ERJ Express. **Published on May 14, 2008 as doi: 10.1183/09031936.00159607**

Pseudomonas aeruginosa, Cyanide Accumulation and Lung Function in Cystic Fibrosis and Non-Cystic Fibrosis Bronchiectasis Patients

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Sources of support: This work was supported by the UK Biotechnology and Biological Sciences Research Council through a studentship to BR.

Running Title: Cyanide and CF lung function.

ABSTRACT

Pseudomonas aeruginosa is the most important respiratory pathogen in patients with cystic fibrosis (CF) and non-CF bronchiectasis. It is able to synthesise hydrogen cyanide, a potent inhibitor of cellular respiration. We investigated whether cyanide is present in the sputum of CF and non-CF bronchiectasis patients infected with *P. aeruginosa*, and whether the detection of cyanide affected lung function.

Cyanide was measured in sputum using a cyanide ion selective electrode.

Cyanide was detected in sputum from 15/25 CF and non-CF bronchiectasis patients with current *P. aeruginosa* infection, whereas it was not detected in any of the 10 patients without this organism ($p<0.01$). Maximum levels were 130μM (mean ±SE: 72 ± 6.6 μM). Concurrent lung function data were available on all 21 *P. aeruginosa*-infected CF patients; the group with measurable sputum cyanide (n=11) was not different from those without (n=10) on the basis of age or gender. However, those with detectable cyanide had significantly poorer lung function than those without (FEV1% predicted: 26.8±3.8% versus 46.0±6.7%, $p<0.01$; FVC% predicted: 44.4±4.9% versus 60.1±7.7, $p<0.05$).

Cyanide is detectable in sputum from CF and non-CF bronchiectasis patients infected with *P. aeruginosa* and is associated with impaired lung function

Introduction

Pseudomonas aeruginosa is the most important respiratory pathogen in cystic fibrosis (CF) and its presence leads to higher morbidity and mortality [1-4]. Chronically infected patients have significantly poorer lung function and increased risk of mortality than their non-infected peers [2, 3, 5]. The end result of chronic infection and inflammation is bronchiectasis, irreversible dilation of the bronchial tree with mucus plugging. Other groups of patients with non-CF bronchiectasis (Bx) also have a high rate of infection with this organism, which is similarly associated with increased disease severity and poorer quality of life [6] .

Factors accepted as explaining the successful establishment of chronic *P. aeruginosa* lung infection in CF patients are impaired mucociliary clearance and induction of mucoidy in *P. aeruginosa*. The induction of high levels of inflammation by bacterial LPS and other factors promotes a vicious cycle of exacerbation which contributes to progressive lung destruction [2, 3, 5]. An interesting aspect of the physiology of *P. aeruginosa* is that it is one of a limited number of bacteria that can synthesise hydrogen cyanide [7]. In culture, the levels of cyanide can reach 300 – 500 μM [7-9]. The purpose of cyanide production by *P. aeruginosa* is unclear, but it has been shown to be the mediating factor in the paralytic killing model of *Caenorhabditis elegans* by *P. aeruginosa* raising the possibility that it may be significant in pathogenicity [10]. Additionally, cyanide has been detected in burn wound infections caused by *P. aeruginosa* [11]. Cyanide is a highly toxic compound that diffuses rapidly into tissues irreversibly binding to targets. Cyanide can inhibit many cellular processes, but its most well recognised effect is to inhibit aerobic respiration through its interaction with the terminal oxidases of aerobic respiratory chains. *P. aeruginosa* may itself avoid the toxic effects of cyanide as it can

synthesise a respiratory chain terminated by a cyanide insensitive terminal oxidase [8, 12, 13], although active detoxification mechanisms are also likely to play an important role [14, 15]. Cyanide production by *P. aeruginosa* occurs maximally with finite but low oxygen concentration; conditions which *P. aeruginosa* are believed to inhabit in the mucus layer of the CF lung [16].

