------------|--------------------------|
| 29. I think my coughing bothers others | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 30. I feel comfortable going out at night | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 31. I often feel lonely..... | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 32. I feel healthy | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 33. It is difficult to make plans for the future (for example, going to college, getting married, getting promoted at work, etc.) | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 34. I lead a normal life..... | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

Section III. School, Work, or Daily Activities

Questions 35 to 38 are about school, work, or other daily tasks.

35. To what extent did you have trouble keeping up with your schoolwork, professional work, or other daily activities during the past **two weeks**?
1. You have had no trouble keeping up
 2. You have managed to keep up but it's been difficult
 3. You have been behind
 4. You have not been able to do these activities at all
36. How often were you absent from school, work, or unable to complete daily activities during the last two weeks because of your illness or treatments?
- Always Often Sometimes Never
37. How often does CF get in the way of meeting your school, work, or personal goals?
- Always Often Sometimes Never
38. How often does CF interfere with getting out of the house to run errands such as shopping or going to the bank?
- Always Often Sometimes Never

Section IV. Symptom Difficulties

Please select a box indicating your answer.

Indicate how you have been feeling during the past two weeks.

	A great deal	Somewhat	A little	Not at all
39. Have you had trouble gaining weight?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
40. Have you been congested?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
41. Have you been coughing during the day?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
42. Have you had to cough up mucus?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Go to
Question 44

43. Has your mucus been mostly:

- Clear
 Clear to yellow
 Yellowish-green
 Green with traces of blood
 Don't know

How often during the past two weeks:

	Always	Often	Sometimes	Never
44. Have you been wheezing?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
45. Have you had trouble breathing?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
46. Have you woken up during the night because you were coughing?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
47. Have you had problems with wind?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
48. Have you had diarrhoea?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
49. Have you had abdominal pain?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
50. Have you had eating problems?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Please make sure you have answered all the questions.

Thank you for your cooperation

Appendix 8

Case Report Forms

Screening and Randomization

The RATNO Study

Reducing Antibiotic Tolerance using low dose Nitric Oxide in cystic fibrosis
A phase 2 pilot study

Screening and enrolment Case Report Form

Screening Date // (DD/MM/YY)

First Name	
Surname	

Date of birth // (DD/MM/YY)

Gender	M/F	Age	<input type="text"/>
---------------	-----	------------	----------------------

Responsible consultant (circle)	Connett / Legg / Evans / Carroll / Daniels / Nightingale Other (please state)
--	--

Height (cm) . **Weight (kg)** .

Study Number	<input type="text"/> <input type="text"/>	Participant Initials	<input type="text"/> <input type="text"/> <input type="text"/>
---------------------	---	-----------------------------	--

Study Number Initials

Inclusion Criteria	Yes (+date of completion)	No (+date of completion)
Cystic Fibrosis		
Age 12 or above		
Colonised with <i>Pseudomonas aeruginosa</i> (50% or more of all cultures in the last 12 months positive for <i>Pseudomonas aeruginosa</i>)		

Exclusion Criteria	Yes	No
Colonisation with <i>Burkholderia. Cepacia</i>		
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 (NIV)		
Patients who cannot tolerate nasal canula e.g. those who cannot breathe through their nose		
Patients who have a pneumothorax		
Patients who are admitted for specific treatment of nontuberculous mycobacteria (NTM)		
Patients who have nasal polyposis that is causing significant blockage of the nasal passages		
Adolescents who are not Gillick competent (and therefore not able to give their own consent in addition to parental consent)		
Children 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 30 days prior to enrolment		
Patients who are pregnant (a pregnancy test will be carried out for females of 11 years and above as is standard practice for clinical trials)		
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.		

- Copy of consent form and information sheet filed in notes (+initials)
- Copy of consent form and information sheet given to participant (+initials)
- Original consent form placed in study file (+initials)
- Letter sent to GP (+initials)

Study Number

Initials

Criteria for enrolment	Yes (+ date)	No
Informed consent received		
Sputum collected for microbiology		
Pseudomonas isolated		
Sputum results attached to this form		
Participant agrees to be enrolled		

Phone call to patient to inform them of enrolment status:

Name of person making call	Date + Time

Outcome of study:

Completed (randomised)

Study Finished before randomisation

Withdrawn (not randomised)

Reason for withdrawal: _____

The RATNO Study

Reducing Antibiotic Tolerance using low dose Nitric Oxide in cystic fibrosis
A phase 2 pilot study

Case Report Form (from randomisation)

Randomisation Date / / (DD/MM/YY)

Pulmonary exacerbation criteria (at least 3 of following)	Yes	No
Increased cough		
Increased sputum production, change in appearance of expectorated sputum production, or both		
Fever (greater than or equal to 38°C for at least 4 hrs in a 24 hrs period) on more than one occasion in the previous week		
Weight loss greater than or equal to 1kg or 5% of bodyweight associated with anorexia and decreased dietary intake		
School or work absenteeism (due to illness) in the previous week		
Increased respiratory rate, increased work of breathing, or both		
New findings on chest examination (e.g. rales, wheezing, crackles)		
Decreased exercise tolerance		
Decrease in FEV ₁ of greater than or equal to 10% from previous baseline study within the past 3 months		
Decrease in haemoglobin saturation (as measured by oximetry) from baseline value within past 3 months of greater than or equal to 10%		
New finding on chest radiograph		

Consent confirmed verbally with participant	Y / N (+ date)
--	-----------------------

Female participants pregnancy test	Positive / negative (+ date) (if test positive, refer back to clinical team)
---	--

Study Number **S** - **R**

Initials

Day 0

Date / / (DD/MM/YY)

Clinical Assessment

Height (cm) . Weight (kg) .

HR (bpm)

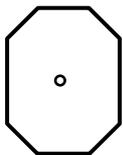
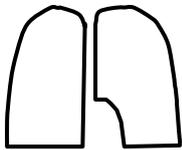
BP (mmHg) /

O2 sats (%) (in air)

RR (/min)

Temp (°C) .

Heart Sounds



Other

Study Number

S			-	R		
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Initials

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IV Antibiotic Therapy (enter start and end date and if any changes made including dose and frequency)

Antibiotics	Start date	End date	Dose	Frequency
Tobramycin				
Ceftazidime				

Researchers Initials

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Study Number

S			-	R		
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Initials

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List all current medications (tick those already listed and specify dose, frequency and route and write any not on list)

Medication	Dose	Route	Frequency	T0	T7	T9-12	T20-25
NO CHANGE TO PREVIOUS MEDICATION							
Creon 10,000/25,000/40,000		Oral					
Ursodeoxycholic acid		Oral	bd				
Vitamin K	10mg	Oral	od				
Vitamin E	_____units	Oral	od				
Vitamin A and D	_____capsules	Oral	od				
Calchichew D3 forte	_____number _____of tabs	Oral	od/bd				
Omeprazole	_____mg	Oral	od/bd				
Movicol/Laxido sachets		Oral	Regular _____ prn				
Carbocysteine	750mg	Oral	bd				
Prednisolone	_____mg	Oral	od				
Bisphosphonate		Oral					
Voriconazole	200mg	Oral	bd				

RATNO Case report Form v 1.2 18/07/2011 NHS REC Number: 11/H0502/7

Researchers Initials

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Study Number

S			-	R		
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 Initials

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Medication	Dose	Route	Frequency	T0	T7	T9-12	T20-25
Itraconazole	200mg	Oral	bd				
Azithromycin	250mg 500mg	Oral	od Mon/Wed/Fri				
Doxycycline	200mg	Oral	od				
Flucloxacillin	1 gram	Oral	bd				
Dornase alpha/RhDNase (Pulmozyme)	2.5mg Other _____	Neb	od/bd Other _____				
Saline 7%	4ml	Neb	bd				
Tobramycin (TOBI or Bramitob)	300 mg	Neb	bd				
Gentamicin	80mg	Neb	bd				
Colomycin	2 MU	Neb	bd				
Salbutamol	_____ puffs/ _____ mg	Inh / neb					
Inhalers/ nebs: can be anything							
Insulin	_____ units	Sub cut					
Heparin	_____ units	Neb					

RATNO Case report Form v 1.2 18/07/2011 NHS REC Number: 11/H0502/7

 Researchers Initials

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Study Number

S			-	R		
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Initials

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Medication	Dose	Route	Frequency	T0	T7	T9-12	T20-25

Has the participant experienced any adverse events since the last visit? Y / N

(if yes please fill in appropriate AE form)

Researchers Initials

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Study Number -

Initials

Lung function

Date / / (DD/MM/YY)

4 hrs after last inhaler or nebuliser

Calibrate Koko spirometer before use

	Best	Predicted	% of predicted
FEV ₁			
FVC			

Calibrate Niox mino before use

	1	2	Average
Exhaled NO levels (ppb)			

Bloods

Date / / (DD/MM/YY)

Clinical bloods taken (FBC, U+E, LFT, bone profile, CRP) Y / N

Clinical blood results printed out and attached to this form Y / N

Research Blood Sample collected Y / N Amount (aim 10 ml) . mls

Priority order for bottles: PAX gene → plain → lithium heparin → EDTA
(please circle bottles sent)

Blood for NO metabolites collected in EDTA tube (3.5ml) Y / N Time :

Blood for NO metabolites spun and stored in WTCRF lab Y / N Time :

Completed HRQOL attached to this form Y / N

Sputum taken for microbiology (Heparin and DNase need to be given AFTER sputum sample – NOT before!) Y / N

Sputum results attached to this form (when available) Y / N

Optional (if consented for) Nasal brushings before NO therapy (PCD team to do) Y / N

Optional (if consented for) Nasal examination Y / N

Findings from nasal examination _____

Study Number -

Initials

Day 1 Date / / (DD/MM/YY)

Calibrate INOvent before commencing delivery

Clarify target O2 saturations for adult participants _____
(paediatric participants should have sats >92%)

1 hour after start of inhaled therapy

Blood for NO metabolites collected in EDTA tube (3.5ml) Y / N Time :

Blood for NO metabolites spun and stored in WTCRF lab Y / N Time :

Blood for methHb levels (venous blood gas taken to med HDU/PAU)

If levels >5% refer to study guidance 'monitoring participants during NO therapy' for further instructions

7 hours after start of inhaled therapy

Blood for NO metabolites collected in EDTA tube (3.5ml) Y / N Time :

Blood for NO metabolites spun and stored in WTCRF lab Y / N Time :

Blood for methHb levels (venous blood gas taken to med HDU/PAU)

If levels >5% refer to study guidance 'monitoring participants during NO therapy' for further instructions

Record of doses of Antibiotic given on day 1

Ceftazidime: 1 2 3

Tobramycin:

Other (please specify including number of doses given) _____

Study Number

S			-	R		
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Initials

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Monitoring during NO/placebo administration – Day 1

	Time	Min	Time	Max
O₂ sats				
Heart Rate				
Blood pressure				
Respiration Rate				
Inspired O₂				
NO₂ levels				

Researchers Initials

--	--	--

Study Number -

Initials

Day 2 Date / / (DD/MM/YY)

Calibrate INOvent before commencing delivery

Clarify target O2 saturations for adult participants _____
(paediatric participants should have sats >92%)

Tobramycin levels

1 hour after 2nd dose .

18 hours after 2nd dose .

(Refer to study guidance: Non-Investigational Medicinal Product (NIMP) Antibiotic Therapy for target levels and advice on altering doses.)

Record of doses of Antibiotic given on day 2

Ceftazidime: 1 2 3

Tobramycin:

Other (please specify including number of doses given) _____

Monitoring during NO/placebo administration – Day 2

	Time	Min	Time	Max
O ₂ sats				
Heart Rate				
Blood pressure				
Respiration Rate				
Inspired O ₂				
NO ₂ levels				

Study Number

S			-	R		
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Initials

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Day 3 Date

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 (DD/MM/YY)

Calibrate INOvent before commencing delivery

Clarify target O2 saturations for adult participants _____
(paediatric participants should have sats >92%)

Record of doses of Antibiotic given on day 3

Ceftazidime: 1 2 3

Tobramycin:

Other (please specify including number of doses given) _____

Monitoring during NO/placebo administration – Day 3

	Time	Min	Time	Max
O ₂ sats				
Heart Rate				
Blood pressure				
Respiration Rate				
Inspired O ₂				
NO ₂ levels				

Study Number

S			-	R		
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Initials

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Day 4 Date

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 (DD/MM/YY)

Calibrate INOvent before commencing delivery

Clarify target O2 saturations for adult participants _____
(paediatric participants should have sats >92%)

Record of doses of Antibiotic given on day 4

Ceftazidime: 1 2 3

Tobramycin:

Other (please specify including number of doses given) _____

Monitoring during NO/placebo administration – Day 4

	Time	Min	Time	Max
O ₂ sats				
Heart Rate				
Blood pressure				
Respiration Rate				
Inspired O ₂				
NO ₂ levels				

Study Number -

Initials

Day 5 Date / / (DD/MM/YY)

Calibrate INOvent before commencing delivery

Clarify target O2 saturations for adult participants _____
 (paediatric participants should have sats >92%)

Record of doses of Antibiotic given on day 5

Ceftazidime: 1 2 3

Tobramycin:

Other (please specify including number of doses given) _____

Monitoring during NO/placebo administration – Day 5

	Time	Min	Time	Max
O ₂ sats				
Heart Rate				
Blood pressure				
Respiration Rate				
Inspired O ₂				
NO ₂ levels				

Study Number **S** - **R**

Initials

Day 1/2/3/4/5 (please circle)

Date // (DD/MM/YY)

Calibrate Niox mino before use

	1	2	Average
Exhaled NO levels (ppb)			

Sputum for NO levels (to be taken immediately to WTCRF lab) Y / N

Results from sputum NO attached to this form (when available) Y / N

Day 1/2/3/4/5 (please circle)

Date // (DD/MM/YY)

**Optional (if consented for) Nasal brushings after NO therapy
(immediately after disconnecting) Y / N**

Study Number **S** - **R**

Initials

Day 6 Date / / (DD/MM/YY)

Calibrate INOvent before commencing delivery

Clarify target O2 saturations for adult participants _____
 (paediatric participants should have sats >92%)

Record of doses of Antibiotic given on day 6

Ceftazidime: 1 2 3

Tobramycin:

Other (please specify including number of doses given) _____

Monitoring during NO/placebo administration – Day 6

	Time	Min	Time	Max
O ₂ sats				
Heart Rate				
Blood pressure				
Respiration Rate				
Inspired O ₂				
NO ₂ levels				

Study Number -

Initials

Day 7 Date / / (DD/MM/YY)

Calibrate INOvent before commencing delivery

Clarify target O2 saturations for adult participants _____
(paediatric participants should have sats >92%)

Tobramycin levels

1 hour after 7th dose .

18 hours after 7th dose .

(Refer to study guidance: Non-Investigational Medicinal Product (NIMP) Antibiotic Therapy for target levels and advice on altering doses.)

Record of doses of Antibiotic given on day 7

Ceftazidime: 1 2 3

Tobramycin:

Other (please specify including number of doses given) _____

Monitoring during NO/placebo administration – Day 7

	Time	Min	Time	Max
O ₂ sats				
Heart Rate				
Blood pressure				
Respiration Rate				
Inspired O ₂				
NO ₂ levels				

Study Number **S** - **R**

Initials

Day 7

Date / / (DD/MM/YY)

Clinical Assessment

HR (bpm)

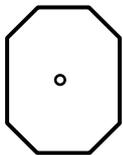
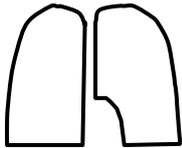
BP (mmHg) /

O2 sats (%) (in air)

RR (/min)

Temp (°C) .

Heart Sounds



Other

Researchers Initials

Study Number

S			-	R		
---	--	--	---	---	--	--

Initials

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IV Antibiotic Therapy (enter start and end date and if any changes made including dose and frequency)

Antibiotics	Start date	End date	Dose	Frequency
Tobramycin				
Ceftazidime				

Other medications – please record on table on pages 4-6 of CRF

Has the participant experienced any adverse events since the last visit? Y / N

(if yes please fill in appropriate AE form)

Study Number **S** - **R**

Initials

Lung function

Date / / (DD/MM/YY)

4 hrs after last inhaler or nebuliser

Calibrate Koko spirometer before use

	Best	Predicted	% of predicted
FEV₁			
FVC			

Calibrate Niox mino before use

	1	2	Average
Exhaled NO levels (ppb)			

Sputum for NO levels (to be taken immediately to WTCRF lab) Y / N

Results from sputum NO attached to this form (when available) Y / N

Sputum for microbiology (Heparin and DNase need to be given AFTER sputum sample – NOT before!) Y / N

Sputum results attached to this form (when available) Y / N

Day 9/10/11/12 (please circle) Date

		/			/		
--	--	---	--	--	---	--	--

 (DD/MM/YY)

Record of doses of Antibiotic given on day 8

Ceftazidime: 1 2 3

Tobramycin:

Other (please specify including number of doses given) _____

Record of doses of Antibiotic given on day 9

Ceftazidime: 1 2 3

Tobramycin:

Other (please specify including number of doses given) _____

Record of doses of Antibiotic given on day 10

Ceftazidime: 1 2 3

Tobramycin:

Other (please specify including number of doses given) _____

Record of doses of Antibiotic given on day 11

Ceftazidime: 1 2 3

Tobramycin:

Other (please specify including number of doses given) _____

Record of doses of Antibiotic given on day 12

Ceftazidime: 1 2 3

Tobramycin:

Other (please specify including number of doses given) _____

Clinical Assessment

HR (bpm)

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BP (mmHg)

--	--	--

 /

--	--	--

O2 sats (%)

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 (in air)

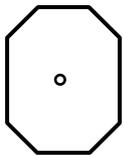
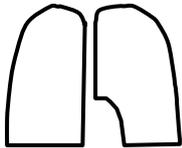
RR (/min)

--	--	--

Temp (°C)

		.	
--	--	---	--

Heart Sounds



Other

Study Number

S			-	R		
---	--	--	---	---	--	--

Initials

--	--	--

IV Antibiotic Therapy (enter start and end date and if any changes made including dose and frequency)

Antibiotics	Start date	End date	Dose	Frequency
Tobramycin				
Ceftazidime				

Other medications – please record on table on pages 4-6 of CRF

Has the participant experienced any adverse events since the last visit? Y / N

(if yes please fill in appropriate AE form)

Study Number -

Initials

Lung function

Date / / (DD/MM/YY)

4 hrs after last inhaler or nebuliser

Calibrate Koko spirometer before use

	Best	Predicted	% of predicted
FEV ₁			
FVC			

Calibrate Niox mino before use

	1	2	Average
Exhaled NO levels (ppb)			

Bloods

Date / / (DD/MM/YY)

Clinical bloods taken (FBC, U+E, LFT, bone profile, CRP) Y / N

Clinical blood results printed out and attached to this form Y / N

Research Blood Sample collected Y / N

Amount (aim 10 ml) . mls

Priority order for bottles: PAX gene → plain → lithium heparin → EDTA
(please circle bottles sent)

Sputum taken for microbiology (Heparin and DNase need to be given AFTER sputum sample – NOT before!) Y / N

Sputum results attached to this form (when available) Y / N

Follow-up (10-15 days after IVs) T 20/21/22/23/24/25 (please circle) Date

--	--

 /

--	--

 /

--	--

 (DD/MM/YY)

Clinical Assessment

Height (cm)

			.	
--	--	--	---	--

 Weight (kg)

			.	
--	--	--	---	--

HR (bpm)

--	--	--

BP (mmHg)

--	--	--

 /

--	--	--

O2 sats (%)

--	--	--

 (in air)

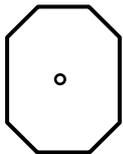
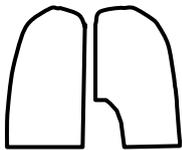
RR (/min)

--	--	--

Temp (°C)

		.	
--	--	---	--

Heart Sounds



Other

Study Number

S			-	R		
---	--	--	---	---	--	--

Initials

--	--	--

IV Antibiotic Therapy (enter start and end date and if any changes made including dose and frequency)

Antibiotics	Start date	End date	Dose	Frequency
Tobramycin				
Ceftazidime				

Other medications – please record on table on pages 4-6 of CRF

Has the participant experienced any adverse events since the last visit? Y / N

(if yes please fill in appropriate AE form)

Study Number **S** - **R**

Initials

Lung function

Date // (DD/MM/YY)

4 hrs after last inhaler or nebuliser

Calibrate Koko spirometer before use

	Best	Predicted	% of predicted
FEV₁			
FVC			

Calibrate Niox mino before use

	1	2	Average
Exhaled NO levels (ppb)			

Bloods

Date // (DD/MM/YY)

Clinical bloods taken (FBC, U+E, LFT, bone profile, CRP) Y / N

Clinical blood results printed out and attached to this form Y / N

Research Blood Sample collected Y / N **Amount (aim 10 ml) . mls**

Priority order for bottles: PAX gene → plain → lithium heparin → EDTA
(please circle bottles sent)

Completed HRQOL attached to this form Y / N

Sputum taken for microbiology (Heparin and DNase need to be given AFTER sputum sample – NOT before!) Y / N

Sputum results attached to this form (when available) Y / N

Study Number

S			-	R		
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Initials

--	--	--

Outcome of study:

Completed (randomised)

Withdrawn (randomised)

Reason for withdrawal: _____

Further follow up (study related) needed Y / N

If Yes please state reason and date of follow-up _____

If further follow-up (study related) is needed has this been arranged? Y / N

Researchers Initials

--	--	--

Appendix 9

Study Protocol

RATNO (Reducing Antibiotic Tolerance using NO)

Reducing antibiotic tolerance using low dose nitric oxide in
cystic fibrosis – a phase 2 pilot study

Protocol version: 1.4: 14/09/11

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List of Abbreviations and Definitions

AE	Adverse Event
AR	Adverse Reaction
ARDS	Acute respiratory Distress Syndrome
BP	Blood Pressure
CF	Cystic Fibrosis
CFQ-UK	Cystic Fibrosis Questionnaire UK
CFTR	Cystic Fibrosis Trans membrane Regulator
CFUs	Colony Forming Units
CI	Chief Investigator
CRF	Case Report Form
CRP	C Reactive Protein
CTIMP	Controlled Trial of Investigational Medicinal Product
CTU	Clinical Trials Unit
ECMO	Extra Corporeal membrane Oxygenation
FBC	Full Blood Count
FEV ₁	Forced Expiratory Volume in 1 second
FiO ₂	Inspired oxygen concentration
GCP	Good Clinical Practice
GMP	Good Manufacturing Practice
HR	Heart Rate
HRQOL	Health Related Quality Of Life
IDSMC	Independent Data Safety Monitoring Committee
IMP	Investigational Medicinal Product
iNO	Inhaled Nitric Oxide
ISF	Investigator Site File
IV	Intravenous
LFT	Liver Function Tests
MCRN	Medicines for Children Research Network
MetHb	Methaemaglobinaemia
MHRA	Medicines and Healthcare products Regulatory Agency
NIMP	Non-investigational Medicinal Product
NIV	Non Invasive Ventilation
NO	Nitric Oxide
NO ₂	Nitrogen Dioxide
O ₂	Oxygen
PI	Principal Investigator
PA	<i>Pseudomonas aeruginosa</i>
ppb	parts per billion
ppm	parts per million
RCT	Randomised Controlled Trial
REC	Research Ethics Committee
SAE	Serious Adverse Event
SAR	Serious Adverse Reaction
Sats	Oxygen saturations
SNP	Sodium Nitroprusside
SPC	Summary of Product Characteristics
SUSAR	Suspected Unexpected Serious Adverse Reaction
TMG	Trial Management Group
TSC	Trial Steering Committee
U+E	Urea and Electrolytes

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1. General Information

1.1 Abstract

The lungs of most patients with cystic fibrosis (CF) become chronically infected with bacteria called *Pseudomonas aeruginosa* during childhood. This infection is now known to consist of free-living bacteria (known as “planktonic bacteria”) and bacteria in colonies on body surfaces known as “biofilms”. The bacteria in biofilms are more resistant and tolerant to antibiotics. Current CF treatment of exacerbations aims to eradicate or control pseudomonal infection using aggressive antibiotic regimes. Despite this treatment many patients develop chronic infection which is never cleared. Chronic infection causes damage to the lungs. Patients colonised with *Pseudomonas* are more unwell and die at a younger age. Our laboratory has established that low dose nitric oxide (NO) can disrupt pseudomonal biofilms in the laboratory. This pilot study will discover whether non-toxic levels of NO administered to participants during an episode of acute infection (exacerbation) will disrupt bacteria from biofilms and increase the effectiveness of antibiotic therapy. This protocol describes a participant-blind randomised controlled pilot study of treatment with nitric oxide gas during an acute infective exacerbation (also known simply as an “acute exacerbation”). Patients with CF aged 12 or above will be asked to take part. They will be randomised to receive 7 days either of inhaled nitric oxide gas or placebo alongside standard therapy during an exacerbation. Sputum samples will be obtained before, during and after the treatment period for microbiological analysis. The primary endpoint will be the microbiological effect on bacterial biofilms before and after NO adjunctive therapy. Secondary microbiological endpoints will include the between group differences in pseudomonal colony forming units (CFU’s), biofilm NO levels and detailed characterisation of biofilms before and after treatment. Secondary clinical endpoints will include lung function and well-established indicators quality of life. The aim of this randomised pilot study is as proof of concept and to guide the design of a large multi-centre trial to definitively evaluate the effectiveness of NO or NO donors as adjunctive therapy in CF.