We set out to develop a method for measuring cyanide in sputum and to then determine whether cyanide was being produced in the lungs of CF and Bx patients infected with *P. aeruginosa* and if present whether it was associated with any impairment in lung function**.**

METHODS

We recruited adult patients from the Royal Brompton Hospital, London. The study was approved by the Ethics Committee of the Royal Brompton, Harefield & NHLI and informed consent was obtained from each subject. Identification of infecting organisms in patients sputum was carried out by the Clinical Microbiology laboratory at the Royal Brompton Hospital using selective media, microscopy and biochemical analysis using the API system (bioMerieux) [17, 18]. All patients that were *P. aeruginosa* infected had been so for at least 2 years and in most cases for 5-10 years, so all had chronic infections. 75% of patients studied had infective exacerbations. A patient was considered infected with *P. aeruginosa* (Pa^+) if the bacterium had been cultured from their sputum for at least two years and was cultured within 1 week of cyanide measurement. Patients were recruited into the non *P. aeruginosa* infected group (Pa^-) if they had not grown *P. aeruginosa* from any sputum sample over the previous 2 years. All patients were adults with an age range

of: 19-58, (mean=31.5, n=27) for CF patients and 52-67, (mean=58.2, n=8) for non-CF bronchiectasis (Bx) patients.

Cyanide concentration in fresh sputum from *P. aeruginosa* positive and *P. aeruginosa* negative patients was assayed using a cyanide electrode. Lung function data for the CF, *P. aeruginosa* cohort was available retrospectively and was used to examine if there was a correlation between detection of cyanide and decreased lung function. Lung function data was only used if it was available for dates within 2 weeks of cyanide measurement (it was usually within a few days).

Fresh sputum samples were collected in 50ml polypropylene test tubes which were sealed immediately with a gas tight rubber, Suba-Seal bung and placed on ice. Cyanide concentration was measured a maximum of 1 hour after collection. 2x v/w 0.1M NaOH was injected into sputum sample through the rubber bung to raise the pH and trap the cyanide and the sample was vortexed. Cyanide measurements followed immediately using an IS-146 Cyanide Ion Sensing Electrode (Lazar Laboratories Inc.) connected to a Cyber Scan pH meter (Eutech Instruments) as previously described [9]. The electrode was calibrated on every day of use in cyanide standards made up in 0.06M NaOH. All cyanide measurements made using a NaOH calibration curve were subsequently converted using the equation actual [cyanide] = 0.6747(apparent [cyanide] – 52.643) (fig. 1).

Spirometry was performed according to American Thoracic Society/ European Society Guidelines [19] on an EasyOne spirometer (NDD Medical technologies). The functional indices measured were forced expiratory volume in one second (FEV_1) and

forced vital capacity (FVC); these were expressed as percentages of values predicted for the patient's age, sex and height [20] When indices where compared from patients over time the data was weighted to reflect this when performing statistical analysis and the p values quoted are based on the weighted means.

Means were compared using student's t-test or ANOVA. A p-value of <0.05 was taken to indicate statistical significance. GraphPad InStat 3 and SPSS software was used.

RESULTS

Development of a method to assay cyanide in sputum

To the authors' knowledge this was the first study that assayed cyanide in sputum using an ion-selective electrode and as such it was necessary to develop an appropriate method. In order to test our method a cyanide spiking experiment was carried out to see if we could accurately measure cyanide in sputum. Cyanide was added to the sputum of a *P. aeruginosa* uninfected patient and cyanide assay clearly demonstrated that it was possible to measure cyanide added to sputum (Fig. 1a). However, the method overestimated cyanide concentration because the electrode was calibrated in NaOH not sputum (it was not practical to routinely calibrate the electrode in sputum due to a limited supply of cyanide negative sputum). The overestimation was identical in 3 separate spiking experiments using sputum from 3 different patients. These data indicated that the relationship between actual and measured cyanide was linear, which allowed an equation describing the relationship between actual cyanide and measured cyanide to be generated (Fig. 1b). This

equation was used to convert all sputum cyanide measurements into actual sputum cyanide concentration.

Cyanide is detected in the sputum of *P. aeruginosa* – infected patients

After having established a method for measuring cyanide in sputum we then assayed sputum from 25 *P. aeruginosa* positive and 10 *P. aeruginosa* negative patients. Sputum from 15/25 Pa^+ patients tested positive for cyanide. In contrast no sample from the any of the 10 Pa^- patients had detectable cyanide (Fig. 2; $p<0.001$). The mean concentration of cyanide in those patients in whom it was detected was 72±6.6 μM with a maximum of 130 μM.