2. Background Information

2.1 Cystic Fibrosis

Cystic fibrosis is one of the UK's most common life-threatening inherited diseases. It affects over 8,500 people in the UK. Around 1 in 25 of the UK population carry a gene mutation for CF (the most common being delta F508). If 2 carriers have a child there is a 1 in 4 chance of the child having CF. Each week 5 babies are born with CF and 3 young lives are lost to CF. The genetic mutation is in the cystic fibrosis transmembrane conductance regulator gene (CFTR) which leads to a defect in a chloride channel that results in sodium and water re-absorption and chloride secretion. This affects a number of the internal organs, particularly the lungs and digestive system due to the increased viscosity of the secretions. In addition goblet cell hyperplasia causes increased mucus production and cilia that should beat to remove bacteria and particles from the airways are clogged up with sticky mucus and are ineffective. As well as making breathing more difficult, the accumulation of airway mucus encourages opportunistic infection that cannot be cleared. If not controlled, infections can lead to damage to the lungs.

In recent years there have been many advances in CF care that have led to improved survival leading to a current life expectancy of about 38 years (www.cff.org). There is still a vital need for further advances in care to improve survival and reduce the burden of the disease. Most disease-related morbidity and mortality in CF is caused by progressive lung disease as a result of bacterial infection and airway inflammation.^{1,2} This provides the biggest burden to patients and results in the greatest strain on the health service (hospital admissions, medications etc.).

Infection with the Gram-negative rod *Pseudomonas aeruginosa* (PA) causes the greatest clinical concern, although multiple bacterial species can be identified in respiratory secretions.³ PA is the commonest cause of chronic lung infection in CF. Chronic infection is found in 9% of pre-school children, 32% of 10-15 year olds and 59% adults with CF.^{4,5} As the lung is colonised, the growth pattern of PA changes and its capacity for survival improves.⁶ The bacteria form biofilms as well as producing large quantities of alginate (the so-called mucoid phenotype) which reduces the effectiveness of phagocytosis and antibiotic therapy.⁷ This leads to chronic colonisation of the lung by PA that is not cleared by conventional antibiotic therapy.⁸ Patients who are colonised with PA show a more rapid decline in lung function,⁹ faster decline in chest radiograph score,¹⁰ poor weight gain, increased hospitalisation rates and an increased need for antibiotic therapy.¹¹ Median survival is significantly reduced and mortality increased.^{1,9}

Long term outcomes in CF have the potential for improvement if new methods can be developed to effectively eradicate PA from the lung and reduce the effect of chronic colonisation.

2.2 Pseudomonas infection and biofilm formation

Bacteria are now known to exist *in vivo* as single cells that float or swim independently in a liquid medium (the planktonic phase) or as structured bacterial

communities known as biofilms. In biofilms, bacteria are encased in a polymeric matrix adherent to a biological or inert surface. Bacteria in biofilms are relatively resistant or tolerant to antibiotics which can be explained in 3 ways:¹²

1. As a consequence of a protective barrier effect of the polymeric matrix.
2. Bacteria in the biofilm may be metabolically quiescent thus reducing the activity of antibiotics that rely upon disrupting energetic processes to be maximally effective.
3. By facilitating the survival of resistant bacterial phenotypes.

A bacterial population in a biofilm may have a degree of antimicrobial resistance up to 1000 times as great as an identical population that is planktonic.^{13 14} Although aggressive early antibiotic treatment can eradicate planktonic bacterial infection, the formation of biofilms, and the antibiotic resistance that they infer, are becoming increasingly recognized as risk factors for chronic infection in diseases such as CF.¹⁵
16

Nitric oxide (NO) is an endogenously produced neurotransmitter with vasodilatory properties. NO is known to down regulate biofilm formation *in vitro* and exhaled NO levels are reduced in CF.^{17 18} which is likely to predispose to biofilm formation *in vivo*.¹⁹ NO also contributes to the regulation of bronchial tone, and a single inhalation of nebulised L-arginine (which stimulates NO production in epithelial cells) has been shown to improve FEV₁ in patients with CF.²⁰ We have shown that biofilm formation by PA is reversed *in vitro* by nanomolar, non-toxic concentrations of NO.²¹ In this model the proportion of PA in the planktonic (vulnerable) phase is increased and a reduction in antibiotic tolerance was observed. Exposure of biofilms to NO also greatly enhanced the ability of antibiotics such as Tobramycin (a typical antibiotic used in CF) to remove the bacteria/biofilms from a glass surface.²¹

Further laboratory work has focussed on the treatment of *ex vivo* biofilms grown from CF sputum (Howlin et al, in preparation). Sputum samples have been collected from participants with CF as part of ongoing respiratory research within the Biomedical Research Unit at Southampton University Hospitals NHS Trust (Investigation of the mechanisms of lung infection and inflammation in children and adults with known or suspected respiratory disease, NHS REC No: 08/H0502/126, Sponsor Code: RHM MED0837). PA has been isolated from these samples and biofilms grown. These biofilms have been treated with Sodium Nitroprusside (SNP), a Nitric Oxide donor. We have shown in a number of different patient isolates that, following addition of the NO donor SNP, dispersal occurs (after approximately 5 hours) in the patients samples that grow PA and which form biofilms easily *ex vivo*.

The next stage of the work that has already been completed assesses biofilm dispersal and bacterial removal using antibiotics combined with SNP. We have shown that treatment of pseudomonal biofilms (grown from CF sputum samples) with a combination of SNP (NO donor) and antibiotic (ceftazidime and tobramycin), results in greater removal of the biofilm from the surface, compared to antibiotic alone, where a significant proportion of biofilm remains. The observed effect is a reduction in surface area occupied by biofilm in the order of 30%.

We also have data that shows a reduction in colony forming units (CFUs) following treatment of pseudomonal biofilms (from CF sputum samples) with the combination of SNP and antibiotic when compared to antibiotic alone.

From these data there is clear evidence that SNP (NO donor) causes dispersal of bacteria from biofilms. When pseudomonas biofilms are treated with antibiotic in combination with SNP, a greater degree of killing is observed than with antibiotic alone. This forms the basis for our hypothesis that introducing low dose NO to CF lungs will disperse bacteria from biofilms and increase antibiotic effectiveness.

2.3 Nitric oxide

Nitric oxide gas has been well researched and used for a number of years in participants. It is not compounded like other drugs and is carried within inert nitrogen.²² Much research regarding use of inhaled NO gas (iNO) has been conducted in preterm neonates. Currently the only licensed indication for use of iNO is for infants >34 weeks gestation with hypoxic respiratory failure associated with evidence of pulmonary hypertension.²³ It is used routinely at concentrations of 20ppm, but at times increased to concentrations of 40ppm. In these patients iNO is often given continuously for a number of days. It acts as a vasodilator and improves oxygenation and reduces the need for extracorporeal membrane oxygenation (ECMO).²⁴ iNO has been used off-label in a number of other patient groups including adults and children with acute pulmonary hypertension and acute respiratory distress syndrome (ARDS) and children with perioperative pulmonary hypertension.^{25 26} It continues to be used regularly to treat such patients on paediatric intensive care units (PICU).

In a study of 13 participants with cystic fibrosis (CF), iNO in concentrations of 100 ppb, 1ppm and 40 ppm had no immediate effect on lung function or oxygenation.²⁷ CF participants undergoing lung transplantation have also been given iNO perioperatively with no significant side effects.²⁸

A pilot study recently completed in Denver, Colorado examined the safety and tolerability of iNO (given at doses of either 20ppm or 40ppm continuously over 44 hours) in clinically stable participants with CF. Although not looking at the effect on biofilms, this study collected induced sputum at baseline and after 44 hours and measured quantitative bacterial counts and markers of inflammation in the sputum. iNO was safe and well tolerated in these participants with no significant antimicrobial or anti-inflammatory effects (Dr Scott Sagel, unpublished data, personal communication, outline data also available on clinical trials.gov: <http://clinicaltrials.gov/ct2/show/NCT00570349?term=nitric+oxide+and+CF&rank=3>).

Side effects

Over the past 10 years iNO has been administered to numerous participants without any apparent significant side effects. Although iNO is considered to be safe in doses of 1-20 parts per million (ppm), there are a number of precautions and safety considerations that must be taken into account. In addition, only medical grade iNO must be used.²⁹ Current recommendations say that scavenging is not necessary if iNO is administered in a well ventilated environment.³⁰

Formation of nitrogen dioxide (NO₂) occurs when NO is mixed with oxygen (O₂). It is also formed from decomposition of peroxyxynitrite (a reactive nitrogen species formed from reaction of NO with reactive oxygen species such as superoxide).³¹ The formation of NO₂ can be reduced by limiting the concentration of O₂ in the inspired gas mixture.³² Levels of NO₂ need to be monitored and kept below 2ppm.

Systemic vasodilation does not occur with use of iNO because NO is rapidly bound and inactivated by haemoglobin (Hb) within the circulation.³³ This results in the formation of methaemoglobin (metHb). High levels of metHb cause a reduction in the O₂ carrying capacity of the blood. Clinically significant levels of metHb are unlikely to result unless concentrations of iNO higher than 20ppm are used for continuous periods over days, or if levels above 80ppm are used.^{34 35} In more than 10 years of successfully treating term infants with iNO, metHb levels have not been a problem.

2.4 Larger microbial community within the CF lung

The lungs of participants with CF and other chronic suppurative lung diseases are colonized with bacteria. The intense host inflammatory response to these bacteria results in damage to the lung and the progressive loss of lung function.³⁶ This is the main cause of mortality in CF participants.³⁶⁻³⁹ Microbiological culture media used routinely are only capable of supporting the growth of a small fraction of the diverse bacterial community present⁴⁰ and only a limited number of important species are identified (including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Burkholderia cepacia*).⁴¹ Culture therefore produces results that are unrepresentative of the actual populations of bacteria present within the lungs. Additionally, conventional antibiotic susceptibility testing only demonstrates how effective single antibiotics are against planktonic bacteria grown in aerobic conditions (not bacteria from biofilms). Participant therapy that is guided by these results is not always effective and participants continue to deteriorate. Hence, treatment of an acute exacerbation in CF is not always based on traditional culture results and antibiotic sensitivities, more often being chosen based on 'standard' therapy and clinical response during previous exacerbations. Current guidelines for antibiotic choices to treat *Pseudomonas* infection do not recommend the use of antibiotic sensitivity testing.

Techniques have been developed to further study the bacterial diversity within specimens provided from participants with CF. Terminal restriction fragment length polymorphism (T-RFLP) is a molecular biology technique for profiling microbial communities based on the position of a restriction site closest to the labeled end of an amplified gene. It has been shown to be an important means of analyzing the composition and diversity of the bacterial community in samples from participants with cystic fibrosis.³ Studies have shown that species not previously reported in CF lung infection have been identified using TRFLP.⁴²

Multilocus sequence typing (MLST) is another technique in molecular biology for characterizing isolates of bacterial and fungal species using the DNA sequences of internal fragments of multiple housekeeping genes. MLST helps increase our knowledge of the genetic variation that occurs within a species. This can provide data for epidemiological surveillance and be used to inform public health policies and develop new treatments and vaccines.⁴³

As part of this study we will collect samples to be able to look at the larger microbial community present in the sputum samples from CF participants. This will allow us to compare with standard culture methods and assess the effect of nitric oxide on the whole community of bacteria. Such information will be crucial to helping inform the design of a phase 3 trial in which a wider group of participants with CF would be included.

3. Trial objective and purpose

3.1 Primary objective

The primary objective of this pilot study is to test the hypothesis that adjunctive low dose nitric oxide concurrently with standard intravenous antibiotic therapy will lead to enhanced antibiotic efficacy due to the disruption and killing of *Pseudomonas aeruginosa* (PA) biofilms. This will be determined by colony forming unit assessment and direct microscopic visualisation of biofilms from sputum, in combination with bacterial live/dead staining, *in-situ* molecular detection of PA, and quantitative computer-based image analysis.

3.2 Secondary objectives

3.2.1 Laboratory

1. To estimate the effect of adjunctive low dose inhaled nitric oxide given with standard antibiotic therapy on the whole community of bacteria within the CF lung by determination of CFU counts on non-selective agar.
2. To estimate the NO levels in sputum in each group.
3. To determine the characteristics in the wider microbial community within the CF lung during an exacerbation and to compare these characteristics between the two groups.
4. To determine the levels of nitric oxide metabolites in the blood and to compare these values between individuals and the two groups.

3.2.2 Clinical

1. To assess the effect of NO on lung function measured by FEV1
2. To assess the effect of low dose inhaled nitric oxide on exhaled nitric oxide levels
3. To assess the effect of low dose inhaled nitric oxide on HRQOL – CFQ-UK?
4. To determine the appropriate clinical endpoints for a trial of nitric oxide in participants with CF undergoing IV antibiotic therapy for an exacerbation?

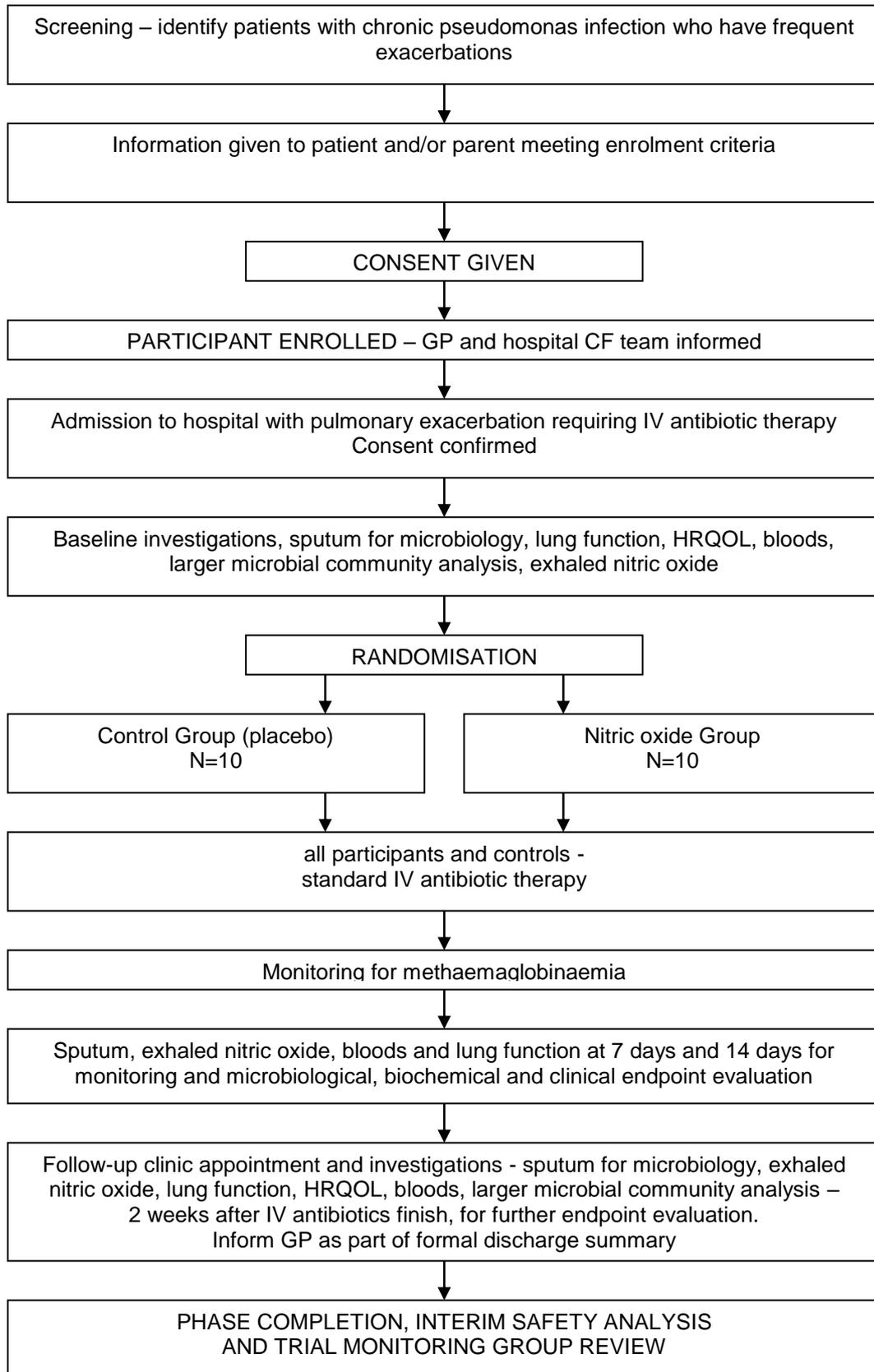
The clinical and laboratory data gathered from this study will be used to inform the design of a large phase 3 double blind randomised controlled trial (RCT). This study will provide basic limited safety data and an assessment of possible clinical and microbiological endpoints for use in the clinical trial.

4. Trial design

4.1 Summary of study design

This is a pilot study using a randomised, placebo controlled design (where participants and primary outcome assessors are blind to treatment group) for proof of concept. 10 CF participants in the treatment group and 10 in the placebo group will receive inhaled therapy (nitric oxide or placebo) alongside standard antibiotic treatment during a pulmonary exacerbation. Outcomes will be measured using a variety of methods including sputum samples, quality of life questionnaires and clinical parameters such as lung function.

RATNO (Reducing Antibiotic Tolerance using NO)



4.2 Detailed study design

This study is a phase 2 pilot study of a licensed drug (inhaled nitric oxide) for a new application. It is a participant blinded randomised controlled trial. The laboratory staff measuring the primary outcome measures and the participants will be blinded to therapy. For practical and cost reasons, the medical and nursing staff will not be blinded to therapy. Participants will be randomised to either the treatment or placebo group.

In order to be eligible to participate, CF patients will be screened for the ability to grow PA *ex vivo* from their sputum to form biofilms. Biofilm-forming PA is a criteria for entry to ensure maximum power for this pilot study of the effect of NO on PA biofilms.

This study is designed to compare 7 days of inhaled nitric oxide therapy with 7 days of placebo (in addition to standard IV antibiotic therapy for pulmonary exacerbation) for participants with CF.

The first 20 participants who have a pulmonary exacerbation will be randomised to one of the following groups:

Group 1: 7 days inhaled nitric oxide therapy, 8 hours continuous therapy through nasal cannula in every 24 hour period (using air or air/oxygen blend according to clinical need).

Group 2: 7 days inhaled placebo (air or air/oxygen blend according to clinical need), 8 hour continuous placebo through nasal cannula in every 24 hour period.

Both groups will receive standard IV antibiotic therapy of tobramycin and ceftazidime for 9-12 days.

A pulmonary exacerbation will be defined according to the criteria published by the 1994 cystic fibrosis foundation microbiology and infectious disease consensus⁴⁴ and used in recent large clinical trials.⁴⁵

The presence of 3 of the following 11 criteria:

- Increased cough
- Increased sputum production, change in appearance of expectorated sputum production, or both
- Fever (greater than or equal to 38°C for at least 4 hrs in a 24 hrs period) on more than one occasion in the previous week
- Weight loss greater than or equal to 1kg or 5% of bodyweight associated with anorexia and decreased dietary intake
- School or work absenteeism (due to illness) in the previous week
- Increased respiratory rate, increased work of breathing, or both
- New findings on chest examination (e.g. rales, wheezing, crackles)
- Decreased exercise tolerance
- Decrease in FEV₁ of greater than or equal to 10% from previous baseline study within the past 3 months

- Decrease in haemoglobin saturation (as measured by oximetry) from baseline value within past 3 months of greater than or equal to 10%
- New finding on chest radiograph

Samples will be collected from the participants over the course of the 10 day period of IV antibiotics as detailed on the study procedures flow sheet (see protocol section 4.2.1). Additionally, participants will be asked to attend a follow-up visit 10-15 days after completing their IV antibiotics for further samples and clinical evaluation.

4.2.1 Study procedures flow sheet

Time	Screening	Randomisation (baseline assessment) T 0	During NO therapy T1-5 (extra)	7 days (end of NO therapy) T 5-7	10 days (end of IV antibiotic therapy) T 9-12	Follow-up (10-15 days after end of IVs) T 20-25
Eligibility assessment and informed consent	x	Consent confirmed				
Sputum for microbiology	x	x		x	x	x
Clinical assessment	x	x		x	x	x
Bloods		x	x x		x	x
Lung function		x		x	x	x
Exhaled nitric oxide measurements		x	x	x	x	x
HRQOL		x				x
Sputum for storage		x		x	x	x
Randomisation		x				
NO levels in sputum		x	x	x		
Additional optional procedures (if consent given) see protocol section 7.1.8 for further details						
Nasal examination		x				
Nasal brushings			x			

Clinical assessment - including height & weight (screening, randomisation and follow-up only), clinical examination, concomitant medication and presence of adverse events.

Sputum collection – Whole sputum will be collected into a 2 sterile universal containers, labelled with the participants initials and study number and transported to the lab (see study guidance for sample handling for full details). In the unlikely event that participants are unable to produce sputum, the standard procedure for sputum induction (as documented in the study guidance for sputum induction) will be used.

Blood sampling – 10ml split between EDTA, Lithium heparin, plain and PAX gene tubes. Note: if insufficient blood is drawn the priority is the clinical samples, then PAX gene, then plain, then lithium heparin then EDTA. Blood samples at T0 and 1-2 and 7 hours after starting inhaled therapy on day 1 will be taken for nitric oxide metabolites. An additional 3.5 ml of blood will be taken into an EDTA or serum tube.

4.3 Study endpoints

4.3.1 Primary endpoint

Between group differences in proportion of bacteria in biofilms (as determined by colony forming unit assessment and live/dead staining, direct visualisation of the biofilm and image analysis) between T0 and T20-25.

4.3.2 Secondary endpoints

4.3.2.1 Laboratory

1. Between group differences in whole colony CFU's at T7, T10 and T20-25.
2. Between group differences in NO levels in fresh sputum at T7.
3. Between group differences in the characteristics of the wider microbial community as assessed by microbial community analysis/molecular analysis using methods such as respiratory panel PCR/16 RNA sequencing/metagenomics/MLST (measured at T7, T10 and T20-25).
4. Between individual and between group differences in the level of nitric oxide metabolites in the blood.

4.3.2.2 Clinical

1. Between group differences in FEV₁ at T7, T10 and T20-25.
2. Between group differences in exhaled NO levels at T7.
3. Between group differences in HRQOL scores at T20-25.
4. Between group differences in length of course of antibiotics.

4.3.3 Storage of samples for future microbiological analysis

All the microbiology samples and bacterial isolates will be anonymised, frozen and stored for future possible microbiological analyses, which may become pertinent to the study group as a result of advances in technology. Explicit consent will be obtained for the future possible use of these specimens in this way and all future studies will have to obtain ethical approval.

4.4 Laboratory investigations

4.4.1 Blood sampling

In order to investigate the inflammatory pathways and observe any possible side effects of nitric oxide therapy, blood samples will be obtained at time points T0, T10 and follow-up/T20-25 (as detailed in the study procedures table protocol section 4.2.1) Only a small sample of blood (10ml) will be required on 3 occasions, designed to coincide with times that participants would usually undergo blood sampling as part of routine clinical care. The third sample at follow-up is in addition to usual clinical samples, however, the majority of patients will have indwelling permanent central venous catheters so it will not involve venepuncture. These volumes of blood are minimal and will not result in any clinical harm. In addition a further 3.5 ml of blood will be taken at T0 and 1-2 and 7 hours after starting inhaled therapy on day 1 to test for nitric oxide metabolites in the blood. These additional blood tests will enable us to see how much nitric oxide is being absorbed systemically and give us an indication of the actual dose of IMP that is being absorbed by the CF lung.

4.4.2 Sputum samples

Sputum will be collected at time points as detailed in the study procedures table (see protocol section 4.2.1) It will be collected in a sterile universal container and labelled with the participants' unique study number and initials. The participant will be asked to provide 2 separate samples on each occasion to allow analysis of all the outcome measures. Transport of sputum samples between departments/buildings/labs will take place using appropriate secure/safe carriers according to WTCRF standard operating procedures for transport of microbiological specimens.

5. Selection and withdrawal of participants

5.1 Screening

Patients aged 12 or above with cystic fibrosis (known to the CF clinicians) who have known chronic *Pseudomonas* infection (50% or more of all cultures in the last 12 months positive for *Pseudomonas aeruginosa*⁴⁶), and regularly produce sputum, will be approached about the study. They will be identified using the Port CF registry database of patients within the adult and paediatric CF services. The initial contact will be by a member of their clinical team either in person (at clinic or on the ward), by letter, e-mail or phone call (see Appendix ... for letter to potential participants). They will be given information sheets and the study will be explained to them. They will be given time to read and consider the information before making a decision about whether to participate or not. If the patient and/or parent agree, they will be asked to sign a consent form (12-18 year olds will be asked to give written assent in addition to parental consent – for more details of the informed consent process see protocol section 12.3).

Once informed consent has been given, we will collect a sputum sample for confirmation of PA biofilm growth. If *Pseudomonas* cannot be isolated from the sample in the lab to grow biofilm, the participant will be informed that they cannot proceed any further with the study. If PA biofilm is grown from the sputum, the participant will be enrolled. The participants will receive a phone call from a member of the research team to inform them whether they have been enrolled into the study or not (as detailed in the participant information sheet). Once enrolled, the participant and the participant's CF physician will be asked to contact the research team if the participant presents with an acute exacerbation requiring IV antibiotic therapy (contact card provided to participant with 24 hour telephone contact number). At this stage, consent will be confirmed, and if the participant agrees, they will be randomised to receive either inhaled nitric oxide therapy or placebo in addition to standard antibiotic therapy.

A log of all potential participants will be kept (screening and enrolment log). All potential participants with CF who are considered eligible for the study, including those who do not decide to participate, or who are found unsuitable for the study, will be documented.

The following assessments will be performed during screening:

1. Confirmation (check with NHS laboratory) of cystic fibrosis diagnosis (sweat test +/- genotype confirmation of disease causing mutations)
2. Confirmation of *P. aeruginosa* infection
3. Fulfilment of inclusion/exclusion criteria
4. Obtain written informed consent

Potential participants who fulfil the screening requirements will be enrolled in the study and be eligible for randomisation.

As per Sponsor guidelines, participants who are enrolled in clinical trials at SUHT will have stickers placed on the front of their medical notes to highlight the fact that they

are enrolled in a clinical trial. Inside the notes will be details of the trial and who to contact if the participant is admitted to hospital for any reason.

5.2 Criteria for enrolment

Enrol: The act of assigning an individual to study procedures.

5.2.1 Inclusion Criteria

- Adolescents and young adults with cystic fibrosis aged 12 or above
- Colonised with *Pseudomonas aeruginosa* (confirmed on sputum sample)

5.2.2 Exclusion Criteria

- Colonisation 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 (NIV)
- Patients who have a pneumothorax
- Patients who are admitted for specific treatment of nontuberculous mycobacteria (NTM)
- Patients who cannot tolerate nasal cannula e.g. those who cannot breathe through their nose
- Patients who have nasal polyposis that is causing significant blockage of the nasal passages
- Adolescents who are 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 are pregnant (a pregnancy test will be carried out for females of 11 years and above as is standard practice for clinical trials)
- 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.

5.3 Randomisation and blinding

5.3.1 Criteria for randomisation

- Requiring antibiotic therapy for a pulmonary exacerbation:

A pulmonary exacerbation will be defined according to the criteria published by the 1994 cystic fibrosis foundation microbiology and infectious disease consensus⁴⁴ and used in recent large clinical trials.⁴⁵

The presence of 3 of the following 11 criteria:

- Increased cough
- Increased sputum production, change in appearance of expectorated sputum production, or both
- Fever (greater than or equal to 38°C for at least 4 hrs in a 24 hrs period) on more than one occasion in the previous week
- Weight loss greater than or equal to 1kg or 5% of bodyweight associated with anorexia and decreased dietary intake
- School or work absenteeism (due to illness) in the previous week
- Increased respiratory rate, increased work of breathing, or both
- New findings on chest examination (e.g. rales, wheezing, crackles)
- Decreased exercise tolerance
- Decrease in FEV₁ of greater than or equal to 10% from previous baseline study within the past 3 months
- Decrease in haemoglobin saturation (as measured by oximetry) from baseline value within past 3 months of greater than or equal to 10%
- New finding on chest radiograph

Once 20 participants have been randomised (and completed their follow-up) the study enrolment and randomisation will finish. Any participants who were enrolled, but did not exacerbate during the period of the study will be contacted by letter to inform them that the study has closed and their participation is completed (as detailed in the participant information sheet).

5.3.2 Randomisation

A computer generated random number list will be generated in a 1:1 ratio using simple block randomisation with random variable block length to ensure that the two groups are well balanced. The randomisation list will be run by BRU statistical department using a 24 hour web-based randomisation service for treatment allocation; this will ensure concealment of treatment allocation.

Emergency telephone randomisation service will be used should a temporary problem be experienced with the web based randomisation system.

5.3.3 Blinding

Laboratory staff carrying out microbiological endpoint evaluation (including primary endpoint) will be blinded as to which therapy the participant has received. Blinding of

medical/nursing staff would be uneconomical for this pilot study without additional scientific benefit but will be considered in a subsequent randomised controlled trial.

Participants will be blinded to therapy by the use of placebo (air or air/oxygen blend according to clinical need). The nitric oxide delivery system has a blinding screen for use in trials so that the participant will not know whether they are breathing nitric oxide or placebo. This is important as a number of the secondary outcome measures are clinical. Numerous studies have shown bias introduced when participants are not blinded. The primary endpoint for this study is microbiological (with laboratory staff blinded to sample treatment allocation), however, the clinical secondary endpoints collected will be used to power a larger phase 3 trial, therefore it is important that any differences are not over/under estimated due to bias from participants not being blinded to therapy.

5.4 Participant withdrawal

5.4.1 Withdrawal from treatment

Participants may be withdrawn from treatment for any of the following reasons:

1. Participant or parent withdraws consent.
2. Change in clinical condition preventing further treatment.
3. Unacceptable toxicity measured by metHb at 1 hour and after 8-10 hrs continuous therapy.
4. Any change in the participant's condition that justifies the discontinuation of treatment in the clinician's opinion.

If a participant wants to withdraw from the trial treatment but not sample follow-up then further microbiological analysis will be carried out and analysed as a separate group. General follow-up will continue unless the participant explicitly also withdraws consent for follow-up (see section 5.4.2).

If there was a participant who could not complete all 7 days of inhaled therapy (for example they were well enough to be discharged after 5 days and wanted to go home) but they had completed a minimum of 5 days, we would carry out the T7 assessment at that point (T5-7) and include their data in the analysis of the results.

5.4.2 Withdrawal from trial completely

Participants are free to withdraw consent at any time without having to provide a reason. Participants who withdraw completely from the trial will have anonymised data collected up to the point of withdrawal included in the analyses. Analysis will be carried out on an intention to treat basis. The participant will then not contribute any further data to the study.

6. Treatment of participants

6.1 Introduction

This study is designed as a single site, randomised participant-blind pilot study comparing 7 days of inhaled nitric oxide therapy with 7 days of placebo (in addition to standard IV antibiotic therapy for pulmonary exacerbation) for participants with CF.

The first 20 participants who have a pulmonary exacerbation will be randomised to one of the following groups:

Group 1: 7 days inhaled nitric oxide therapy, 8 hours continuous therapy through nasal cannula overnight in every 24 hour period for 7 days.

Group 2: 7 days inhaled placebo (air or air/oxygen blend), 8 hour continuous placebo (air/oxygen) through nasal cannula in every 24 hour period for 7 days.

Both groups will receive standard IV antibiotic therapy of tobramycin and ceftazidime for 10 days.

6.2 Dosage and administration

Standard medical gas cylinders will be used with no additional clinical trial labelling.

6.2.1 Nitric oxide

Finished product (Brand) name	Inomax 400ppm mol/mol inhalation gas
Active ingredients	Nitric Oxide (NO) 400ppm mol/mol
Excipients	Nitrogen
Nature and contents of cylinder	10 litre aluminium gas cylinder (identification with aquamarine shoulder and white body) filled under a pressure of 155 bar, equipped with a stainless steel positive pressure (residual) valve with a specific outlet connection)
Route of Administration	Inhalation gas
Manufacturer's name	INO Therapeutics AB SE-181 81 Lidingo Sweden
Storage temperature/time	Shelf life 2 years All regulations concerning handling of pressure vessels must be followed. Store gas cylinders indoors in well-ventilated rooms. The gas cylinder should be put in an

equipped site with appropriate material in order to hold the gas cylinder vertically

Supplier's name

INO Therapeutics UK
Kent Science Park
Sittingbourne
Kent
ME9 8PX

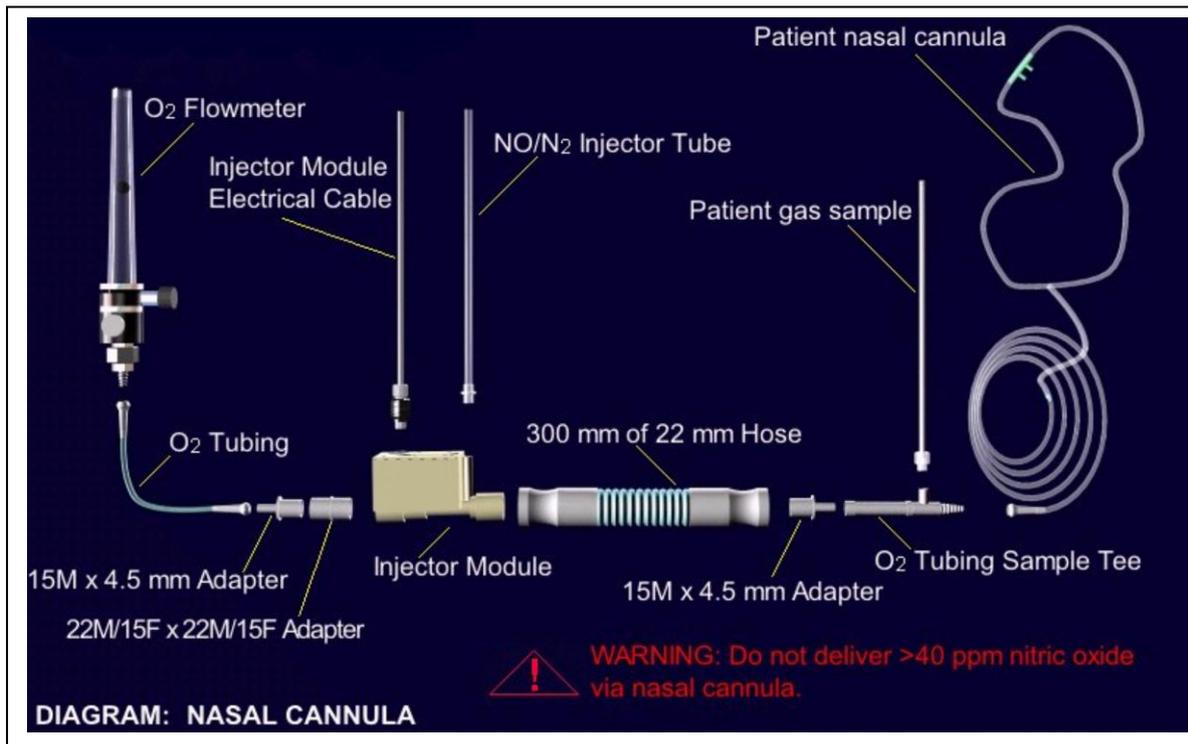
6.2.1.1 Labelling of IMP

Nitric Oxide gas canisters will be labelled by the study team as for clinical trial use in accordance with Part 7 of The Medicines for Human Use (Clinical Trials) Regulations 2004, LABELLING OF INVESTIGATIONAL MEDICINAL PRODUCTS, and therefore in accordance with Article 15 of European Commission Directive 2003/94/EC.

The study label will read as follows:

For Clinical Trials Use Only
RATNO – Reducing Antibiotic Tolerance using NO
Nitric Oxide gas for inhalation
Use as directed by protocol
EudraCT 2010-023529-39
Investigator *Dr Gary Connett/Dr Thomas Daniels (delete as appropriate)*
Patient Name.....
Date of supply.....
Southampton University Hospital NHS Trust

6.2.1.2 Preparation, dosage and administration



Nitric oxide will be given at a dose of 10ppm.

Nitric Oxide is delivered to the participant via nasal cannula after dilution with an oxygen/air mixture using an approved (CE marked) Nitric Oxide delivery system. The inspired INOmax concentration will be measured continuously in the inspiratory limb of the circuit near the participant. The nitrogen dioxide (NO₂) concentration and inspired oxygen (FiO₂) concentration will also be measured at the same site using calibrated and approved (CE marked) monitoring equipment. For participant safety, appropriate alarms will be set for INOmax (\pm 2ppm of the prescribed dose), NO₂ (1ppm) and FiO₂ (\pm 0.05). The INOmax gas cylinder pressure will be displayed allowing timely gas cylinder replacement without inadvertent loss of therapy. Backup gas cylinders will be available to provide timely replacement.

All staff involved in administering NO to participants will receive training in administration from a company representative. This will cover correct set-up and connections, operation and monitoring.

Nitric oxide will be given via nasal cannula. The NO will be added to air or air/oxygen mix (selected on the air/O₂ blender) and run at approximately 4L/min. This allows adequate mixing of NO before delivery to the participant.

Once connected to the NO, participants will receive 8 hours of uninterrupted flow. If a participant needs to be removed from the NO at any stage then the weaning protocol must be implemented (see study guidance: weaning of NO).

NO must be weaned before stopping, as there is a theoretical risk of increased pulmonary hypertension and reduced oxygenation when the NO is stopped. This

data is based on neonates with significant pulmonary hypertension and poor oxygenation receiving doses around 20 ppm. The weaning protocol is documented in the 'weaning of NO therapy' study guidance.

Participants will be monitored closely during the administration of inhaled therapy as documented in the 'monitoring during NO administration' study guidance. Levels of methHb will also be measured according to the 'monitoring during NO administration' study guidance.

Participants in the placebo group will have "sham" adjustments made to the NO/placebo delivery device at time points equivalent to weaning doses.

6.2.1.3 Accountability procedures for study treatment

Use of NO or placebo will be recorded on medical gas cylinder accountability records completed by trial staff.

6.2.1.4 Assessment of compliance

All trial treatment will be administered during hospital inpatient visits which will ensure accurate monitoring of IMP compliance.

6.2.1.5 Precautions required

All female participants will have a pregnancy test before randomisation. If they are found to be pregnant they will not be able to continue with the study. Reporting of pregnancy will be carried out according to protocol procedures outlined in section 8.4.

There is no adequate data from the use of nitric oxide in pregnant women. The potential risk for humans is unknown. Passive exposure to nitric oxide in humans during pregnancy and lactation should be avoided. Therefore, pregnant and breast-feeding staff will not look after participants receiving nitric oxide during the period of NO delivery to the participant. They can however look after participants at all other times during the study.

The upper limit of exposure (mean exposure) to nitric oxide for personnel defined by worker's legislation is 25ppm for 8 hours (30mg/m³) and the corresponding limit for NO₂ is 2-3ppm (4-6mg/m³).

6.2.2 Placebo

Participants randomised to the placebo arm of the trial will receive air or air/oxygen blend according to clinical need (determined by oxygen saturation monitoring as per standard clinical practice). This will be administered through nasal cannula in the same manner as the nitric oxide so that participants will not know whether they are receiving the trial treatment or placebo, including sham weaning procedures as defined in the 'weaning protocol for NO' study guidance. The use of a placebo is felt important in this study to minimize bias. There is no additional risk to participants receiving the placebo.⁴⁷

6.2.3 Non investigational medicinal product

6.2.3.1 Tobramycin

For the purposes of this study tobramycin is being used in line with normal clinical care, and as such will be used in the trial as a Non Investigational Medicinal Product (NIMP) in accordance with EU directive 2001/20/EC Article 13 and 14.

Tobramycin will be used at a dose of 7.5mg/kg once a day (as a 30min infusion). This treatment will be given for 10 days.

Tobramycin should be dispensed from normal pharmacy stock in accordance with local practice. The majority of treatment will be administered during hospital visits which will ensure accurate monitoring of NIMP compliance. For the few adult participants who usually administer their own IV antibiotics at home, they will be allowed to continue with this (after the 7 days of inhaled therapy have ended) and will be asked to complete a diary of dose and time administered and return empty vials at each visit so as to ensure compliance with the NIMP.

Tobramycin levels will be monitored according to standard clinical practice (as documented in the SOP for NIMP antibiotic therapy).

6.2.3.2 Ceftazidime

For the purposes of this study Ceftazidime is being used in line with normal clinical care, and as such will be used in the trial as a Non Investigational Medicinal Product (NIMP) in accordance with EU directive 2001/20/EC Article 13 and 14.

Ceftazidime will be used at a dose of 2g three times a day (for adults) and 50mg/kg three times a day (for children - maximum 2g). This treatment will be given for 10 days. The participants who would usually be at home will receive 2 doses in hospital and administer one dose themselves at home.

Ceftazidime should be dispensed from normal pharmacy stock in accordance with local practice. The majority of treatment will be administered during hospital visits which will ensure accurate monitoring of NIMP compliance. For the few adult participants who usually administer their own IV antibiotics at home, they will be allowed to continue with this and will be asked to complete a diary of dose and time administered and return empty vials at each visit so as to ensure compliance with the NIMP.