There was no relationship between patient age and sputum positivity for cyanide (mean ages ± SD; cyanide-positive = 35±3.2 years, cyanide-negative = 32± 3.3 years) or levels of cyanide measured. We found no correlation between the length of colonisation and HCN levels. Also while *P. aeruginosa* loads in the CN^+ cohort range from 10^3 to 10^7 cfu/ml, there was no correlation between bacterial load and cyanide levels (data not shown). About 75% of patients (in the Pa+ group) studied had infective exacerbations, but similar proportions of cyanide negative and cyanide positive samples were found in patients with or without infective exacerbations. Patients in this study were prescribed a range of antipseudomonal antibiotics, based on sensitivity testing and clinical considerations, but there were no patterns to distinguish between cyanide-positive and cyanide-negative patent groups.

A wide range of other microbes were detected in addition to *P. aeruginosa* in sputum samples. There was no significant difference between the mean microbial loads for the sputum cyanide-positive and the sputum cyanide-negative groups (10^6 cfu ml^{-1} for both groups). It is possible that the presence or absence of co-infecting microbes influenced the cyanide levels in sputum, but we found no evidence of this.

The data in Fig. 3, which shows how the mean sputum cyanide concentrations were affected by the presence or absence of a specific infecting microbe, demonstrate that: (i) the presence of *P. aeruginosa* is an absolute requirement for detecting sputum cyanide, that is, if *P. aeruginosa* is absent no sputum cyanide was detected, (ii) that the detection of cyanide was not associated with any other co-infecting microbe indicating no indirect effect of *Burkholderia cenocepacia*, *Staphylococcus aureus*, *Candida albicans* or *Aspergillus fumigatus* on cyanide accumulation.

The presence of cyanide in sputum is associated with decreased pulmonary function in CF patients

P. aeruginosa infection is widely accepted as having a negative affect on lung function. The mix of cyanide sputum positive and cyanide sputum negative patients in the *P. aeruginosa* positive cohort enabled us to examine if cyanide in sputum *per se* had any affect on lung function. Lung function data was available retrospectively for all the CF patients in this study. In order to minimise differences in other factors that could affect lung function we analysed lung function data from the CF Pa^+ cohort only. The cyanide-positive group (n=11) had significantly lower mean lung function indices than the cyanide-negative group subjects (FEV_1% predicted: 26.8±3.8% *versus* 46.0±6.7%, $p<0.01$; FVC% predicted: 44.4±4.9% *versus* 60.1±7.7, $p<0.05$, Fig. 4a,b). This could not be explained by differences in age (Fig. 4d), gender or total microbial pathogen counts between the two groups (Fig. 4c). When the lung function data of these patients from 6 months prior to 6 months post the time of cyanide measurement were examined the cyanide-positive group still had significantly lower mean lung function indices than the cyanide-negative group subjects (FEV_1%

predicted: 35±4.6% *versus* 54.0±7.0%, p<0.05; FVC% predicted: 44±3.08% *versus* 64±7.05, p<0.05) (Fig. 5).

DISCUSSION

We have demonstrated for the first time the presence of cyanide in the sputum of CF and non-CF bronchiectasis patients infected with *P. aeruginosa*. Up to 130μM cyanide was measured in sputum from CF and Bx patients with an average concentration of 72μM. This concentration is potentially of clinical significance and compares with a concentration of 40 μM, which is considered a toxic or lethal blood cyanide level [21].

P. aeruginosa is well established as a cyanogenic bacterium and during laboratory cultures accumulates cyanide at concentrations of up to 300-500 μM [7, 9] The conditions of low oxygen and high population density that *P. aeruginosa* is believed to experience in the CF lung is conducive to maximal cyanide production [8, 16]. The only factor associated with the presence of cyanide in sputum was *P. aeruginosa* infection; cyanide was only detected when *P. aeruginosa* was present, and was never detected in its absence. Neither patient age nor co-infection with other microbes had any effect on the likelihood of cyanide being present or its levels. Therefore, the most probable explanation for our findings is that *P. aeruginosa* is synthesising cyanide in the lungs of CF and Bx patients.

Some *P. aeruginosa* infections did not lead to cyanide accumulation in the sputum, but this was not simply a reflection of bacterial load; there was no difference in *P. aeruginosa* CFU counts between patients with and without detectable cyanide. Therefore, the reason for this finding remains unknown, but one explanation is that some strains have lost or have a significantly reduced ability to make hydrogen

cyanide. It is certainly clear that bacterial populations in sputum samples from CF patients are mixed with respect to their genotype and phenotype [22-25]. In respect of cyanide, a study of 167 clinical isolates of *P. aeruginosa* from CF patients found that 74% of strains could produce HCN, and 83% of 103 patients were infected with at least 1 strain capable of producing cyanide *in vitro* [26], but clearly cyanide non-producing strains are found in the CF lung.