6.2.3.3 Clinically driven changes to concomitant antibiotic therapy

Ceftazidime and tobramycin will be given for 9-12 days as standard IV antibiotic therapy to all participants. As per standard clinical practice, this will be reviewed by the participant's consultant on a daily basis. If the CF clinician in charge of the participants' clinical care feels the participant is not improving, they can adjust the antibiotics. This will be recorded on the CRF and in the participant's medical records.

6.2.3.4 Accountability procedures for study non investigational medicinal product

Study staff will keep formal records of NIMP antibiotics used during the course of the study.

6.2.3.5 Assessment of adherence with study non investigational medicinal product

The majority of treatment will be administered during hospital visits which will ensure accurate monitoring of NIMP adherence. For the few adult participants who usually administer their own IV antibiotics at home, they will be allowed to continue with this and will be asked to complete a diary of dose and time administered and return empty vials at each visit so as to ensure compliance with the NIMP.

6.3 Ordering of investigational medicinal product

The investigational product for this study will be provided by INOtherapeutics UK. SUHT local procedures for ordering via pharmacy will be followed.

6.4 Packaging and labelling of study drug

No formal label will be applied to the IMP (medical gas) as detailed in the CTA application to the MHRA.

6.5 Dose modifications

The decision to interrupt or discontinue trial treatment is at the discretion of the treating clinician. Therapy may be interrupted or discontinued at any time during the trial period for reasons such as unacceptable adverse events, intercurrent illness, development of a serious disease or any change in the participants' condition that the clinician believes warrants a change in medication. Any changes in medication must be documented in the CRF along with justification for those changes.

CF physicians will be free to change the participant's antibiotics at any stage if they feel that a change is needed based on the participant's clinical condition. This will not affect treatment with the IMP.

6.6 Other concomitant medications

Nebulised antibiotics will be continued while participants are receiving IV antibiotics (as is standard clinical practice). These will all be recorded in the CRF.

All other treatments (including fluids) will be given at the discretion of the lead clinician in accordance with standard inpatient management of cystic fibrosis. The name and dose of all concomitant medications should be documented on the CRF throughout the study.

6.7 Physiotherapy

All participants will undergo twice daily physiotherapy regimes while on IV antibiotics. This is standard procedure for CF participants, especially during a pulmonary exacerbation.

6.8 Co-enrolment guidelines

Participants should not be recruited into other trials at the same time as this study. Potential participants who have participated in a trial testing a medicinal product likely to influence *P. aeruginosa* isolation (such as oral quinolones or intravenous or nebulised beta-lactams or aminoglycosides) within the 3 months prior to enrolment will be ineligible for this study. Where recruitment into another trial is considered to be appropriate and will not have a detrimental effect on this study this must be discussed and agreed with the Chief Investigator (Saul Faust).

7. Assessment of efficacy

7.1 Trial schedule

For full details of protocol procedure please see study procedures table – protocol section 4.2.1.

7.1.1 Recruitment strategy

Patients aged 12 or above with cystic fibrosis (known to the CF clinicians) who have known chronic PA infection (50% or more of all cultures in the last 12 months positive for *Pseudomonas aeruginosa*⁴⁶), and regularly produce sputum, will be approached about the study. They will be identified using the Port CF registry database of patients within the adult and paediatric CF services. The initial contact will be by a member of their clinical team either in person (at clinic or on the ward), by letter, e-mail or phone call (see Appendix ... for letter to potential participants). They will be given information sheets and the study will be explained to them. They will be given the opportunity to ask questions and consider all the information. Patients will ideally be given at least 24 hours to consider the information before they are asked to give consent to take part in the study. Due to the nature of clinical medicine, it may be possible that a patient has less than 24 hours to consider the information, if waiting for that period of time would jeopardise their clinical care and their option of taking part in the study.

If the patient and/or parent agree, they will be asked to sign a consent form (12-17 year olds will be asked to give written assent in addition to parental consent – for more details of the informed consent process see protocol section 12.3).

7.1.2 Diagnosis of pseudomonas

Before a participant is recruited into the study confirmation of chronic pseudomonas colonisation will be needed (50% or more of all cultures in the last 12 months positive for *Pseudomonas aeruginosa*⁴⁶). After they have given informed consent to participate, they will provide a sputum sample that will be used to confirm growth of mucoid PA that forms biofilms. Biofilm-forming PA is a criteria for entry to ensure maximum power for this pilot study of the effect of NO on PA biofilms. Participants will receive a phone call (as detailed on the participant information sheet) to inform them of whether they have been enrolled into the study (as detailed in the participant information sheet).

7.1.3 Baseline assessment/Randomisation

When a participant has been enrolled in the study they will then be asked to contact their CF clinician when they feel they are experiencing symptoms of a pulmonary exacerbation. They may also be identified in routine CF clinic appointment as having a pulmonary exacerbation requiring IV antibiotic therapy. At this point the CF clinician will inform the research team that the participant has an exacerbation and they will be randomised.

They will have the full baseline assessment (also detailed on the study procedures table, see protocol section 7.1):

1. Sputum for microbiology
2. Clinical assessment – height, weight, clinical examination
3. Lung function
4. Bloods – FBC, U+E, CRP, LFT, NO metabolites
5. Exhaled nitric oxide levels
6. HRQOL
7. NO levels in sputum

7.1.4 Planned assessments whilst receiving IV antibiotic therapy for a pulmonary exacerbation

During the treatment course of IV antibiotics and treatment with study drug or placebo, the participant will undergo assessment at 5-7 and 9-12 days (T5-7 and T9-12). This assessment will include:

1. Sputum for microbiology
2. Clinical assessment – clinical examination
3. Lung function
4. Bloods – FBC, U+E, CRP, LFT (T10 only)
5. Exhaled nitric oxide levels (T7 only)
6. NO levels in sputum (T7 only)

In addition participants will have Tobramycin levels checked after the second dose along with renal function monitoring (as is standard clinical practice).

MetHb levels will be checked 1 hour after starting NO therapy on the first night and before stopping NO therapy the next morning (see study guidance for monitoring during inhaled therapy for full details). If levels are not raised (above 5%) then there is no further need for monitoring during the trial. In addition, at these 2 time points blood will be taken for NO metabolite levels.

7.1.5 Planned assessments during study visit (follow-up)

10-15 days after completing their course of IV antibiotics the participant will return for a follow-up visit (T20-25). At this visit they will undergo assessment of the following:

1. Sputum for microbiology
2. Clinical assessment – height, weight, clinical examination
3. Lung function
4. Exhaled nitric oxide levels
5. HRQOL

7.1.6 Unscheduled assessments

If a participant is re-admitted to hospital or re-started on IV antibiotics during the 15 days following the completion of IV antibiotic therapy (before their scheduled follow-up visit) they will undergo a further assessment of the following:

1. Sputum for microbiology
2. Clinical assessment – clinical examination
3. Lung function
4. Bloods – FBC, U+E, CRP

The CF clinician responsible for their care will be asked to inform the research team if this occurs. Once the follow-up visit has been completed the participant will have completed the study and no further assessments will be necessary.

7.1.7 Assessments for participants who are withdrawn

If a participant withdraws from the study before they have experienced a pulmonary exacerbation and been randomised they will not require any assessment. However, if a participant withdraws from the study after randomisation they will undergo a withdrawal assessment which will include the following (if the participant agrees):

1. Sputum for microbiology
2. Clinical assessment – clinical examination
3. Lung function
4. HRQOL

In any case, reasons for withdrawing will be documented. Participants who have already been randomised and who may have received the study drug will be asked if they will consider attending the follow-up visit. Participants will be included in the analysis on an intention to treat basis.

7.1.8 Optional additional assessments

There are a number of additional assessments and samples that could provide useful information for other ongoing research studies at the University of Southampton and within the Respiratory Biomedical Research Unit. However, consent to these additional procedures is not necessary for enrolment. There may however be some participants who would like to give these additional samples or undergo additional assessments. All participants will be given the option of consenting to these additional procedures/samples. It will be made completely clear that they are optional 'extras'.

These assessments/procedures are as follows:

1. Nasal examination by ENT surgeon (Including flexible nasendoscopic examination if deemed necessary) – It is reasonable to consider nasal examination in those with a significant history of nasal blockage, symptoms suggestive of chronic rhino sinusitis (nasal blockage, facial pain, chronic post nasal drip and loss of sense of smell) and those who have had previous sinonasal surgery. This is in order to exclude significant blockage by nasal polyps which may affect delivery of the inhaled treatment to the lungs. Participants may benefit if nasal polyps are discovered as appropriate treatment will be instituted by the ENT team.
2. Nasal brushing to examine muco-ciliary clearance – Nasal brushings will be performed by a nurse or doctor trained in the technique. This procedure does not require sedation or anaesthetic, and is a common quick outpatient procedure. It takes a few seconds and is uncomfortable but well tolerated. A nasal brushing, using a cytology brush (Olympus) will be taken from the middle third of the inferior turbinate. The cytology brush will be advanced into the nose to the mid-point of the turbinate and then quickly agitated along the surface of the turbinate, moving forwards and backwards for about 1 cm. Samples will be transferred into suitable tissue culture medium at room

temperature and transported to the laboratory immediately. The procedure will be undertaken before and after treatment with iNO/placebo.

7.2 Procedures for assessing efficacy

7.2.1 Proportion of *Pseudomonas aeruginosa* in biofilm

The primary outcome measure will be the difference in proportion and viability of PA in biofilms between T0 and T20-25, as determined by colony forming unit assessment and direct microscopic visualisation of biofilms in combination with bacterial live/dead staining, *in-situ* molecular detection of PA, and quantitative computer-based image analysis.

7.3 Other assessments

7.3.1 *Pseudomonas* colony forming unit (CFUs)

This is a microbiological endpoint and will be analysed by laboratory researchers using standardised procedures. CFUs will be measured at randomisation (before treatment), 7 days, 10 days and follow-up (T0, T7, T10 and T20-25).

7.3.2 Quality of life

This will be assessed using the Cystic Fibrosis Questionnaire (CFQ-UK). This is a disease specific, developmentally appropriate, quality of life (QOL) questionnaire designed to measure the physical, emotional and social impact of CF on participants and their families. There is a growing recognition that the measurement of health-related QOL provides unique information about the impact of an illness and the effectiveness of current and newly developed treatments. This is particularly true of chronic illnesses like CF, where an important goal for treatment is to improve daily functioning and participant well-being. Although more conventional measures of efficacy (such as lung function) are important, they do not fully gauge the broader impact of the disease on the participant's physical, social and psychological functioning. There are different versions of this questionnaire for different ages, for children aged 12-13 and for teen/adults (14 years or older). Both versions are completed by the participant with no input from parents/family. They are scored according to the CFQ-UK scoring system.⁴⁸ We will measure HRQOL before and after treatment (T0 and T20-25). The questions in the questionnaire refer to the previous 2 weeks, so subjects will be asked to complete the questionnaire when treatment for a pulmonary exacerbation is initiated (at randomisation) and at the follow-up visit. This will allow comparison of scores before and after treatment between groups (see Appendix for full versions of the CFQ-UK that will be used in this study).

7.3.3 Lung function

FEV₁ is a well established predictor of survival and the most widely used surrogate outcome measure for CF lung disease. This will be very important as a secondary outcome measure as any differences between the 2 groups will be used for statistical power calculations to estimate sample size in the large phase 3 RCT. It is unlikely that in this pilot study (with a small sample size) we will see any significant

differences in FEV₁ between the 2 groups. Lung function will be measured before start of treatment (randomisation), at 7 and 10 days and at follow-up (T0, T7, T10 and T20-25). During routine treatment of CF participants with pulmonary exacerbations, lung function is measured on admission and completion of IV antibiotics (as a marker of success of treatment). We will carry out 2 additional measurements of lung function as part of the study, at 7 days and at follow-up.

7.3.4 Pulmonary exacerbations

Data will be collected regarding length of antibiotic treatment. Most participants will receive 10 days IV antibiotics, as per standard procedure, however, if their CF clinician feels at the end of 10 days they need further treatment, they can authorise this. This will be recorded as total number of days of IV antibiotic treatment.

7.3.5 Microbial community CFU counts on non-selective agar

Sputum samples will be analysed in the laboratory for microbial community CFU counts of non-selective agar. This is in addition to the *pseudomonas* CFUs. Although the majority of the background data regarding the use of nitric oxide to disrupt bacteria from biofilms is based on PA isolates, there is some preliminary data that suggests the effect is similar on the whole population of bacteria within the CF lung. By measuring whole colony CFUs we hope to show a change in bacterial density overall. This will help inform the design of a larger trial, in particular the inclusion criteria and whether participants who do not have chronic PA infection would also benefit from nitric oxide therapy. Microbial community CFU counts will be measured before the start of treatment (randomisation), at 7 days, 10 days and at follow-up (T0, T7, T10 and T20-25).

7.3.6 Exhaled nitric oxide levels

Participants with CF are known to have reduced exhaled nitric oxide levels.^{17 18 49 50} There are no studies to date that show whether giving inhaled nitric oxide can increase exhaled nitric oxide levels. The aim of administering low dose inhaled Nitric Oxide in this study is increase airway NO levels. By measuring exhaled Nitric Oxide levels in participants during the study we can get an idea of the NO levels within the CF lung and whether administration of NO to participants actually does result in an increase in airway NO levels. Exhaled NO levels will be measured at T0 and T7 (at the same time as NO levels in sputum (see protocol section 7.3.7).

7.3.7 Nitric oxide levels in sputum

Nitric Oxide levels in sputum will be measured using a Nitric Oxide micro sensor. This will be done in fresh sputum close to the bedside, immediately following production of sputum by the participant, using a new technique not previously used in clinical samples. We hope that by measuring NO levels in fresh sputum we can show that we have increased the NO levels where the biofilms are found. NO levels in sputum will be measured in a sputum sample provided by the participant during the time they are receiving the inhaled therapy (This will be done at T0 and T7 and one additional sample between T1-5).

7.3.8 Nitric oxide metabolites in blood

NO metabolites in blood will be measured at T0 and 1-2 and 7 hours after starting inhaled therapy on day 1. These 3 additional blood tests will enable us to see how

much nitric oxide is being absorbed systemically and give us an indication of the actual dose of IMP that is being absorbed by the CF lung.

7.3.9 Health economics

Despite low manufacture costs, nitric oxide gas is currently very expensive to use in clinical practice. Pilot data gathered in this study will inform the cost-benefit analysis of a future large RCT. It may also be possible that other NO donors become available for use in a future study.

7.4 Loss to follow-up

If any of the study participants are lost to follow up, contact will initially be attempted through the PI or designated research staff for the study. Follow up will also be attempted by the participant's usual CF clinician (if different from the research team). If these attempts are unsuccessful, the participants GP will be asked to contact the participant and/or family. Wherever possible, information about the reason for loss to follow up will be recorded.

8. Assessment of safety

Investigators are responsible for monitoring the safety of participants who have entered this study, and for alerting the co-investigators and local ethics committee about any event that seems unusual, whether this event is considered an unanticipated benefit or harm to the participant. The investigator is responsible for the appropriate medical care of study participants during the study in connection with protocol procedures.

8.1 Terms and definitions

The Medicines for Human Use (Clinical Trials Regulations 2004 (SI 2004/1031)) definitions:

Adverse Event (AE)

Any untoward medical occurrence in a participant to whom a medicinal product has been administered, including occurrences which are not necessarily caused by or related to that product.

Adverse Reaction (AR)

Any untoward and unintended response in a participant to an investigational medicinal product which is related to any dose administered to that participant.

Unexpected Adverse Reaction

An Adverse Reaction the nature and severity of which is not consistent with the applicable product information.

Serious Adverse Event (SAE), Serious Adverse Reaction (SAR) or Suspected Unexpected Serious Adverse Reaction (SUSAR)

Any Adverse Event, Adverse Reaction or Unexpected Adverse Reaction, that:

- Results in death
- Is life threatening (participant is at immediate risk of death)*
- Requires inpatient hospitalisation or prolongation of existing hospitalisation**
- Results in persistent or significant disability or incapacity
- Consists of a congenital anomaly or birth defect
- Other***

* 'life-threatening' in the definition of 'serious' refers to an event in which the participant was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.

** Hospitalisation is defined as an inpatient admission, regardless of length of stay, even if the hospitalisation is a precautionary measure for continued observation. Hospitalisations for a pre-existing condition, including elective procedures that have not worsened, do not constitute an SAE.

*** Other important medical events that may not result in death, be life-threatening, or require hospitalisation may be considered a serious adverse event when, based upon appropriate medical judgement, they may jeopardise the participant and may

require medical or surgical intervention to prevent one of the outcomes listed in this definition.

8.2 Adverse Events

Adverse events will be reported following randomisation.

8.2.1 Adverse Event Reporting specific to this trial

During the period between enrolment and randomisation (pulmonary exacerbation) adverse events will not be reported. This is because the participant will not have received any treatment at this stage and the only protocol procedures that will have been carried out are screening, informed consent and providing one sputum sample.

8.2.2 Adverse events include

- An exacerbation of a pre-existing illness.
- An increase in frequency or intensity of a pre-existing episodic event/condition.
- A condition (even though it may have been present prior to the start of the trial) detected after study drug administration.
- Continuous persistent disease or symptoms present at baseline that worsen following the administration of the study drug.
- Laboratory abnormalities that require clinical intervention or further investigation (unless they are associated with an already reported clinical event).
- Abnormalities in physiological testing or physical examination that require further investigation or clinical intervention.
- Injury or accidents.

Specifically in the case of participants with CF enrolled in this study, Adverse Events will include:

- Re-hospitalisation for CF related lung problems.

8.2.3 Adverse events do not include

- Medical or surgical procedures – the condition which leads to the procedure is the adverse event.
- Pre-existing disease or conditions present before treatment that do not worsen.
- Any elective surgery which was planned before the participant was enrolled into the study.
- The disease being treated or associated symptoms/signs unless more severe than expected for the participant's condition.

8.2.4 Severity/grading of Adverse Events

The assessment of intensity (severity) will be based on the investigator's clinical judgement using the following definitions:

Mild: an event that is easily tolerated by the participant, causing minimal discomfort and not interfering with everyday activities.

Moderate: an event that interferes with normal everyday activities.

Severe: an event that prevents normal everyday activities.

A distinction is drawn between serious and severe AE's. Severity is a measure of intensity (see above) whereas seriousness is defined using the criteria in section 8.1 and is based on the outcome or action criteria.

8.3 Causality

The assignment of causality should be made by the investigator responsible for the care of the participant using the definitions below:

Unrelated: There is no evidence of any causal relationship. N.B. An alternative cause for the AE should be given.

Unlikely: There is little evidence to suggest there is a causal relationship (e.g. the event did not occur within a reasonable time after administration of the trial medication). There is another reasonable explanation for the event (e.g. the participant's clinical condition, other concomitant treatment).

Possibly*: There is some evidence to suggest a causal relationship (e.g. because the event occurs within a reasonable time after administration of the trial medication). However, the influence of other factors may have contributed to the event (e.g. the participant's clinical condition, other concomitant treatments).

Probably*: There is evidence to suggest a causal relationship and the influence of other factors is unlikely.

Almost certainly*: There is clear evidence to suggest a causal relationship and other possible contributing factors can be ruled out.

If any doubt about the causality exists the investigator should inform the Chief Investigator. In the case of discrepant views on causality the MHRA will be informed of both points of view.

* Where an event is assessed as possibly, probably or almost certainly related, the event is an Adverse Reaction.

8.4 Expectedness

The expectedness of an Adverse Reaction shall be determined according to the SPC for nitric oxide.

- **Expected:** reaction previously identified and described in the protocol and/or SPC.
- **Unexpected:** reaction not previously documented in the protocol or SPC.

All events judged by the investigator to be possibly, probably or almost certainly related to the IMP, graded as serious and unexpected (see section 8.1 and the SPC for Nitric Oxide – Appendix) should be reported as a SUSAR.

8.5 Follow-up after Adverse Events

After a study participant's completion or discontinuation from the study, the investigator remains responsible to follow, through an appropriate health care option, adverse events that are serious or that caused the participant to discontinue before completing the study. All adverse events should be followed up until there is satisfactory resolution or until the investigator responsible for the care of the participant makes a decision that no further follow-up is needed. Frequency of follow-up is left to the discretion of the investigator.

When reporting SAEs and SUSARs the investigator responsible for the care of the participant should apply the following criteria to provide information relating to event outcomes:

- Resolved
- Resolved with sequelae (specify in additional notes)
- Not resolved/ongoing
- Ongoing at final follow-up
- Fatal
- Unknown

8.6 Reporting Procedures

Adverse Events will be reported in accordance with the SUHT Research Related Adverse Event Reporting Policy.

All Adverse Events fulfilling the reporting criteria should be recorded on the CRF and submitted to the Sponsor within the defined timelines. This begins from the time that written informed consent is obtained (i.e. prior to undergoing any study related procedure and/or receiving any investigational medicinal product) and continuing until the final follow-up visit. Participants will receive a phone call from a member of the research team 28 days after their last dose of IMP to check for any Adverse Events that have not otherwise been recorded. Depending on the nature of the event the reporting procedures listed in sections 8.6.1 and 8.6.2 should be followed.

If a participant experiences an Adverse Event after informed consent has been received (entry) but they have not experienced a pulmonary exacerbation (i.e. randomisation has not taken place), the event will not be reported unless the investigator feels that the event may have been caused by a protocol procedure.

8.6.1 Adverse Reactions/Adverse Events

All such events, whether expected or not, should be recorded on an Adverse Event form which should be submitted to the sponsor within 7 days. They will also be recorded in the CRF.

8.6.2 Serious ARs/AEs/SUSARs

SARs, SAEs and SUSARs should be reported within 24 hours of the investigator becoming aware of the event. The SAE form asks for the nature of the event, date of onset, severity, corrective therapies given, outcome and causality. The responsible investigator should assign the causality of the event. Additional information should

be sent within 5 days if the reaction has not resolved at the time of reporting. The sponsor will notify the MHRA and REC of all 'fatal' or life-threatening' SUSARs occurring during the study within 7 days after the sponsor became aware of the event and within 15 days where the SUSAR was not assessed as 'life-threatening' or 'fatal'. The sponsor will report any additional relevant information to the MHRA and REC within 8 days of the last report.

8.6.3 Other expedited safety reports

The European Commission guidance recommends that expedited reports on the following occurrences should also be sent to the MHRA and the REC according to the same timelines as SUSARs:

- single case reports of an expected serious adverse reaction with an unexpected outcome (e.g. death).
- an increase in the rate of occurrence of an expected serious adverse reaction, which is judged to be clinically important.
- post-study SUSARs that occur after the participant has completed a trial.
- a new event, related to the conduct of the trial or the development of nitric oxide that is likely to affect the safety of participants, such as:
 - a serious adverse event which could be associated with the trial procedures and which could modify the conduct of the trial.
 - a significant hazard to the participant population.

8.7 Safety Monitoring

The trial management group (see protocol section 10.1.1) will monitor safety throughout the course of the study. All deaths and serious adverse event reports will be reviewed by the trial safety committee (see protocol section 10.1.3) during the study period to ensure completeness and accuracy.

8.8 Periodic Safety Reporting

The Chief Investigator will provide the MHRA, REC and sponsor with an annual safety report on participants, including any SUSARs occurring during this time period. Annual safety reports should contain analysis of the participants' safety in the study with an appraisal of its on-going risk: benefit.

8.9 Reporting of pregnancy

Any pregnancy which occurs during the study should be reported as an SAE to the Sponsor within 24 hours of the site becoming aware of its occurrence and the participant should stop taking study drugs. All pregnancies that occur during treatment need to be followed up until after the outcome using an SAE form. Consent to report information regarding these pregnancy outcomes should be obtained from the mother prior to completing and faxing of the SAE form.

The investigator should contact the participant to discuss the risks of continuing with the pregnancy and possible effect to the foetus. Appropriate obstetric care should be arranged.

9. Statistical consideration

9.1 Method of randomisation

A computer generated random number list will be generated in a 1:1 ratio using simple block randomisation with random variable block length to ensure that the two groups are well balanced. The randomisation list will be run by BRU statistical department using a 24 hour web-based randomisation service for treatment allocation; this will ensure concealment of treatment allocation.

Emergency telephone randomisation service will be used should a temporary problem be experienced with the web based randomisation system.

9.2 Outcome measures

The primary outcome measure is proportion of bacteria in biofilms. Secondary outcome measures include microbiological outcomes such as pseudomonal CFU's and clinical parameters such as FEV₁ and Health Related Quality of Life scores (CFQ-UK). Any differences in these clinical outcome measures will be used to power a subsequent large randomised controlled trial.

9.3 Sample size

This is a pilot study and it is feasible to recruit 20 participants during one year. The primary aim of this study is to gain evidence that NO will reduce the proportion of bacteria in biofilms (with regards to reduction in surface area and reduction in average colony size) in participants treated with NO.

In order to demonstrate that the treatment with NO is better than the control we have calculated the probability of observing the correct ordering of the proportion of bacteria in biofilms for each group, for a given sample size and effect size (estimated taking into account the results observed from the laboratory experiments).⁵¹

It is estimated that the proportion of bacteria in biofilms with regards to surface area in the patients treated with will be 0.7 for placebo and 0.4 for patients treated with NO. A sample size of 10 participants in each treatment group will be sufficient to determine that the NO treatment arm is superior to the control group (by reducing the proportion of bacteria) with 90% probability assuming a change from 0.7 to 0.4.⁵¹

It is recognised that this study will have limited ability to detect important but rare treatment-related adverse events which will be identified in a larger RCT.

9.4 Analysis

The primary analysis will be an intention-to-treat analysis. All randomised participants will be included, this includes participants who do not receive the assigned treatment or who withdraw.

The change in proportion of bacteria in biofilms between baseline and follow-up (T20-25) will be calculated for each treatment group, and the mean difference of this change between the groups will be estimated with 95% confidence intervals. This analysis will be repeated for day 7 and 10.

The analysis of secondary outcomes comparing the two treatment groups will consist of estimating the difference in proportions and means (where appropriate) with 95% confidence intervals. Hypothesis tests will not be performed.

The use of appropriate data transformations (such as log transformation) will be decided upon for each endpoint individually. If appropriate multiple imputation methods will be used for missing data values and a sensitivity analysis with complete case information will be performed.

10. Quality control and quality assurance

This study will be conducted in accordance with The Medicine for Human Use (Clinical Trial) Amendment Regulations 2006 and subsequent amendments; the International Conference for Harmonisation of Good Clinical Practice (ICH GCP) guidelines and the Research Governance Framework for Health and Social Care.

The study will be monitored and audited in accordance with Southampton University Hospitals NHS Trust procedures. All trial related documents will be made available on request for monitoring and audit by SUHT, the Research Ethics Committee and for inspection by the MHRA or other licensing bodies.

Quality assurance includes all the planned and systematic actions established to ensure the trial is performed and data generated, documented/recorded and reported in compliance with applicable regulatory requirements. Quality control includes the operational techniques and activities done within the quality assurance system to verify that the requirements for quality of trial-related activities are fulfilled e.g. state what clinical site monitoring and audit is planned, if any.

This section should indicate the plans for quality control:

- Data will be evaluated for compliance with protocol and accuracy in relation to source documents
- The study will be conducted in accordance with procedures identified in the protocol
- The types of materials to be reviewed, who is responsible, and the schedule for reviews may be specified or referenced in other documents.
- Types and mechanism of training of staff for the study should be specified.

10.1 Trial Committees

10.1.1 Trial Management Group

The TMG will be responsible for the day-to-day running and management of the trial and will meet approximately every 4 months.

The Trial Management Group (TMG) will comprise:

Dr Saul Faust
Dr Gary Connett
Dr Katrina Cathie
Dr Thomas Daniels
Dr Jeremy Webb

This team will provide appropriate training for the clinicians and nurses involved in the study. Parents, guardians and the clinicians will have the right to withdraw the child from the study.

10.1.2 Trial Steering Committee (TSC)

The role of the Trial Steering Committee (TSC) is to provide overall supervision for the trial and provide advice. The TSC will comprise of all named investigators, plus additional members of the trial safety committee (see protocol section 10.1.3).

10.1.3 Trial Safety Committee

The Trial Safety Committee will comprise of independent respiratory and CF experts along with the trial statistician, chief investigator and principal investigator (Dr Victoria Cornelius, Dr Saul Faust, Dr Gary Connett, Dr Julia Nightingale and Dr Hazel Evans). In the event of a high number of SAEs or any death, the trial safety committee will provide a recommendation to the Trial Steering Committee concerning the continuation of the study.

10.2 Audit

To ensure that the trial is conducted according to ICH GCP guidelines, audits will be carried out on a random basis. The investigator will be required to demonstrate knowledge of the trial protocol and procedures and Good Clinical Practice. The accessibility of the site file to trial staff and its contents will be checked to ensure all trial records are being properly maintained. Adherence to requirements for consent will be examined. In the event of non-compliance the Trial Steering Committee will address the specific issues to ensure that relevant training and instruction is given.

10.3 Termination of the study

The study will be terminated when the last participant has completed follow-up, unless there are significant safety concerns that necessitate the early termination of the study. If the trial is terminated early then the procedures detailed in protocol section 11.4.2 must be followed.

11. Trial Contacts, Study Sites, Form Information and Regulatory Reports

11.1 Trial Contacts

Following a reportable AE or SAE, investigators must inform the lead study nurse and Chief Investigator immediately or within 24 hours of being made aware of the event. It will then be the responsibility of the Chief Investigator to inform the Research and Development Department at Southampton University Hospital NHS Trust.

Lead study nurses:

Ms Sandy Pink, Senior Research Nurse
Southampton Centre for Biomedical Research (Respiratory BRU),
Southampton University Hospitals NHS Trust,
Southampton SO16 6YD.
Tel: 023 8079 4989.

Chief Investigator:

Dr Saul Faust, Senior Lecturer in Paediatric Immunology and Infectious Diseases,
Director of WTCRF
Wellcome Trust Clinical Research Facility
C Level, West Wing, Mailpoint 218,
Southampton University Hospitals NHS Trust,
Tremona Road,
Southampton, SO16 6YD
Tel: + 44 (0) 23 8079 4989
Email: s.faust@southampton.ac.uk

MHRA, Pharmacovigilance Unit: 020 7084 2000 (Weekday 9am-5pm)
020 7210 3000 (all other times)

Ethics Committee: Southampton Research Ethics Committee A

11.2 Study Sites

Single site pilot study – Southampton Centre for Biomedical Research (Respiratory BRU), Southampton University Hospitals NHS Trust.

11.3 Forms

All reporting will be done on forms available from the Sponsor's website at:
<http://www.suht.nhs.uk/Research/Safetyandgovernance/Safetyreporting.aspx>

11.4 Reports

11.4.1 Annual Progress Reports and End of Study Reports

Ethics Committee

NHS research ethics committees are required to monitor research with a favourable opinion. A progress report should be submitted to the REC 12 months after the date on which the favourable opinion on which the form was given using the form available at:

<http://www.nres.npsa.nhs.uk/applications/after-ethical-review/annual-progress-reports/>

Annual progress reports should be submitted thereafter until the end of the study, when the following form should be used, which should also be submitted to the MHRA no later than 12 months after the end of the study:

http://ec.europa.eu/health/files/eudralex/vol-10/declaration_end_trial_form.doc

An overview of all safety reporting responsibilities can be found at:

<http://www.mhra.gov.uk/Howweregulate/Medicines/Licensingofmedicines/Clinicaltrials/Safetyreporting-SUSARsandASRs/index.htm>

MHRA

An annual report is required to be sent to the MHRA 12 months after the CTA is granted, then annually until the end of the study. All SAEs should also be reported. At the end of the study the sponsor is responsible for notifying the MHRA that the trial has ended. This notification should be sent by the sponsor within 90 days of the trial conclusion. An end of trial notification form is available from the EudraCT website. Reports should be sent to: Clinical Trials Unit, MHRA, 10-2 Market Towers, 1 Nine Elms Lane, London SW8 5NQ

The end of the trial is defined as when the last participant recruited has completed their scheduled involvement in the trial (after their follow-up visit, 10-15 days following last dose of IMP).

11.4.2 Early termination

If the trial is terminated before the specified date of its conclusion then the investigators should notify the Southampton R&D Office immediately so that Southampton University Hospitals NHS Trust, as sponsor, can notify the MHRA and ethics committee within 15 days of the trial termination.

12. Ethics

12.1 Ethical considerations

12.1.2 The use of minors in a Controlled trial of an Investigational Medicinal Product (CTIMP)

The inclusion of children aged 12 or over in this study has been considered and felt essential by the research team. For lung diseases that start in childhood, there may be windows of opportunity in which intervention with novel therapeutic options may reduce or prevent progression to chronic lung infection or inflammation that might otherwise occur. Chronic *Pseudomonas* infection in the lungs of participants with CF becomes more problematic the older the participant. If the use of nitric oxide is found to be beneficial to participants it may become a key new therapy for children to try and prevent the lung damage caused by chronic *Pseudomonas* infection.

12.1.2 Confidentiality

The study requires trained researchers not directly involved with the participants' medical care to have access to confidential information. It is necessary to gain consent from the participant and/or their parents (if they are <18 years) to link anonymised clinical information with the laboratory data generated. This will be done by using study numbers to identify specimens and clinical data.

Please see protocol section 13 for full details.

12.1.3 Risk to participants

As this is a trial of an Investigational Medicinal Product (IMP) there are some unknown risks to the participants. This new use is outside the current licence for nitric oxide gas and therefore the exact risks are unknown. However, at the low doses we propose to use, we do not anticipate any significant side effects or risk to participants. We will be monitoring very closely to identify any potential problems, side effects or additional risk to participants. Please see protocol section 8 for full details of safety monitoring and reporting during the trial.

12.2 Ethical approval

This study will be performed participant to Research Ethics Committee (REC) approval and local Research and Development (R&D) approval.

12.3 Informed consent process

Informed consent is a process initiated prior to an individual agreeing to participate in a study and continues throughout that individual's participation. Informed consent is a requirement for all participants participating in a CTIMP. During the informed consent process, the investigator or delegate will comply with all the applicable regulatory requirements and adhere to Good Clinical Practice (GCP) and to ethical principles that have their origin in the Declaration of Helsinki.

Patients and/or parents will have opportunity to discuss the objectives, risks and inconveniences of the study. Staff with experience in obtaining informed consent will explain to potential participants the details of how the study is to be conducted. Age appropriate information sheets will be provided to participants and/or their parents. These will describe in detail the study interventions, procedures and risks. Once the individual has had time to read the document there will be opportunity for the investigator (or delegate) to explain the study to the individual. At all times staff will emphasise that participation in the study is completely voluntary and that the participant may withdraw from the study at any time and for any reason.

All potential participants will be given the opportunity to ask questions and will have time (ideally 24 hours or longer) to consider the information they have received and discuss it with their family prior to consent being given. All participants will be provided with the appropriate contact details for further information about the trial.

If, after these discussions, the patient and/or parent is happy to take part, they will be asked to sign and date the informed consent document. This will be signed and dated by both the participant and the person receiving consent. The participant and/or parent will be given a copy of the consent form to keep for their own records. The original will be filed in the participant's clinical notes and another copy will be kept in the Investigator Site File.

12.3.1 Assent in minors

All children aged 12-17 will be asked to give their assent to the study in addition to parental consent. They will be approached by a member of the research team with experience in paediatrics and communicating with children. They will receive age-appropriate information regarding the study, procedures and risks, and will have the opportunity to have any questions answered. If they agree to take part, the child will then be asked to write/sign their name on the consent form, which is then to be signed by their parent and the researcher. We have decided to only enrol children into this study 12 years and above so that they can fully understand the study themselves and give informed assent to take part.

12.4 Study discontinuation

In the event that the study is discontinued, participants will be treated according to usual standard clinical care.

13. Data handling and record keeping

13.1 Confidentiality

Individual participant medical information obtained as a result of this study is considered confidential and disclosure to third parties is prohibited with the exception of the participant's medical team and all appropriate personnel responsible for the participant's welfare. Case report forms will be labelled with the participant's initials and unique trial screening and/or randomisation number.

Participants will be identified by their trial number to ensure confidentiality. All electronic and personal data will be stored in accordance with the Data Protection Act 1998.

13.2 Direct access to source data/documents

Source data: All information in original records and certified copies of original records of clinical findings, observations or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in source documents (original records or certified copies).

Source documents: Original documents, data and records (e.g. hospital records, clinical and office charts, laboratory notes, memoranda, pharmacy dispensing records, recorded data from automated instruments, records kept at the laboratories).

In order to perform their role effectively, monitors and persons involved in quality assurance and inspection will need direct access to primary participant data e.g. participant records, laboratory reports, appointment books etc. Because this affects the participant's confidentiality this fact is included on the participant information sheet and consent form.

13.3 Records retention

All essential documents including source documents will be retained for a minimum period of 15 years following the end of the study, A 'DO NOT DESTROY' label stating the time after which documents can be destroyed will be placed on the inside cover of the medical records of trial participants (as is standard practice in SUHT).

Study documents (paper and electronic) will be retained in a secure location during and after the trial has finished.

14. Financial and insurance matters

14.1 Funding

This project is funded as part of the core National Institute of Health Research Respiratory Biomedical Research Unit grant.

14.1.1 Participant payment

Participants and their parents will not be paid to participate in the study. According to European and UK Law, children are not allowed to receive any incentive for taking part in research. Adult participants will be given £150 for the added inconvenience of staying in hospital overnight for 7 nights. Payment will be sent following a participants completion of follow-up in the study along with a cover letter (see Appendix).

14.2 Indemnity

This is an NHS-sponsored research study, For NHS sponsored research HSG(96)48 reference number 2 refers. If there is negligent harm during the clinical trial when the NHS body owes a duty of care to the person harmed, NHS Indemnity covers NHS staff, medical academic staff with honorary contracts, and those conducting the trial. NHS Indemnity does not offer no-fault compensation and is unable to agree in advance to pay compensation for non-negligent harm.

15. Publication policy

Results from this study will be published in national and international, medical and scientific journals. Participant confidentiality will be maintained in any publication.

16. References

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17. Appendices

17.1 Letter to potential participants

Southampton
University Hospitals NHS Trust



UNIVERSITY OF
Southampton

Southampton Respiratory
Biomedical Research Unit

The RATNO Study

Reducing Antibiotic Tolerance using low dose Nitric Oxide in cystic fibrosis
A phase 2 pilot study

Dear

The new Respiratory National Institute for Health Research Biomedical Research Unit in Southampton has provided NHS clinical research resources for the CF clinical teams to work with University colleagues to investigate new treatments that might be of benefit to people with cystic fibrosis. We are writing to you as part of a group of researchers who are doing a study that might be of interest to you.

We are writing to you because you have Cystic Fibrosis and you fit the criteria for taking part in this study. If you want to know more, please read the enclosed information sheet which will give you lots of information about the study, what is involved if you decide to take part and what to do if you are interested. The research team would like to hear from you if this study is of interest to you or if you have further questions. Please contact them on 023 8079 4989. Alternatively we can explain a bit more about the study to you and ask the research team to speak to you if you are interested.

Thank you for taking the time to read this letter and we hope you will read the information enclosed about the study and consider taking part.

Yours Faithfully

Dr Gary Connett (on behalf of the Paediatric CF team)/Dr Thomas Daniels (on behalf of the adult CF team)

Research team contact details (RATNO study team)

Southampton Centre for Biomedical Research
Southampton University Hospitals NHS Trust
Mailpoint 218
Tremona Road
Southampton
SO16 6YD

Tel: 023 8079 4989

17.2 Letter to GP

Southampton 
University Hospitals NHS Trust

UNIVERSITY OF
Southampton

Southampton Respiratory
Biomedical Research Unit

The RATNO Study

Reducing Antibiotic Tolerance using low dose Nitric Oxide in cystic fibrosis
A phase 2 pilot study

Dear Dr

RE: Patient Name and Date of Birth

The lungs of most patients with cystic fibrosis become chronically infected with *pseudomonas aeruginosa* during childhood. This infection is now known to consist of free-living bacteria (planktonic) and bacteria in micro-colonies (biofilms). The bacteria in biofilms are more resistant to antibiotics and lead to chronic infection which causes damage to the lungs. This pilot study will discover whether nitric oxide administered to patients during an episode of acute infection (exacerbation) will disrupt bacteria from biofilms and increase the effectiveness of antibiotic therapy. This randomised pilot study will guide the design of a large multi-centre trial which will be crucial to evaluate the effectiveness of this potential new therapy.

Your patient has agreed to participate in the above clinical trial.

Participants will be randomised to receive either inhaled nitric oxide or placebo during a pulmonary exacerbation in addition to intravenous antibiotics and all other standard therapy. All study medication will be administered in hospital. As such your patient's participation in this trial should not impact on your own prescribing or that of your practice in any way.

If you have any questions please feel free to contact the study team at any time.

Yours sincerely

RATNO study team (Chief Investigator: Dr Saul Faust)

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Mailpoint 218
Tremona Road
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17.3 Information Sheets

The RATNO Study

Reducing Antibiotic Tolerance using low dose Nitric Oxide in cystic fibrosis
A phase 2 pilot study

INFORMATION SHEET FOR PARTICIPANTS Version 1.1

We would like to invite you to take part in a research study which aims to find out if a potential treatment for pseudomonal lung infection can benefit patients with Cystic Fibrosis. Before you decide we would like you to understand why the research is being done and what it would involve. One of our team will talk through the information sheet with you and answer any questions you have.

- Part 1 of this information sheet tells you the purpose of the study and what will happen if you take part.
- Part 2 gives you more detailed information about how the study will be organized.

Please take your time to read the information carefully and discuss it with your family or GP if you wish. If anything is not clear or if you would like more information, please ask us or telephone the study team on 023 8079 4989.

Part 1

What is the purpose of the study?

One of the main bacteria causing lung infection in CF is a bug called *Pseudomonas*. This causes a chronic (long term) lung infection. Whilst intravenous antibiotics can be used to treat this bug they are not completely effective and the infection is often a recurring problem. We have been researching new ways to treat *Pseudomonas* infection. To do this, we are studying a medicine called nitric oxide. Nitric oxide is currently used to treat blood vessel contraction in premature babies and in some lung diseases in adult and children's intensive care, but we have found in the laboratory that very low doses of nitric oxide can help kill *Pseudomonas* from patients with CF. Nitric oxide is given by inhalation and works by bringing the bacteria out of the mucous in which they are embedded so that they are easier for the antibiotics to kill. This small study has been designed to find out whether nitric oxide can help make our usual intravenous antibiotics (given during an episode of acute infection or exacerbation) more effective in killing *Pseudomonas* and improve the health of CF patients.

Why have you been chosen?

We have asked you to take part because you have CF and you also have *Pseudomonas* infection. We are asking adults and children aged 12 or above to take part. You may be well at the moment, but if you agree, when you have an exacerbation we will give you an additional inhaled treatment with your usual IV antibiotics.

Research study design

Sometimes we don't know which way of treating patients is best. To find out, we need to compare different treatments. We put people into groups and give each group a different treatment. The results are compared to see if one is better. To try to make sure the groups are the same to start with, each patient is put into a group by chance (randomly).

In this study half the patients will receive the study therapy (nitric oxide) and the other half will receive air (this is called a placebo because it is made to look the same as for the patients being given the study therapy). The study medicine is tasteless and it will not be possible for you to know what you will be receiving, although you will find out at the end of the study. Both groups will receive intravenous antibiotics as usual for an exacerbation and all other standard treatment.

During the trial period, if you become unwell with symptoms of a respiratory infection (exacerbation) and need intravenous antibiotics you will be randomly allocated to one of the 2 groups. Neither you nor the doctors or study team will be able to choose which group you will be in.

What is the drug that is being tested?

The drug that we will be studying is called "nitric oxide". This will be given by inhalation through nasal cannula (similar to giving oxygen). Although this is the first time that nitric oxide has been used specifically to treat infection, it has been widely used in other ways to treat illnesses in both preterm babies and children and adults with breathing difficulties on intensive care units.

What are the alternatives for diagnosis or treatment?

All standard treatment will be given to you at all times. This study is testing nitric oxide as an extra medicine to be given alongside all the standard treatments.

What are the possible disadvantages and risks of taking part?

When you are admitted with an exacerbation you will receive the inhaled therapy overnight for the first 7 nights of your course of antibiotics. You will be monitored carefully by research or ward nurses who will keep a very close eye on you. If you would usually give your own antibiotics at home you will need to stay in hospital for the first 7 nights of your course of antibiotics to receive the inhaled therapy. During the day you will be on the ward or allowed home as is your usual practice.

You will have some blood tests taken as part of the study, but these should be done at the same time as the ones they would have taken anyway for your exacerbation.