Quorum sensing is a regulatory mechanism employed by many bacteria, including *P. aeruginosa*, which involves the use of extracellular signal molecules to regulate phenotypes in response to population density. It is known that cyanide production is in part regulated by quorum sensing in this bacterium [27-29], its synthesis being induced at high population densities. However, although the *P. aeruginosa* load ranged from 10^3 to 10^7 cfu/ml in the sputum of the cyanide positive cohort, there was no correlation between bacterial load and cyanide levels. There are a number of issues here. Firstly, major airway cultures, such as those from sputum, may not be reflective of all the bacteria present in the lung, and in particular may not reflect those from the periphery of the lung [30]. While we did not type the strains present in the sputum sample analysed for cyanide, it is an interesting possibility that cyanide production is associated with the presence of specific clones in the bacterial population of a patient's lung rather than simply the total *P. aeruginosa* load. In this context it is interesting to note that some isolates of the Liverpool cystic fibrosis epidemic strain of *P. aeruginosa* overproduce certain quorum sensing regulated exoproducts, such as pyocyanin and LasA protease, but cyanide was not determined [25, 31]. However, *lasR* mutants with decreased quorum sensing activity were also found frequently in *P. aeruginosa* during CF lung infections [22]. Furthermore, it is not straightforward to extrapolate between data obtained from

culture and data from sputum. 10^3 bacterial cfu/ml homogeneously distributed in liquid culture is very different to the heterogeneous environment of the CF lung that yields the sputum samples with the same net population density. Contributing to this heterogeneity will be the fact that the bacteria will be present in colonial or biofilm structures [16], which result in high specific population densities that stimulate quorum sensing regulated pathways, leading to favourable conditions for cyanide production.

In addition to quorum sensing, low O_2 levels also regulate HCN synthesis *in vitro* via the action of the O_2 sensing regulator Anr. Various O_2 levels are expected to exist within the mucus of the CF lung, which could further modulate HCN production [7, 16, 32].

While it is unlikely, we cannot entirely rule out the possibility that cyanide in the sputum results from non-microbial sources. Hydrogen cyanide can be produced by neuronal tissue in response to specific μ-opiate agonists, leading to an average concentration of cyanide in rat brain of 6.9 μM [33]. Cyanide has also been detected in the breath of healthy, non-smoking subjects with most of it being formed by saliva in the oropharynx [34]. Most interestingly human leucocytes challenged with *Staphylococcus epidermidis* have been reported to produce hydrogen cyanide *in vitro* [35-37]. This work has not been followed up and in particular there have been no studies to indicate that leukocyte–mediated cyanide production occurs *in vivo.* We cannot rule out that cyanide results from the host response to *P. aeruginosa*, whereby increased airway inflammation caused by *P. aeruginosa* infection results in cyanide production by leucocytes. However, if the cyanide we detected in sputum resulted from leukocyte activation then we would expect to see cyanide accumulate irrespective of the nature of the infecting microbe and we do not; cyanide production

is *P. aeruginosa* specific. In addition the fact cyanide was not detected in sputum from 10 *P. aeruginosa* positive patients is not consistent with this explanation

The demonstration that cyanide is produced *in situ* during *P. aeruginosa* lung infections could be a clinically significant finding. It raises the issue of which symptoms of the diseases are due to prolonged exposure of the tissues of the lung to cyanide. Cyanide inhibits many metabolic processes but, the most commonly recognised mechanism of toxicity is its binding to and inhibition of cytochrome *c* oxidase, leading to a cessation of cellular respiration, a shift to anaerobic metabolism and a reduction in cellular ATP levels [21, 38-40]. This is invariably associated with cytotoxic hypoxia and lactic acidosis [41]. Cyanide at the concentrations found in this study would be expected to inhibit cellular respiration in the local environment of the CF lung, including in airway epithelial cells and alveolar macrophages, as well as in other invading microbes. Cyanide will inhibit other haem-containing and metallo enzymes including superoxide dismutase and xanthine oxidase [21] and may also inhibit key immune protection enzymes such iNOS, with consequences for the ability of *P. aeruginosa* to cause chronic infections. However, cyanide (2 mM) has been found to stimulate the respiratory burst of PMNs upon phagocytosis [42, 43], which if it occurred in the lung during chronic *P. aeruginosa* infection may lead to an increased inflammatory response and increased tissue damage. In humans it is thought that rhodanese (thiosulfate sulfurtransferase) and α-mercaptopyruvate sulfurtransferase contribute to the main pathway for cyanide elimination by converting it to the less toxic thiocyanate [44-46].