Although we do not anticipate any major risk to you, we are monitoring to detect any unexpected serious safety issues with the use of nitric oxide in people with CF (see "side effects" paragraph below).

What are the possible benefits of taking part?

We hope that this potential treatment will help clear infection from the lungs better than intravenous antibiotics given alone, so there is a small chance you may benefit from this.

However, only one course of this treatment will be given and so the actual benefit from this study is likely to be small. The information we get from this study will help us find out whether this potential treatment could be used for patients with CF in the future and reduce the amount they have to come into hospital.

What are the side effects of nitric oxide treatment?

Although side effects from nitric oxide at the low dose we are using are rare, they are possible. You will be monitored very closely while you are on the treatment to look out for any side effects. The most common are caused if the nitric oxide therapy is stopped too quickly, which can cause headaches and/or a reduction in blood pressure and oxygen levels in the blood. To avoid this we will slowly reduce the levels before stopping the therapy each morning and you will be monitored very closely for any of these potential problems. If troublesome side effects occur then you will be reviewed by a nurse or doctor involved in the study. We will record carefully anything we think may be related to the study treatment. You have the option of withdrawing from the study at any time without affecting your standard treatment. There is also a special study committee who will monitor the effects and if it is felt the treatment is causing problems the study will be stopped.

It is not known whether nitric oxide is harmful to unborn babies, therefore all female participants will have a pregnancy test before being allocated to a treatment group. If this test is positive, they will not be able to take part.

What will happen to me if I take part?

Initial assessment:

1. We will examine you (like you would have done at clinic) and take some details about the medications you are on.
2. We will ask you to cough up some sputum into a pot for us to test in the lab.

Exacerbations/acute respiratory infection:

We will ask you and your CF doctor to let us know when you have an exacerbation (increased cough/sputum/breathlessness/fever). In addition to the usual tests you would have if you came in for IV antibiotics (lung function, bloods, height and weight) you will be asked to do the following:

1. Give an extra sample of sputum and blood (taken at the same time as the routine samples).
2. Fill out a questionnaire about how your CF has been affecting you over the last 2 weeks.
3. If/when you have an exacerbation you will be allocated to one of 2 groups:
 - a. Nitric oxide
 - b. Placebo (air or air+oxygen – depending on your needs)

The treatment is breathed by the patient via nasal cannula. So if you take part you will have to wear nasal cannula overnight for the first 7 nights of your admission.

You will receive all the other standard care that you would usually have when you have an exacerbation including IV antibiotics, physiotherapy and monitoring on the ward.

Follow-up:

We would like to review all patients approximately 2 weeks after they finish their IV antibiotics. At this appointment we will:

1. Examine you (like you would have done at clinic) and take some details about the medications you are on.
2. Ask you to cough up some sputum into a pot for us to test in the lab.
3. Ask you to fill in a questionnaire about how your CF has been affecting you since you finished your intravenous antibiotics.
4. Do some lung function tests like you would usually do in clinic.

Not all patients who agree to take part will have an exacerbation while we are doing the study. If this is the case for you, we will write to you to let you know that the study has ended and you will continue to receive standard clinical care as before.

Additional optional procedures:

These procedures are optional and you do not have to consent to these to take part in the trial:

1. Nasal examination – We will suggest a nasal examination if you have a history or symptoms of nasal blockage or if you have had previous sinonasal surgery. This will allow us to exclude significant blockage which may affect delivery of the inhaled treatment to the lungs.
2. Nasal brushings – This is a quick procedure involving a small brush of the inside of the nose. It is slightly uncomfortable, but only lasts a few seconds. It allows us to look at the cells that help clear particles from the nose.

Do I have to take part?

It is up to you to decide whether or not you take part in this research. It is entirely voluntary. If you do not want to take part please be assured the care you receive will not be affected in any way. If you agree to take part in this study then you will be asked to sign a consent form. You are free to withdraw your consent at any time and without giving a reason. This will not affect the standard of care you receive.

What if there is a problem?

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

Who should I contact if I have any questions or concerns or if I want more information?

Please contact a member of the study team on 023 8079 4989, or you can discuss your concerns with your CF doctor.

If you want more general information about taking part in research the following websites may be helpful: <http://www.nres.npsa.nhs.uk/aboutus/protecting-participant-safety/> and <http://www.peopleinresearch.org/>

This completes part 1.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

Part 2

Will my taking part be kept confidential?

Yes, all information about you will be kept confidential. We will tell your GP that you have taken part in this study. All information about you will be coded with a unique study number. This allows us to link your results with your medical information, but keep the details confidential. Data collected during the study may be shared with our research colleagues in other hospitals (possibly abroad); however they will not know who the information belongs to, as your name and address will only be kept at Southampton University Hospitals NHS Trust. Only the research team will have access to your personal details. The sponsor of the study (Southampton University Hospitals NHS Trust) or regulatory authorities may also wish to access the records as part of their monitoring of ongoing research, but no identifiable information with leave Southampton University Hospitals NHS Trust. You will not be individually identified in any reports or publications resulting from the study.

What will happen to any samples I give?

The sputum samples will be analysed for the amount of *Pseudomonas* bacteria. The blood samples will look at markers of infection. Some of the sputum and blood samples will be stored securely at Southampton University Hospitals NHS Trust or the University of Southampton for later tests to be done in connection with this study or other CF or lung infection studies. It may be that researchers from this hospital or other groups in the UK or abroad ask us for small amounts of the stored samples to use in additional research on children and adults with cystic fibrosis. The samples will not be labelled with your name or address so that other researchers analysing them will not know who the sample belongs to. All future studies will be approved by a research ethics committee before being able to use the samples.

What if relevant new information becomes available?

Sometimes we get new information about the treatment being studied. If this happens, the doctors will tell you and discuss whether you should continue in the study. If you or your doctors decide you will not carry on, the standard clinical care will not be affected in any way. If the study is stopped for any other reason, we will tell you.

What will happen if I don't want to carry on with the study?

You can decide to withdraw from the study at any time and are not obliged to give any reasons. Whatever you decide, the standard of clinical care you receive will not be affected in any way. If you withdraw from treatment you can keep in contact with us to let us know about your progress. Information collected before withdrawal will still be used. Any stored blood and sputum will be destroyed if you wish.

What if there is a problem?

If you have a concern about any part of this study you should ask to speak to a member of the research team who will do their best to answer any of your questions (Tel 023 8079 4989). You can also discuss any concerns you have with your CF doctor.

If you still have questions or concerns you can contact Research and Development at Southampton University Hospitals NHS Trust. In the very unlikely event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for legal action for compensation against Southampton University Hospital NHS Trust but you may have to pay your legal costs.

Regardless of this, if you have concerns about any aspect of the way you have been approached or treated during the course of this study you may wish to contact the hospital's Patient Advice and Liaison Service (PALS) on 023 8079 8498, email PALS@suht.swest.nhs.uk or write to PALS, C Level, Centre Block, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD).

What will happen to the results of the research study?

All the results of the study will be made available by publication in medical journals. You will not be identified in any report/publication to do with this trial.

What happens when the research study stops?

If this study finds that nitric oxide has potentially beneficial effects for patients with cystic fibrosis, a much larger and definitive trial will be designed to determine whether this treatment should become standard for all patients with cystic fibrosis.

Who is organising and funding the research?

This study has been designed by a group of researchers from the National Institute of Health Research Respiratory Biomedical Research Unit at Southampton University Hospitals NHS Trust and the University of Southampton. All the researchers have been involved in trying to determine better treatments for patients with cystic fibrosis for many years. None of the doctors, nurses or researchers are being paid to include patients in this study. This project is being paid for by a grant from the National Institute for Health Research, the body that funds clinical research in the NHS.

Expenses and payments

Because of the additional inconvenience for a number of adult patients of staying in hospital overnight for the first 7 nights of their course of IV antibiotics, you will be given a gift of £150 (at the final visit) if you have an exacerbation during the study period and receive the inhaled therapy.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by Research Ethics Committee. Prior to submission to the ethics committee, the study was reviewed by doctors and scientists independent from our team. The study was also reviewed as part of the application to the National Institute of Health Research (NIHR) for the Southampton Respiratory Biomedical Research Unit.

How long do I have to decide whether I should take part?

Your decision to participate in this study is entirely voluntary. You should take as much time as you need.

Thank you for taking time to read this information sheet.

You will be given a copy of this information sheet to keep, together with a copy of the signed consent form if you decide that you can take part.

The RATNO Study

Reducing Antibiotic Tolerance using low dose Nitric Oxide in cystic fibrosis
A phase 2 pilot study

INFORMATION SHEET FOR PARENTS Version 1.1

We would like to invite your son/daughter to take part in a research study which aims to find out if a potential treatment for pseudomonal lung infection can benefit patients with Cystic Fibrosis. Before you decide we would like you to understand why the research is being done and what it would involve. One of our team will talk through the information sheet with you and answer any questions you have.

- Part 1 of this information sheet tells you the purpose of the study and what will happen if your son/daughter takes part.
- Part 2 gives you more detailed information about how the study will be organized.

Please take your time to read the information carefully and discuss it with your family or GP if you wish. If anything is not clear or if you would like more information, please ask us or telephone the study team on 023 8079 4989.

Part 1

What is the purpose of the study?

One of the main bacteria causing lung infection in CF is a bug called *Pseudomonas*. This causes a chronic (long term) lung infection. Whilst intravenous antibiotics can be used to treat this bug they are not completely effective and the infection is often a recurring problem. We have been researching new ways to treat *Pseudomonas* infection. To do this, we are studying a medicine called nitric oxide. Nitric oxide is currently used to treat blood vessel contraction in premature babies and in some lung diseases in adult and children's intensive care, but we have found in the laboratory that very low doses of nitric oxide can help kill *Pseudomonas* from patients with CF. Nitric oxide is given by inhalation and works by bringing the bacteria out of the mucous in which they are embedded so that they are easier for the antibiotics to kill. This small study has been designed to find out whether nitric oxide can help make our usual intravenous antibiotics (given during an episode of acute infection or exacerbation) more effective in killing *Pseudomonas* and improve the health of CF patients.

Why has your son/daughter been chosen?

We have asked your son/daughter to take part because they have CF and they also have *Pseudomonas* infection. We are only asking children aged 12 or above to take part. This is because we want them to be able to understand about the study and agree to take part.

Your son/daughter may be well at the moment, but if you agree, when they have an exacerbation we will give them an additional inhaled treatment with their usual IV antibiotics.

Research study design

Sometimes we don't know which way of treating patients is best. To find out, we need to compare different treatments. We put people into groups and give each group a different treatment. The results are compared to see if one is better. To try to make sure the groups are the same to start with, each patient is put into a group by chance (randomly).

In this study half the patients will receive the study therapy (nitric oxide) and the other half will receive air (this is called a placebo because it is made to look the same as for the patients being given the study therapy). The inhaled medicine is tasteless and it will not be possible for you or your son/daughter to know what they will be receiving, although you will find out at the end of the study. Both groups will receive intravenous antibiotics as usual for an exacerbation and all other standard treatment.

During the trial period, if your son/daughter becomes unwell with symptoms of a respiratory infection (exacerbation) and needs intravenous antibiotics he/she will be randomly allocated to one of the 2 groups. Neither you nor the doctors or study team will be able to choose which group they will be in.

What is the drug that is being tested?

The drug that we will be studying is called "nitric oxide". This will be given by inhalation through nasal cannula (similar to giving oxygen). Although this is the first time that nitric oxide has been used specifically to treat infection, it has been widely used in other ways to treat illnesses in both preterm babies and children and adults with breathing difficulties on intensive care units.

What are the alternatives for diagnosis or treatment?

All standard treatment will be given to your son/daughter at all times. This study is testing nitric oxide as an extra medicine to be given alongside all the standard treatments.

What are the possible disadvantages and risks of taking part?

When your son/daughter is admitted with an exacerbation they will receive the inhaled therapy overnight for the first 7 nights of your course of antibiotics, and they may have to sleep overnight in a special area in the hospital dedicated to research. They will be monitored carefully by research or ward nurses who will keep a very close eye on them. If you would usually stay with them you will still be able to do this. During the day they will be on the ward or allowed home as is your usual practice.

Your son/daughter will have some blood tests taken as part of the study, but these should be done at the same time as the ones they would have taken anyway for their exacerbation.

Although we do not anticipate any major risk to your son/daughter, we are monitoring to detect any unexpected serious safety issues with the use of nitric oxide in people with CF (see "side effects" paragraph below).

What are the possible benefits of taking part?

We hope that this potential treatment will help clear infection from the lungs better than intravenous antibiotics given alone, so there is a small chance your son/daughter may benefit from this. However, only one course of this treatment will be given and so the

actual benefit from this study is likely to be small. The information we get from this study will help us find out whether this treatment could be used for patients with CF in the future and reduce the amount they have to come into hospital.

What are the side effects of nitric oxide treatment?

Although side effects from nitric oxide at the low dose we are using are rare, they are possible. Your son/daughter will be monitored very closely while they are on the treatment to look out for any side effects. The most common are caused if the nitric oxide therapy is stopped too quickly, which can cause headaches and/or a reduction in blood pressure and oxygen levels in the blood. To avoid this we will slowly reduce the levels before stopping the therapy each morning and your son/daughter will be monitored very closely for any of these potential problems. If troublesome side effects occur then your son/daughter will be reviewed by a nurse or doctor involved in the study. We will record carefully anything we think may be related to the study treatment. You and your son/daughter have the option of withdrawing from the study at any time without affecting your standard treatment. There is also a special study committee who will monitor the effects and if it is felt the study treatment is causing problems the study will be stopped.

It is not known whether nitric oxide is harmful to unborn babies, therefore all female participants will have a pregnancy test before being allocated to a treatment group. If this test is positive, they will not be able to take part.

What will happen to my son/daughter if they take part?

Initial assessment:

3. We will examine your son/daughter (like they would have done at clinic) and take some details about the medications they are on.
4. We will ask them to cough up some sputum into a pot for us to test in the lab.

Exacerbations/acute respiratory infection:

We will ask you and your son/daughter's CF doctor to let us know when your son/daughter has an exacerbation (increased cough/sputum/breathlessness/fever). In addition to the usual tests they would have if they came in for IV antibiotics (lung function, bloods, height and weight) they will be asked to do the following:

4. Give an extra sample of sputum and blood (taken at the same time as the routine samples).
5. Fill out a questionnaire about how their CF has been affecting them over the last 2 weeks.
6. If/when your son/daughter has an exacerbation they will be allocated to one of 2 groups:
 - a. Nitric oxide
 - b. Placebo (air or air+oxygen – depending on their needs)

The treatment is breathed by the patient via nasal canula. So if your son/daughter takes part they will have to wear nasal canula overnight for the first 7 nights of their admission. Your son/daughter will receive all the other standard care that they would usually have when they have an exacerbation including IV antibiotics, physiotherapy and monitoring on the ward.

Follow-up:

We would like to review all patients approximately 2 weeks after they finish their IV antibiotics. At this appointment we will:

5. Examine your son/daughter (like they would have done at clinic) and take some details about the medications they are on.

6. Ask them to cough up some sputum into a pot for us to test in the lab.
7. Ask them to fill in a questionnaire about how their CF has been affecting them since they finished their intravenous antibiotics.
8. Do some lung function tests like they would usually do in clinic.

Not all children who agree to take part will have an exacerbation while we are doing the study. If this is the case for your son/daughter, we will write to you to let you know that the study has ended and they will continue to receive standard clinical care as before.

Additional optional procedures:

These procedures are optional and you do not have to consent to these to take part in the trial:

3. Nasal examination – We will suggest a nasal examination if your son/daughter has a history or symptoms of nasal blockage or if they have had previous sinonasal surgery. This will allow us to exclude significant blockage which may affect delivery of the inhaled treatment to the lungs.
4. Nasal brushings – This is a quick procedure involving a small brush of the inside of the nose. It is slightly uncomfortable, but only lasts a few seconds. It allows us to look at the cells that help clear particles from the nose.

Does my son/daughter have to take part?

It is up to you and your son/daughter to decide whether or not they take part in this research. It is entirely voluntary. If you do not want them to take part please be assured the care they receive will not be affected in any way. If you agree to your son/daughter taking part in this study then you will be asked to sign a consent form. They will also be asked to sign to say they agree. You and your son/daughter are free to withdraw your consent at any time and without giving a reason. This will not affect the standard of care they receive.

What if there is a problem?

Any complaint about the way your son/daughter has been dealt with during the study or any possible harm they might suffer will be addressed. The detailed information on this is given in Part 2.

Will my son/daughter taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

Who should I contact if I have any questions or concerns or if I want more information?

Please contact a member of the study team on 023 8079 4989, or you can discuss your concerns with your son/daughter's CF doctor.

If you want more general information about taking part in research the following websites may be helpful: <http://www.nres.npsa.nhs.uk/aboutus/protecting-participant-safety/> and <http://www.peopleinresearch.org/>

This completes part 1.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

Part 2

Will my son/daughter taking part be kept confidential?

Yes, all information about your son/daughter will be kept confidential. We will tell their GP that they have taken part in this study. All information about your son/daughter will be coded with a unique study number. This allows us to link their results with their medical information, but keep the details confidential. Data collected during the study may be shared with our research colleagues in other hospitals (possibly abroad); however they will not know who the information belongs to, as your son/daughter's name and address will only be kept at Southampton University Hospitals NHS Trust. Only the research team will have access to your son/daughter's personal details. The sponsor of the study (Southampton University Hospitals NHS Trust) or regulatory authorities may also wish to access the records as part of their monitoring of ongoing research, but no identifiable information will leave Southampton University Hospitals NHS Trust. Your son/daughter will not be individually identified in any reports or publications resulting from the study.

What will happen to any samples given by my son/daughter?

The sputum samples will be analysed for the amount of *Pseudomonas* bacteria. The blood samples will look at markers of infection. Some of the sputum and blood samples will be stored securely at Southampton University Hospitals NHS Trust or the University of Southampton for later tests to be done in connection with this study or other CF or lung infection studies. It may be that researchers from this hospital or other groups in the UK or abroad ask us for small amounts of the stored samples to use in additional research on children with cystic fibrosis. The samples will not be labelled with your son/daughter's name or address so that other researchers analysing them will not know who the sample belongs to. All future studies will be approved by a research ethics committee before being able to use the samples.

What if relevant new information becomes available?

Sometimes we get new information about the treatment being studied. If this happens, the doctors will tell you and discuss whether your son/daughter should continue in the study. If you or your doctors decide your son/daughter will not carry on, the standard clinical care will not be affected in any way. If the study is stopped for any other reason, we will tell you.

What will happen if I don't want my son/daughter to carry on with the study?

You can decide to withdraw your son/daughter from the study at any time and are not obliged to give any reasons. Whatever you decide, the standard of clinical care they receive will not be affected in any way. If your son/daughter withdraws from treatment you can keep in contact with us to let us know about their progress. Information collected before withdrawal will still be used. Any stored blood and sputum will be destroyed if you wish.

What is there is a problem?

If you have a concern about any part of this study you should ask to speak to a member of the research team who will do their best to answer any of your questions (Tel 023 8079 4989). You can also discuss any concerns you have with your son/daughter's CF doctor.

If you still have questions or concerns you can contact Research and Development at Southampton University Hospitals NHS Trust. In the very unlikely event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence

then you may have grounds for legal action for compensation against Southampton University Hospital NHS Trust but you may have to pay your legal costs.

Regardless of this, if you have concerns about any aspect of the way your son/daughter has been approached or treated during the course of this study you may wish to contact the hospital's Patient Advice and Liaison Service (PALS) on 023 8079 8498, email PALS@suht.swest.nhs.uk or write to PALS, C Level, Centre Block, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD).

What will happen to the results of the research study?

All the results of the study will be made available by publication in medical journals. Your son/daughter will not be identified in any report/publication to do with this trial.

What happens when the research study stops?

If this study finds that nitric oxide has potentially beneficial effects for patients with cystic fibrosis, a much larger and definitive trial will be designed to determine whether this treatment should become standard for all patients with cystic fibrosis.

Who is organising and funding the research?

This study has been designed by a group of researchers from the National Institute of Health Research Respiratory Biomedical Research Unit at Southampton University Hospitals NHS Trust and the University of Southampton. All the researchers have been involved in trying to determine better treatments for patients with cystic fibrosis for many years. None of the doctors, nurses or researchers are being paid to include patients in this study. This project is being paid for by a grant from the National Institute for Health Research, the body that funds clinical research in the NHS.

Expenses and payments

No extra expense payments will be made. According to European and UK Law, children are not allowed to receive any payment for taking part in research.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by Research Ethics Committee. Prior to submission to the ethics committee, the study was reviewed by doctors and scientists independent from our team. The study was also reviewed as part of the application to the National Institute of Health Research (NIHR) for the Southampton Respiratory Biomedical Research Unit.

How long do I have to decide whether my son/daughter should take part?

Your decision to participate in this study is entirely voluntary. You should take as much time as you need.

Thank you for taking time to read this information sheet.

You will be given a copy of this information sheet to keep, together with a copy of the signed consent form if you decide that your son/daughter can take part.

The RATNO Study

Reducing Antibiotic Tolerance using low dose Nitric Oxide in cystic fibrosis
A phase 2 pilot study

SIMPLIFIED INFORMATION SHEET FOR YOUNG PERSON AGED 12-18 YEARS **Version 1.1**

Why are we doing this research?

We are trying to find ways to help children and young people with cystic fibrosis (CF) like you. Many children and young people with CF get problems with their lungs. When your lungs are not working properly you may find it hard to breathe. This stops you being able to do everything you want to and you may have to come to hospital.

We are doing a study to see if giving you an extra treatment when you come into hospital for antibiotics will reduce the chance of you getting problems with your lungs.

What will happen if I take part?

If you and your parents agree to take part in the study, we will ask you to write your name on a form called a consent form, and if you are under 18 your parents or guardian will also sign this form. This is to say you understand the study and what will happen. You and your parents will keep a copy of the consent form and you can keep this information sheet.

What will happen to me during the study?

A lot of what we are doing in the study is exactly the same as what would usually happen to you when you come into hospital for IV antibiotics.

Doctors will examine you and ask you to cough up sputum into a pot a few times during your stay. When you have your blood tests done we will take an extra teaspoon of blood for the study. When you come into hospital for intravenous (IV) antibiotics you might stay overnight in a special area of the hospital where we do research. It is very much like the ward you usually stay on. Your mum or dad can stay there with you if you want. In the day you will be on your normal ward and everything else will be the same (including your treatment and physio). Overnight you will need to wear nasal cannula to get the extra treatment. Half of the patients in the study will get the study treatment and half will get just air/oxygen (called a placebo). You will not know what you are getting. This is important so we can compare the study treatment fairly.