It is of interest that *P. aeruginosa* infection along with cyanide accumulation in the lung is associated with a significant decline in pulmonary function. Both indices of lung function we analysed were significantly reduced in *P. aeruginosa*–infected CF

patients with cyanide-positive sputum compared to similarly infected patients with cyanide-negative sputum. The greatest effect was on FEV_1 and it is well recognised that in obstructive lung diseases FEV_1 is reduced disproportionately to the FVC and is an indicator of flow limitation. While the correlation between the presence of HCN and lung function does not prove a direct effect of cyanide on lung function, it is plausible to suggest that this is a direct consequence of the action of cyanide in the lung environment. It is also relevant that within 18 months of the cyanide measurements being taken 5 of the patients studied died and 4 of these were from the cyanide positive group.

The failure of host defences to eliminate *P. aeruginosa* infection from the CF lung is poorly understood and it is possible that cyanide production and its effects on epithelial and immune cell function may play a role. Cyanide production by *P. aeruginosa* may be a factor in its ability to dominate the bacterial flora the CF lung and establish life-long infection which accelerates decline in lung functional capacity [1, 2]. The possible systemic effects of long term cyanide exposure from *P. aeruginosa* infection need to be considered.

This study, though preliminary, has produced interesting and significant data. To further investigate these findings future work will use longitudinal measurements to observe how cyanide levels change over time and how this in turn affects lung function. Following patients through a cycle from *P. aeruginosa* non-infected to infected will help to determine between causation and association with respect to *P. aeruginosa*, inflammation, sputum cyanide and lung function.

In conclusion, the present study shows that infection with the cyanogenic bacterium *P. aeruginosa* in the lungs of CF and Bx patients is associated with the

accumulation of cyanide in sputum. In addition the presence of cyanide is associated with a decrease in lung function.

***Conflict of Interest Statement**:* None of the authors have a financial relationship with a commercial entity that has an interest in the subject of the manuscript.

***Acknowledgement*:** We would like to thank the patients who participated in this study and the staff of the Clinical Microbiology Laboratory of the Royal Brompton Hospital. This work was supported by the UK Biotechnology and Biological Sciences Research Council through a studentship to BR.

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FIGURE LEGENDS

FIGURE 1. Cyanide spiking of sputum. a) Cyanide was added to sputum sample from a non-*P. aeruginosa* infected patient and the added and measured cyanide in their sputum plotted. b) Plot of actual cyanide against measured cyanide is linear, allowing an equation describing this relationship (actual [cyanide] = 0.6747(measured [cyanide] – 52.643) to be derived, which was subsequently used to convert all sputum cyanide measurements made with a NaOH calibration.