We would like you to fill out a questionnaire about how your CF has been affecting you as part of the study. We would also like you to come in for a follow-up visit 2 weeks after your IV antibiotics so we can see how you are getting on.

Does I have to take part?

No, it is totally up to you and your parents whether you take part or not.

Will taking the medicine upset me?

We don't expect the study medication to upset you, but the nurses will keep a close eye on you to make sure you are ok. If you think you feel unwell or funny then you must tell the nurse.

Why is this form not as detailed as the one for my parents?

We know that young people want different amounts of information depending on how they are feeling, how old they are, what they have done so far in school and for many other reasons. We need to be able to make sure that you get the information you want, and that you can understand any information we give you.

You and your parents have also been given a more detailed information sheet that explains the study in more detail,

The doctors and nurses will be pleased to spend any time you want explaining the treatment or the study to you in more detail if you would like this.

Thank you for reading this – please let your doctor or nurse know if you would like more information.

**RATNO - Reducing Antibiotic Tolerance using low dose Nitric Oxide in cystic fibrosis – a phase 2 pilot study
Consent Form Version 1.0**

Ethics Study Number:

Participant Identification Number for this trial:

9. I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.
10. I understand that my son/daughter's participation is voluntary and that I am free to withdraw this participation at any time without giving any reason, without my son/daughter's medical care or legal rights being affected.
11. a. I agree to my son/daughter's anonymised data relevant to this trial being collected on case report forms and stored on electronic databases in accordance with the Data Protection Act 1998.
- b. I agree to this anonymised information being shared with other researchers in the UK or abroad.
12. a. I agree to blood and sputum samples taken from my son/daughter being stored anonymously for later testing relevant to cystic fibrosis and infection in the UK or abroad. I understand that I may not be given the results of these tests.
- b. I agree to anonymised samples being shared with other research groups in the UK or abroad.
13. I give permission for relevant sections of my son/daughter's medical notes and data collected during the study to be looked at by individuals from the study sponsor (Southampton University Hospital NHS Trust) and from regulatory authorities where it is relevant to my son/daughter taking part in this research.
14. I agree to the optional additional procedures:
- a. Nasal examination
- b. Nasal brushings
15. I agree to my GP being informed of my son/daughter's participation in the study.
16. I agree to my son/daughter taking part in the RATNO study.

Name of Parent/Guardian Date Signature

Name of Researcher taking consent Date Signature

I understand the information I have been given and I agree to taking part in this study:

Name of Patient Date Signature

When completed, 1 for parent/guardian; 1 for researcher site file; 1 (original) to be kept in medical notes.

17.5 End of Trial letter to participants

Southampton 
University Hospitals NHS Trust

UNIVERSITY OF
Southampton

Southampton Respiratory
Biomedical Research Unit

The RATNO Study

Reducing Antibiotic Tolerance using low dose Nitric Oxide in cystic fibrosis
A phase 2 pilot study

Dear

Thank you so much for taking part in the RATNO study. We have now completed the study and had 20 patients who have had an exacerbation and received the inhaled therapy. We do not need any more patients for this part of the study. From now, if you have an exacerbation you will receive standard care as usual.

We thank you for your time and commitment to this research. We are now in the stage of analysing the results to see if there was any positive effect from the nitric oxide. We will write to you to let you know the results when they are available. We may contact you again in the future if a larger study is planned to look into this treatment further, to ask if you would consider taking part.

Yours Faithfully

RATNO study team

Southampton Centre for Biomedical Research
Southampton University Hospitals NHS Trust
Mailpoint 218
Tremona Road
Southampton
SO16 6YD

Tel: 023 8079 4989

17.6 24 hour contact card for participants

Southampton **NHS** University Hospitals NHS Trust

UNIVERSITY OF Southampton

Southampton Respiratory Biomedical Research Unit

The RATNO Study

Reducing Antibiotic Tolerance using low dose Nitric Oxide in cystic fibrosis
A phase 2 pilot study

Southampton Centre for Biomedical Research
Southampton University Hospitals NHS Trust
Mailpoint 218
Tremona Road
Southampton
SO16 6YD

Tel: 023 8079 4989
24 hour mobile: XXXXX XXXXXX

17.7 Summary of Product Characteristics for Nitric Oxide

ANNEX I

SUMMARY OF PRODUCT CHARACTERISTICS

1. NAME OF THE MEDICINAL PRODUCT

INOmax 400 ppm mol/mol inhalation gas

2. QUALITATIVE AND QUANTITATIVE COMPOSITION

Nitric oxide (NO) 400 ppm mol/mol.

A 2 litre gas cylinder filled at 155 bar absolute brings 307 litres of gas under pressure of 1 bar at 15°C.

A 10 litre gas cylinder filled at 155 bar absolute brings 1535 litres of gas under pressure of 1 bar at 15°C.

For a full list of excipients, see section 6.1.

3. PHARMACEUTICAL FORM

Inhalation gas

4. CLINICAL PARTICULARS

4.1 Therapeutic indications

INOmax, in conjunction with ventilatory support and other appropriate agents, is indicated for the treatment of newborns \geq 34 weeks gestation with hypoxic respiratory failure associated with clinical or echocardiographic evidence of pulmonary hypertension, in order to improve oxygenation and to reduce the need for extracorporeal membrane oxygenation.

4.2 Posology and method of administration

Prescription of nitric oxide should be supervised by a physician experienced in neonatal intensive care. Prescription should be limited to those neonatal units that have received adequate training in the use of a nitric oxide delivery system. INOmax should only be delivered according to a neonatologist's prescription.

INOmax should be used in ventilated infants expected to require support >24 hours. INOmax should be used only after respiratory support has been optimised. This includes optimising tidal volume/pressures and lung recruitment (surfactant, high frequency ventilation, and positive end expiratory pressure).

Posology

INOmax should be used only after respiratory support is optimised, including the use of surfactant. The maximum recommended dose of INOmax is 20 ppm and this dose should not be exceeded. In the pivotal clinical trials, the starting dose was 20 ppm. Starting as soon as possible and within 4-24 hours of therapy, the dose should be weaned to 5 ppm provided that arterial oxygenation is adequate at this lower dose. Inhaled nitric oxide therapy should be maintained at 5 ppm until there is improvement in the neonate's oxygenation such that the FiO_2 (fraction of inspired oxygen) < 0.60 .

Treatment can be maintained up to 96 hours or until the underlying oxygen desaturation has resolved and the neonate is ready to be weaned from INOmax therapy. The duration of therapy is variable, but typically less than four days. In cases of failure to respond to inhaled nitric oxide, see section 4.4.

Weaning

Attempts to wean INOmax should be made after the ventilator support is substantially decreased or after 96 hours of therapy. When the decision is made to discontinue inhaled nitric oxide therapy, the dose should be reduced to 1 ppm for 30 minutes to one hour. If there is no change in oxygenation during administration of INOmax at 1 ppm, the FiO₂ should be increased by 10%, the INOmax is discontinued, and the neonates monitored closely for signs of hypoxaemia. If oxygenation falls >20%, INOmax therapy should be resumed at 5 ppm and discontinuation of INOmax therapy should be reconsidered after 12 to 24 hours. Infants who cannot be weaned off INOmax by 4 days should undergo careful diagnostic work-up for other diseases.

Method of Administration

Nitric oxide is delivered to the patient via mechanical ventilation after dilution with an oxygen/air mixture using an approved (CE-marked) nitric oxide delivery system.

The delivery system must provide a constant inhaled INOmax concentration irrespective of the ventilator. With a continuous flow neonatal ventilator, this may be achieved by infusing a low flow of INOmax into the inspiratory limb of the ventilator circuit. Intermittent flow neonatal ventilation may be associated with spikes in nitric oxide concentration. The nitric oxide delivery system for intermittent flow ventilation should be adequate to avoid spikes in nitric oxide concentration.

The inspired INOmax concentration must be measured continuously in the inspiratory limb of the circuit near the patient. The nitrogen dioxide (NO₂) concentration and FiO₂ must also be measured at the same site using calibrated and approved (CE-marked) monitoring equipment. For patient safety, appropriate alarms must be set for INOmax (± 2 ppm of the prescribed dose), NO₂ (1 ppm), and FiO₂ (± 0.05). The INOmax gas cylinder pressure must be displayed to allow timely gas cylinder replacement without inadvertent loss of therapy and backup gas cylinders must be available to provide timely replacement. INOmax therapy must be available for manual ventilation such as suctioning, patient transport, and resuscitation.

In the event of a system failure or a wall-outlet power failure, a backup battery power supply and reserve nitric oxide delivery system should be available. The power supply for the monitoring equipment should be independent of the delivery device function.

The upper limit of exposure (mean exposure) to nitric oxide for personnel defined by worker's legislation is 25 ppm for 8 hours (30 mg/m³) in most countries and the corresponding limit for NO₂ is 2-3 ppm (4-6 mg/m³).

Training in Administration

The key elements that need to be covered in training hospital personnel are as follows.

Correct set-up and connections

- Connections to the gas cylinder and to the ventilator patient breathing circuit

Operation

- Pre-use check list procedure (a series of steps required immediately prior to each patient initiation to ensure that the system is working properly and that the system is purged of NO₂)
- Setting the device for the correct concentration of nitric oxide to be administered
- Setting the NO, NO₂ and O₂ monitors for high and low alarm limits
- Using the manual backup delivery system
- Procedures for correctly switching gas cylinders and purging system
- Troubleshooting alarms
- NO, NO₂ and O₂ monitor calibration
- Monthly system performance check-up procedures

Monitoring formation of Methaemoglobin

Neonates are known to have diminished MetHb reductase activity compared to adults. Methaemoglobin level should be measured within one hour after initiation of INOmax therapy, using an analyser which can reliably distinguish between foetal haemoglobin and methaemoglobin. If it is > 2.5%, the INOmax dose should be decreased and the administration of reducing agents such as methylene blue may be considered. Although it is unusual for the methaemoglobin level to increase significantly if the first level is low, it is prudent to repeat methaemoglobin measurements every one to two days.

Monitoring formation of Nitrogen Dioxide

Immediately prior to each patient initiation, proper procedure must be applied to purge the system of NO₂. The NO₂ concentration should be maintained as low as possible and always < 0.5 ppm. If the NO₂ is > 0.5 ppm, the delivery system should be assessed for malfunction, the NO₂ analyser should be recalibrated, and the INOmax and/or FiO₂ should be reduced if possible. If there is an unexpected change in INOmax concentration, the delivery system should be assessed for malfunction and the analyser should be recalibrated.

4.3 Contraindications

Neonates known to be dependent on right-to-left, or significant left-to-right, shunting of blood.
Hypersensitivity to the active substance or any of the excipients.

4.4 Special warnings and precautions for use

If it is judged that clinical response is inadequate at 4-6 hours after starting INOmax, the following should be considered.

For patients who are to be referred to another hospital, to prevent worsening of their condition on acute discontinuation of INOmax, the availability of nitric oxide during transport should be assured. Rescue, such as ECMO where available, should be considered based on continued deterioration or failure to improve, defined by criteria based on local circumstances.

In clinical trials, no efficacy has been demonstrated with the use of inhaled nitric oxide in patients with Congenital Diaphragmatic Hernia.

Discontinuation of Therapy: The INOmax dose should not be discontinued abruptly as it may result in an increase in pulmonary artery pressure (PAP) and/or worsening of blood oxygenation (PaO₂). Deterioration in oxygenation and elevation in PAP may also occur in neonates with no apparent response to INOmax. Weaning from inhaled nitric oxide should be performed with caution. For patients transported to other facilities for additional treatment, who need to continue with inhaled nitric oxide, arrangements should be made to ensure the continuous supply of inhaled nitric oxide during transportation. The physician should have access at the bedside to a reserve nitric oxide delivery system.

A large portion of nitric oxide for inhalation is absorbed systemically. The end products of nitric oxide that enter the systemic circulation are predominantly methaemoglobin and nitrate. The concentrations of methaemoglobin in the blood should be monitored (see section 4.2).

NO₂ rapidly forms in gas mixtures containing nitric oxide and O₂, and nitric oxide may in this way cause airway inflammation and damage. The dose of nitric oxide should be reduced if the concentration of nitrogen dioxide exceeds 0.5 ppm.

Treatment with inhaled nitric oxide might aggravate cardiac insufficiency in a situation with left-to-right shunting. This is due to unwanted pulmonary vasodilation caused by inhaled nitric oxide, resulting in a further increase of already existing pulmonary hyperperfusion. It, therefore, is recommended that prior to the

administration of nitric oxide, pulmonary artery catheterisation or echocardiographic examination of central haemodynamics be performed.

Animal models have shown that nitric oxide may interact with haemostasis, resulting in an increased bleeding time. Data in adult humans are conflicting, and there has been no increase in bleeding complications in randomised controlled trials in term and near-term neonates with hypoxic respiratory failure. INOmax has not been shown to be of benefit in premature (< 34 weeks) infants.

4.5 Interaction with other medicinal products and other forms of interaction

No interaction studies have been performed. A clinically significant interaction with other medications used in the treatment of hypoxic respiratory failure cannot be excluded based on the available data. There may be an additive effect with INOmax on the risk of developing methaemoglobinemia with nitric oxide donor compounds, including sodium nitroprusside and nitroglycerin. INOmax has been safely administered with tolazoline, dopamine, dobutamine, steroids, surfactant, and high-frequency ventilation. Experimental studies have suggested that nitric oxide and also nitrogen dioxide may react chemically with surfactant and/or surfactant proteins.

There is an increased risk of methaemoglobin formation if substances with a known tendency to increase methaemoglobin concentrations are administered concomitantly with nitric oxide (e.g. alkyl nitrates and sulphonamides). Substances known to cause increased methaemoglobin levels should thus be used with caution during therapy with inhaled nitric oxide. Prilocaine, whether administered as oral, parenteral, or topical formulations may cause methaemoglobinaemia. Care must be taken when INOmax is given at the same time as medicines containing prilocaine.

In the presence of oxygen, nitric oxide is rapidly oxidised to derivatives which are toxic to the bronchial epithelium and alveolo-capillary membrane. nitrogen dioxide (NO₂) is the main compound formed, and during treatment with nitric oxide, the NO₂ concentration should be < 0.5 ppm in the nitric oxide dose range < 20 ppm. If at any time the NO₂ concentration exceeds 1 ppm, the nitric oxide dose should immediately be reduced. See section 4.2 for information on monitoring for NO₂.

4.6 Pregnancy and lactation

There are no adequate data from the use of nitric oxide in pregnant women. The potential risk for humans is unknown.

INOmax is not intended for adults. Passive exposure to nitric oxide in humans during pregnancy and lactation should be avoided.

4.7 Effects on ability to drive and use machines

Not relevant.

4.8 Undesirable effects

Formation of methaemoglobinaemia > 5% has been observed despite administration at appropriate concentrations. Neonates have diminished MetHb reductase activity and could therefore be at greater risk of developing methaemoglobinaemia.

Rapid rebound reactions such as intensified pulmonary vasoconstriction and hypoxaemia after sudden withdrawal of inhaled nitric oxide therapy have been described, precipitating cardiopulmonary collapse. The patient should be treated with increased FiO₂ and/or by reinstatement of therapy with inhaled nitric oxide. When possible, inhaled nitric oxide should be continued until the underlying disease has resolved.

NO₂ rapidly forms in gas mixtures containing nitric oxide and O₂, and NO₂ may in this way cause airway inflammation and damage. There are also animal data suggesting an increased susceptibility to airway infection upon exposure to low levels of NO₂.

In one clinical study (NINOS), treatment groups were similar with respect to the incidence and severity of intracranial haemorrhage, Grade IV haemorrhage, periventricular leukomalacia, cerebral infarction, seizures requiring anticonvulsant therapy, pulmonary haemorrhage, or gastrointestinal haemorrhage.

The table below shows reactions that occurred in at least 5% of patients receiving INOmax in the CINRGI study. None of the differences in these adverse reactions were statistically significant when inhaled nitric oxide patients were compared to patients receiving placebo. The reporting rate is classified as very common (>1/10), common (>1/100, <1/10), uncommon (>1/1,000<1/100), rare (>1/10,000, <1/1,000), very rare (<1/10,000) including isolated reports.

Blood and Lymphatic System Disorders
<i>Very Common:</i> Thrombocytopenia
Hepatobiliary Disorders
<i>Common:</i> Hyperbilirubinemia
Metabolism and Nutrition Disorders
<i>Common:</i> Hypokalemia
Respiratory, thoracic and Mediastinal Disorders
<i>Common:</i> Atelectasis
Vascular Disorders
<i>Common:</i> Hypotension

Post Marketing Experience

The following adverse reactions have been reported as part of the post-marketing surveillance: headaches associated with environmental exposure, hypotension associated with acute withdrawal of the drug, hypoxaemia associated with acute withdrawal of the drug, and dose errors associated with the delivery system.

4.9 Overdose

Overdose with INOmax will be manifest by elevations in methaemoglobin and NO₂. Elevated NO₂ may cause acute lung injury. Elevations in methaemoglobinaemia reduce the oxygen delivery capacity of the circulation. In clinical studies, NO₂ levels > 3 ppm or methaemoglobin levels > 7% were treated by reducing the dose of, or discontinuing, INOmax.

Methaemoglobinaemia that does not resolve after reduction or discontinuation of therapy can be treated with intravenous vitamin C, intravenous methylene blue, or blood transfusion, based upon the clinical situation.

5. PHARMACOLOGICAL PROPERTIES

5.1 Pharmacodynamic properties

Pharmacotherapeutic group: Other respiratory system products, ATC code R07 AX

Nitric oxide is a compound produced by many cells of the body. It relaxes vascular smooth muscle by binding to the haeme moiety of cytosolic guanylate cyclase, activating guanylate cyclase and increasing intracellular levels of cyclic guanosine 3',5'-monophosphate, which then leads to vasodilation. When inhaled, nitric oxide produces pulmonary vasodilation.

INOMax appears to increase the partial pressure of arterial oxygen (PaO₂) by dilating pulmonary vessels in better ventilated areas of the lung, redistributing pulmonary blood flow away from lung regions with low ventilation/perfusion (V/Q) ratios toward regions with normal ratios.

Persistent pulmonary hypertension of the newborn (PPHN) occurs as a primary developmental defect or as a condition secondary to other diseases such as meconium aspiration syndrome (MAS), pneumonia, sepsis, hyaline membrane disease, congenital diaphragmatic hernia (CDH), and pulmonary hypoplasia. In these states, pulmonary vascular resistance (PVR) is high, which results in hypoxemia secondary to right-to-left shunting of blood through the patent ductus arteriosus and foramen ovale. In neonates with PPHN, INOMax can improve oxygenation (as indicated by significant increases in PaO₂).

The efficacy of INOMax has been investigated in term and near-term newborns with hypoxic respiratory failure resulting from a variety of etiologies.

In the NINOS trial, 235 neonates with hypoxic respiratory failure were randomised to receive 100% O₂ with (n=114) or without (n=121) nitric oxide most with an initial concentration of 20 ppm with weaning as possible to lower doses with a median duration of exposure of 40 hours. The objective of this double-blind, randomised, placebo controlled trial was to determine whether inhaled nitric oxide would reduce the occurrence of death and/or initiation of extracorporeal membrane oxygenation (ECMO). Neonates with less than a full response at 20ppm were evaluated for a response to 80ppm nitric oxide or control gas. The combined incidence of death and/or initiation of ECMO (the prospectively defined primary endpoint) showed a significant advantage for the nitric oxide treated group (46% vs. 64%, p=0.006). Data further suggested a lack of additional benefit for the higher dose of nitric oxide. The adverse events collected occurred at similar incidence rates in both groups. Follow-up exams at 18-24 months of age were similar between the two groups with respect to mental, motor, audiological, and neurologic evaluations.

In the CINRGI trial, 186 term- and near-term neonates with hypoxic respiratory failure were randomised to receive either INOMax (n=97) or nitrogen gas (placebo; n=89) with an initial dose of 20 ppm weaning to 5 ppm in 4 to 24 hours with median duration of exposure of 44 hours. The prospectively defined primary endpoint was the receipt of ECMO. Significantly fewer neonates in the INOMax group required ECMO compared to the control group (31% vs 57%, p<0.001). The INOMax group had significantly improved oxygenation as measured by PaO₂, OI, and alveolar-arterial gradient (p<0.001 for all parameters). Of the 97 patients treated with INOMax, 2(2%) were withdrawn from study drug due to methaemoglobin levels >4%. The frequency and number of adverse events were similar in the two study groups.

Nitric oxide chemically reacts with oxygen to form nitrogen dioxide.

Nitric oxide has an unpaired electron, which makes the molecule reactive. In biological tissue, nitric oxide may form peroxynitrite with superoxide (O₂⁻), an unstable compound which may cause tissue damage through further redox reactions. In addition, nitric oxide has affinity to metalloproteins and may also react with SH-groups in protein forming nitrosyl compounds. The clinical significance of the chemical reactivity of nitric oxide in tissue is unknown. Studies show that nitric oxide exhibits pulmonary pharmacodynamic effects at intra-airway concentrations as low as 1 ppm.

5.2 Pharmacokinetic properties

The pharmacokinetics of nitric oxide has been studied in adults. Nitric oxide is absorbed systemically after inhalation. Most of it traverses the pulmonary capillary bed where it combines with haemoglobin that is 60% to 100% oxygen-saturated. At this level of oxygen saturation, nitric oxide combines predominantly with oxyhaemoglobin to produce methaemoglobin and nitrate. At low oxygen saturation, nitric oxide can combine with deoxyhaemoglobin to transiently form nitrosylhaemoglobin, which is converted to nitrogen oxides and methaemoglobin upon exposure to oxygen. Within the pulmonary system, nitric oxide can combine with oxygen and water to produce nitrogen dioxide and nitrite, respectively, which interact with oxyhaemoglobin to produce methaemoglobin and nitrate. Thus, the end products of nitric oxide that enter the systemic circulation are predominantly methaemoglobin and nitrate.

Methaemoglobin disposition has been investigated as a function of time and nitric oxide exposure concentration in neonates with respiratory failure. Methaemoglobin concentrations increase during the first 8 hours of nitric oxide exposure. The mean methaemoglobin levels remained below 1% in the placebo group and in the 5 ppm and 20 ppm INOmax groups, but reached approximately 5% in the 80 ppm INOmax group. Methaemoglobin levels > 7% were attained only in patients receiving 80 ppm, where they comprised 35% of the group. The average time to reach peak methaemoglobin was 10 ± 9 (SD) hours (median, 8 hours) in these 13 patients; but one patient did not exceed 7% until 40 hours.

Nitrate has been identified as the predominant nitric oxide metabolite excreted in the urine, accounting for > 70% of the nitric oxide dose inhaled. Nitrate is cleared from the plasma by the kidney at rates approaching the rate of glomerular filtration.

5.3 Preclinical safety data

Effects in non-clinical studies were observed only at exposures considered sufficiently in excess of the maximum human exposure indicating little relevance to clinical use.

Acute toxicity is related to anoxia resulting from elevated methaemoglobin levels.

Nitric oxide is genotoxic in some test systems. A low incidence in uterine adenocarcinomas in rats following daily exposure to the recommended human dose for two years was tentatively considered treatment related.

No reproduction toxicity studies have been conducted.

6. PHARMACEUTICAL PARTICULARS

6.1 List of excipients

Nitrogen

6.2 Incompatibilities

All equipment, including connectors, tubing, and circuits, used in the delivery of nitric oxide must be made of materials compatible with the gas. From a corrosion point of view the supply system can be divided into two zones: 1) From the gas cylinder valve to the humidifier (dry gas) and 2) From the humidifier to outlet (moist gas which may contain NO₂). Tests show that dry nitric oxide mixtures can be used with most materials. However, the presence of nitrogen dioxide and moisture creates an aggressive atmosphere. Among metallic construction materials, only stainless steel can be recommended. Tested polymers which can be used in nitric oxide administration systems include polyethylene (PE) and polypropylene (PP). Butyl rubber, polyamide, and polyurethane should not be used. Polytrifluorochloroethylene, hexafluoropropene-vinylidene copolymer and polytetrafluorethylene have been used extensively with pure nitric oxide and other corrosive gases. They were considered so inert that testing was not required.

6.3 Shelf life

2 years

6.4 Special precautions for storage

All regulations concerning handling of pressure vessels must be followed.

Store gas cylinders indoors in well-ventilated rooms or outdoors in ventilated sheds where they are protected from rain and direct sunlight.

Protect the gas cylinders from shocks, falls, oxidising and flammable materials, moisture, sources of heat or ignition.

The installation of a nitric oxide pipeline system with supply station of gas cylinders, fixed network and terminal units is forbidden.

Storage in the pharmacy department The gas cylinders should be stored in an airy, clean and locked place, for storage of medicinal gas only. Inside this place, a separate premise should be dedicated to the storage of nitric oxide gas cylinders.

Storage in the medical department

The gas cylinder should be put in an equipped site with appropriate material in order to hold the gas cylinder vertically.

Transport of gas cylinders

The gas cylinders should be transported with appropriate material in order to protect them from risks of shocks and falls.

During inter- or within-hospital transfers of patients treated with INOmax, the gas cylinders should be fixedly stowed away in order to hold the gas cylinders vertically and to avoid the risk of fall or untimely modifying output. A particular attention should be also turned to the fastening of the pressure regulator so as to avoid the risks of accidental failures.

6.5 Nature and contents of container

2 litre aluminium gas cylinder

10 litre aluminium gas cylinder

A 2 litre and 10 litre aluminium gas cylinder (identification with aquamarine shoulder and white body) filled under a pressure of 155 bar, equipped with a stainless steel positive pressure (residual) valve with a specific outlet connection.

6.6 Special precautions for disposal

Instructions for use/handling INOmax

In order to avoid all incidents, the following instructions should be absolutely respected.

- the good condition of the material should be checked before use
- the gas cylinders should be fixedly stowed away in order to avoid untimely fall
- the valve should not be violently opened
- a gas cylinder whose valve is not protected by a cap or a shell should not be used
- a specific connection, with a 30 mm thread which is designated for medical use, complying with ISO 5145 and a pressure regulator which admits a pressure at least equal to 1.5 the maximum operating pressure (155 bar) of the gas cylinder should be used
- the pressure regulator should be purged by the nitrogen-nitric oxide mixture before each new use in order to preclude nitrogen dioxide inhalation
- a defective valve should not be repaired
- the pressure regulator should not be tightened with pliers, at the risk of crushing the gasket

Instruction for Disposal of Gas Cylinder

When the gas cylinder is empty, do not discard. Empty gas cylinders will be collected by the supplier.

7. MARKETING AUTHORISATION HOLDER

INO Therapeutics AB
SE-181 81 Lidingö
Sweden

8. MARKETING AUTHORISATION NUMBER(S)

EU/1/01/194/001, EU/1/01/194/002

9. DATE OF FIRST AUTHORISATION/RENEWAL OF THE AUTHORISATION

Date of first authorisation: 1 August 2001
Date of last renewal: 1 August 2006

10. DATE OF REVISION OF THE TEXT

29 October 2009

ANNEX II

- A. MANUFACTURING AUTHORISATION HOLDER RESPONSIBLE FOR BATCH RELEASE**
- B. CONDITIONS OF THE MARKETING AUTHORISATION**

A. MANUFACTURING AUTHORISATION HOLDER RESPONSIBLE FOR BATCH RELEASE

AGA Medical
Zone Industrielle de Limay Porcheville
3, avenue Ozanne
78440 Gargenville
France

B. CONDITIONS OF THE MARKETING AUTHORISATION

• **CONDITIONS OR RESTRICTIONS REGARDING SUPPLY AND USE IMPOSED ON THE MARKETING AUTHORISATION HOLDER**

Medicinal product subject to restricted medical prescription (see Annex I: Summary of Product Characteristics, section 4.2).

• **CONDITIONS OR RESTRICTIONS WITH REGARD TO THE SAFE AND EFFECTIVE USE OF THE MEDICINAL PRODUCT**

Not applicable.

ANNEX III
LABELLING AND PACKAGE LEAFLET

A. LABELLING

PARTICULARS TO APPEAR ON THE IMMEDIATE PACKAGING

2 litres

1. NAME OF THE MEDICINAL PRODUCT

INOmax 400 ppm mol/mol inhalation gas
Nitric oxide.

2. STATEMENT OF ACTIVE SUBSTANCE(S)

INOmax contains nitric oxide 400 ppm mol/mol.

3. LIST OF EXCIPIENTS

Also contains nitrogen.

4. PHARMACEUTICAL FORM AND CONTENTS

A 2 litre gas cylinder filled at 155 bar absolute brings 307 litres of gas under pressure of 1 bar at 15°C.

5. METHOD AND ROUTE(S) OF ADMINISTRATION

Endotracheopulmonary use.

Read the package leaflet before use.

6. SPECIAL WARNING THAT THE MEDICINAL PRODUCT MUST BE STORED OUT OF THE REACH AND SIGHT OF CHILDREN

Keep out of the reach and sight of children.

7. OTHER SPECIAL WARNING(S), IF NECESSARY

Ensure that the parent or guardian has read and is aware of the indications and cautions presented in the package leaflet prior to administration to their baby.

8. EXPIRY DATE

Expiry date: {month/year}

9. SPECIAL STORAGE CONDITIONS

All regulations concerning handling of pressure vessels must be followed.

Store gas cylinders vertically in well-ventilated rooms.

Protect the gas cylinders from shocks, falls, oxidising and flammable materials, moisture, sources of heat or ignition.

10. SPECIAL PRECAUTIONS FOR DISPOSAL OF UNUSED MEDICINAL PRODUCTS OR WASTE MATERIALS DERIVED FROM SUCH MEDICINAL PRODUCTS, IF APPROPRIATE

Do not discard used gas cylinders. All gas cylinders should be returned to the supplier for disposal.

11. NAME AND ADDRESS OF THE MARKETING AUTHORISATION HOLDER

INO Therapeutics AB
SE-181 81 Lidingö
Sweden

12. NUMBER(S) IN THE COMMUNITY REGISTER OF MEDICINAL PRODUCTS

EU/1/01/194/002

13. MANUFACTURER'S BATCH NUMBER

Batch Number: {number}

14. GENERAL CLASSIFICATION FOR SUPPLY

Medicinal product subject to medical prescription

15. INSTRUCTIONS ON USE

PARTICULARS TO APPEAR ON THE IMMEDIATE PACKAGING

10 litres

1. NAME OF THE MEDICINAL PRODUCT

INOmax 400 ppm mol/mol inhalation gas
Nitric oxide.

2. STATEMENT OF ACTIVE SUBSTANCE(S)

INOmax contains nitric oxide 400 ppm mol/mol.

3. LIST OF EXCIPIENTS

Also contains nitrogen.

4. PHARMACEUTICAL FORM AND CONTENTS

A 10 litre gas cylinder filled at 155 bar absolute brings 1535 litres of gas under pressure of 1 bar at 15°C.

5. METHOD AND ROUTE(S) OF ADMINISTRATION

Endotracheopulmonary use
Read the package leaflet before use.

6. SPECIAL WARNING THAT THE MEDICINAL PRODUCT MUST BE STORED OUT OF THE REACH AND SIGHT OF CHILDREN

Keep out of the reach and sight of children.

7. OTHER SPECIAL WARNING(S), IF NECESSARY

Ensure that the parent or guardian has read and is aware of the indications and cautions presented in the package leaflet prior to administration to their baby.

8. EXPIRY DATE

Expiry date: {month/year}

9. SPECIAL STORAGE CONDITIONS

All regulations concerning handling of pressure vessels must be followed.

Store gas cylinders vertically in well-ventilated rooms.

Protect the gas cylinders from shocks, falls, oxidising and flammable materials, moisture, sources of heat or ignition.

10. SPECIAL PRECAUTIONS FOR DISPOSAL OF UNUSED MEDICINAL PRODUCTS OR WASTE MATERIALS DERIVED FROM SUCH MEDICINAL PRODUCTS, IF APPROPRIATE

Do not discard used gas cylinders. All gas cylinders should be returned to the supplier for disposal.

11. NAME AND ADDRESS OF THE MARKETING AUTHORISATION HOLDER

INO Therapeutics AB
SE-181 81 Lidingö
Sweden

12. MARKETING AUTHORISATION NUMBER(S)

EU/1/01/194/001

13. MANUFACTURER'S BATCH NUMBER

Batch Number: {number}

14. GENERAL CLASSIFICATION FOR SUPPLY

Medicinal product subject to medical prescription

15. INSTRUCTIONS ON USE

B. PACKAGE LEAFLET

PACKAGE LEAFLET: INFORMATION FOR THE USER

INOmax 400 ppm mol/mol inhalation gas
Nitric oxide

Read all of this leaflet carefully before you start using this medicine.

- Keep this leaflet. You may need to read it again.
- If you have any further questions, ask your baby's doctor
- If any of the side effects gets serious, or if you notice any side effects not listed in this leaflet, please tell your baby's doctor.

In this leaflet:

1. What INOmax is and what it is used for
2. Before your baby begins treatment with INOmax
3. How your baby is treated with INOmax
4. Possible side effects
5. How to store INOmax
6. Further Information

1. WHAT INOmax IS AND WHAT IT IS USED FOR

INOmax is a gas mixture for inhalation.

INOmax is for the treatment of newborn babies with lung failure associated with high blood pressure in the lungs, a condition known as hypoxic respiratory failure. When inhaled, nitric oxide can improve the flow of blood through the lungs, which may help to increase the amount of oxygen that reaches your baby's blood.

2. BEFORE YOUR BABY BEGINS TREATMENT WITH INOmax

Do not use INOmax

- If your baby is allergic (hypersensitive) to nitric oxide or any other ingredients of INOmax.
- INOmax is not to be given to babies who have an abnormal circulation within the heart.

Using other medicines

The doctor will decide when to treat your baby with INOmax and with other medicines, and will carefully supervise the treatment. Some medicines can affect the ability of blood to carry oxygen. INOmax 400 ppm gas should be given with some medicines called "nitric oxide donors" only if care is taken to make sure that the blood can carry enough oxygen. This need for care is present whether the medicines are administered by mouth, by injection, or even when applied as a cream to the skin. For example, care should be taken when INOmax is given at the same time as other medicines containing prilocaine.

Please tell your doctor if your baby is taking or has recently taken any other medicines, including medicines obtained without a prescription.

3. HOW INOmax IS ADMINISTERED

Your doctor will decide the correct dose of INOmax and will administer INOmax to your baby's lungs through a system designed for delivering nitric oxide. This delivery system will ensure that the correct amount of nitric oxide is delivered. The amount of nitrogen dioxide and oxygen delivered will be constantly monitored.

INOMax is administered to your baby using an approved (CE-marked) delivery system designed to deliver nitric oxide. This system delivers the prescribed concentration of nitric oxide to your baby's lungs by diluting INOMax with an oxygen/air mixture immediately before delivery.

For your baby's safety, the delivery systems intended for administration of INOMax are fitted with devices that constantly measure the amount of nitric oxide, nitrogen dioxide, and oxygen being delivered to your baby's lungs.

Your baby's doctor will decide how long your baby should be treated with INOMax.

If your baby stops using INOMax

At the end of therapy, the doctor will slowly lower the dose of INOMax being given to your baby. Treatment with INOMax should not be stopped suddenly, so that the circulation in your baby's lungs is able to adjust to oxygen/air without INOMax. So when your baby's treatment with INOMax is almost finished, a gradual reduction in the amount of INOMax being administered to your baby will be supervised by your baby's doctor. Low blood pressure has been known to occur if treatment with INOMax is stopped suddenly without first lowering the dose.

If you have any other questions on the use of this product, ask your doctor.

4. POSSIBLE SIDE EFFECTS

Like all medicines, INOMax can cause side effects, although not everybody gets them. Your baby's doctor will examine your baby closely for all side effects.

The most common side effects relate to a very high concentration of nitric oxide reaching your baby's blood, or side effects associated with formation of nitrogen dioxide:

- If too much nitric oxide reaches your baby's blood, the capacity of his/her blood to carry oxygen may be reduced. If this happens, your baby's doctor will immediately reduce the dose of nitric oxide being given, and your baby's blood will regain its ability to carry oxygen.
- When nitric oxide and oxygen combine, they form a chemical called nitrogen dioxide. Nitrogen dioxide is associated with airway inflammation and damage, and should be maintained as low as possible.

Furthermore, although both nitric oxide and the presence of nitrogen dioxide at high nitric oxide concentrations may cause tissue damage, the long-term clinical significance is unknown. Therefore, your baby's doctor will consider this potential risk in balance with the immediate benefits, which the short-term treatment with INOMax may provide and will carefully monitor the dose of nitric oxide being given.

Side effects with INOMax include deficient oxygenation of the blood due to sudden withdrawal of the treatment, low blood pressure, blood in urine, high blood sugar, blood poisoning, infection, and skin infection.

If any of the side effects gets serious, or if you notice any side effects not listed in this leaflet, even after your baby leaves the hospital, please tell your baby's doctor.

5. HOW TO STORE INOmax

Keep out of the reach and sight of children

Do not use INOmax after the expiry date which is stated on the label.

All regulations concerning handling of pressurised gas cylinders must be followed. Storage is supervised by the specialists at the hospital. Gas cylinders are to be stored in well-ventilated rooms or in ventilated sheds where they are protected from rain and direct sunlight.

Protect the gas cylinders from shocks, falls, oxidising and flammable materials, moisture, sources of heat or ignition.

Storage in the pharmacy department

The gas cylinders should be stored in an airy, clean and locked place, for storage of medicinal gas only. Inside this place, a separate premise should be dedicated to the storage of nitric oxide gas cylinders.

Storage in the medical department

The gas cylinder should be put in an equipped site with appropriate material in order to hold the cylinder vertically.

6. FURTHER INFORMATION

What INOmax contains

The active substance in INOmax is nitric oxide. The other ingredient is nitrogen.

What INOmax looks like and contents of the pack

INOmax is available in 2 litre and 10 litre aluminium gas cylinder.

Marketing Authorisation Holder and Manufacturer

Marketing Authorisation Holder

INO Therapeutics AB
SE-181 81 Lidingo
Sweden

Manufacturer

AGA Medical
Zone Industrielle de Limay Porcheville
3, avenue Ozanne
78440 Gargenville
France

This leaflet was last approved in {date}

17.8 Cystic Fibrosis UK Questionnaire

CFQ-UK

CYSTIC FIBROSIS QUESTIONNAIRE – UK

Adolescents and Adults

Understanding the impact of your illness and treatments on your everyday life can help your healthcare team keep track of your health and adjust your treatments. For this reason, this questionnaire was specifically developed for people who have cystic fibrosis. Thank you for your willingness to complete this form.

Instructions: The following questions are about the current state of your health, as you perceive it. This information will allow us to better understand how you feel in your everyday life.

Please answer all the questions. There are **no** right or wrong answers! If you are not sure how to answer, choose the response that seems closest to your situation.

Section I. Demographics

Please fill-in the information or tick the box indicating your answer.

A. What is your date of birth?

Date

--	--	--	--	--	--	--	--

Day Month Year

B. What is your gender?

Male Female

C. During the **past two weeks**, have you been on holiday or out of school or work for reasons **NOT** related to your health?

Yes No

D. What is your current marital status?

- Single/never married
- Married
- Widowed
- Divorced
- Separated
- Remarried
- With a partner

E. Which of the following best describes your racial background?

- White - UK
- White - other
- Indian/ Pakistani
- Chinese/ Asian
- African
- Caribbean
- Other [not represented above or people whose predominant origin cannot be determined/ mixed race]

F. What is the highest level of education you have completed?

- Some secondary school or less
- GCSEs/ O-levels
- A/AS-levels
- Other higher education
- University degree
- Professional qualification or post-graduate study

G. Which of the following best describes your current work or school status?

- Attending school outside the home
- Taking educational courses at home
- Seeking work
- Working full or part time (either outside the home or at a home-based business)
- Full time homemaker
- Not attending school or working due to my health
- Not working for other reasons



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Section II. Quality of Life

Please tick the box indicating your answer.

	A lot of difficulty	Some difficulty	A little difficulty	No difficulty
<i>During the past two weeks, to what extent have you had difficulty:</i>				
1. Performing vigorous activities such as running or playing sports.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
2. Walking as fast as others	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
3. Carrying or lifting heavy things such as books, shopping, or school bags.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
4. Climbing one flight of stairs.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
5. Climbing stairs as fast as others	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
<i>During the past two weeks, indicate how often:</i>				
6. You felt well	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
7. You felt worried.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
8. You felt useless.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
9. You felt tired.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
10. You felt full of energy	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
11. You felt exhausted	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
12. You felt sad.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Please circle the number indicating your answer. Please choose only one answer for each question.

Thinking about the state of your health over the last two weeks:

13. To what extent do you have difficulty walking?
1. You can walk a long time without getting tired
 2. You can walk a long time but you get tired
 3. You cannot walk a long time because you get tired quickly
 4. You avoid walking whenever possible because it's too tiring for you
14. How do you feel about eating?
1. Just thinking about food makes you feel sick
 2. You never enjoy eating
 3. You are sometimes able to enjoy eating
 4. You are always able to enjoy eating



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15. To what extent do your treatments make your daily life more difficult?
1. Not at all
 2. A little
 3. Moderately
 4. A lot
16. How much time do you currently spend each day on your treatments?
1. A lot
 2. Some
 3. A little
 4. Not very much
17. How difficult is it for you to do your treatments (including medications) each day?
1. Not at all
 2. A little
 3. Moderately
 4. Very
18. How do you think your health is now?
1. Excellent
 2. Good
 3. Fair
 4. Poor

Please select a box indicating your answer.

*Thinking about your health during the past **two weeks**, indicate the extent to which each sentence is true or false for you.*

	Very true	Somewhat true	Somewhat false	Very false
19. I have trouble recovering after physical effort	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
20. I have to limit vigorous activities such as running or playing sports.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
21. I have to force myself to eat	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
22. I have to stay at home more than I want to	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
23. I feel comfortable discussing my illness with others	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
24. I think I am too thin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
25. I think I look different from others my age	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
26. I feel bad about my physical appearance	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
27. People are afraid that I may be contagious	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
28. I get together with my friends a lot.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>



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- | | | | | |
|---|--------------------------|--------------------------|--------------------------|--------------------------|
| 29. I think my coughing bothers others | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 30. I feel comfortable going out at night | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 31. I often feel lonely..... | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 32. I feel healthy | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 33. It is difficult to make plans for the future (for example, going to college, getting married, getting promoted at work, etc.) | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 34. I lead a normal life..... | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

Section III. School, Work, or Daily Activities

Questions 35 to 38 are about school, work, or other daily tasks.

35. To what extent did you have trouble keeping up with your schoolwork, professional work, or other daily activities during the past two weeks?
1. You have had no trouble keeping up
 2. You have managed to keep up but it's been difficult
 3. You have been behind
 4. You have not been able to do these activities at all
36. How often were you absent from school, work, or unable to complete daily activities during the last two weeks because of your illness or treatments?
- Always Often Sometimes Never
37. How often does CF get in the way of meeting your school, work, or personal goals?
- Always Often Sometimes Never
38. How often does CF interfere with getting out of the house to run errands such as shopping or going to the bank?
- Always Often Sometimes Never



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Section IV. Symptom Difficulties

Please select a box indicating your answer.

Indicate how you have been feeling during the past two weeks.

	A great deal	Somewhat	A little	Not at all
39. Have you had trouble gaining weight?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
40. Have you been congested?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
41. Have you been coughing during the day?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
42. Have you had to cough up mucus?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Go to
 Question 44

43. Has your mucus been mostly:

- Clear
 Clear to yellow
 Yellowish-green
 Green with traces of blood
 Don't know

How often during the past two weeks:

	Always	Often	Sometimes	Never
44. Have you been wheezing?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
45. Have you had trouble breathing?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
46. Have you woken up during the night because you were coughing?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
47. Have you had problems with wind?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
48. Have you had diarrhoea?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
49. Have you had abdominal pain?	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
50. Have you had eating problems?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Please make sure you have answered all the questions.

Thank you for your cooperation



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 CFQ-UK English Language Version 1.0

17.9 Letter to participants to send with payment



Dear

Please find enclosed the cheque for payment to thank you for your participation in the RATNO study. We are very grateful to you for taking part. We will make the results available on the Southampton CF website once we have completed the study and analysed the data.

Once again, many thanks for your participation,

Signed

Name

Date

RATNO study team

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