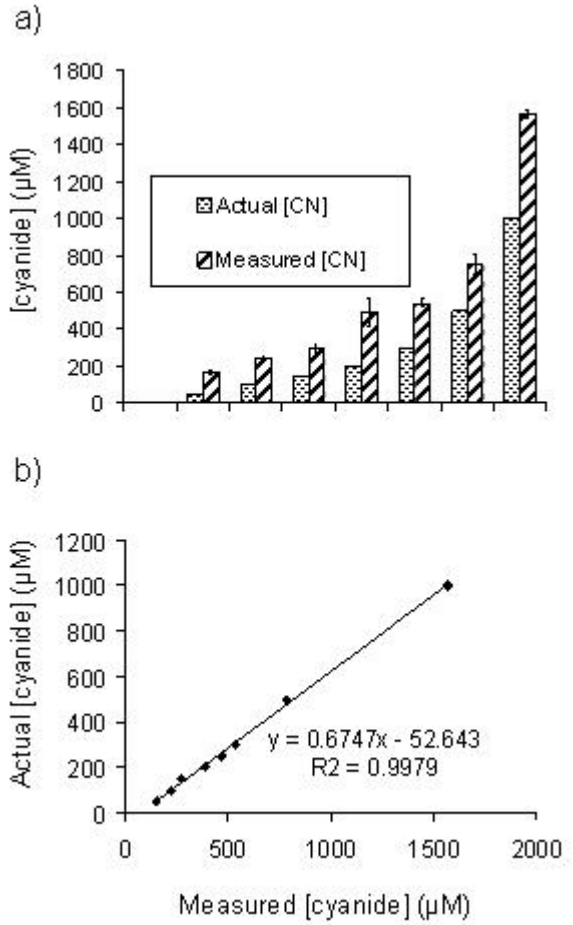


FIGURE 2. *P. aeruginosa* infection is associated with cyanide in sputum. Cyanide concentrations in sputum samples from cystic fibrosis (CF) (1-4 & 11-33) and non CF bronchiectasis (Bx) (5-10 & 34-35) patients with (11-35) and without (1-10) *P.*

aeruginosa lung infection. Error bars are SE of 3 measurements. 15 of 25 in the *P. aeruginosa* positive group had quantifiable levels of cyanide in their sputum (mean=72 μM, SD=24μM) whereas cyanide was not detected in any of the *P. aeruginosa* negative group.

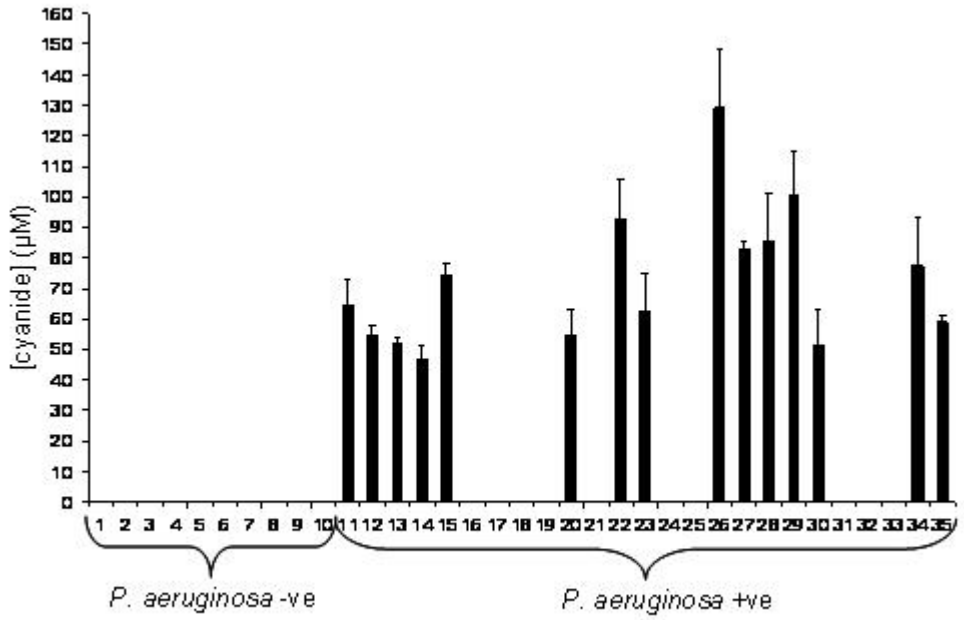


FIGURE 3. Cyanide concentration in sputum is not affected by the presence of any organism other than *P. aeruginosa*. A wide range of microbes was detected in addition to *P. aeruginosa* in sputum samples. The average cyanide concentration in sputum samples from patients in which a specific organism was present or absent is shown. In all cases where cyanide was detected in sputum *P. aeruginosa* was the sole or a co-infecting organism and the presence of other organisms had no significant affect on the cyanide concentration detected. +/- indicates presence/absence of organism, numbers underneath are the number of patients with or with out infection of the particular organism, error bars are SD.

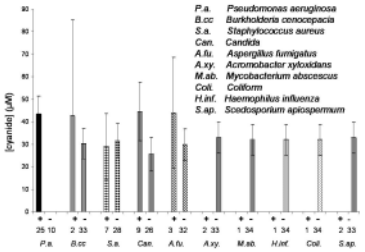


FIGURE 4. Cyanide in the sputum of *P. aeruginosa*-infected CF patients is associated with impaired lung function. Cyanide was associated with a significant decrease in FEV1 % predicted (a) and FVC % predicted (b). This could not be explained by differences in bacterial load of either *P. aeruginosa* alone or total pathogens (c) or by difference in age (d). Horizontal bars show mean value (long bars) and SD (short bars).

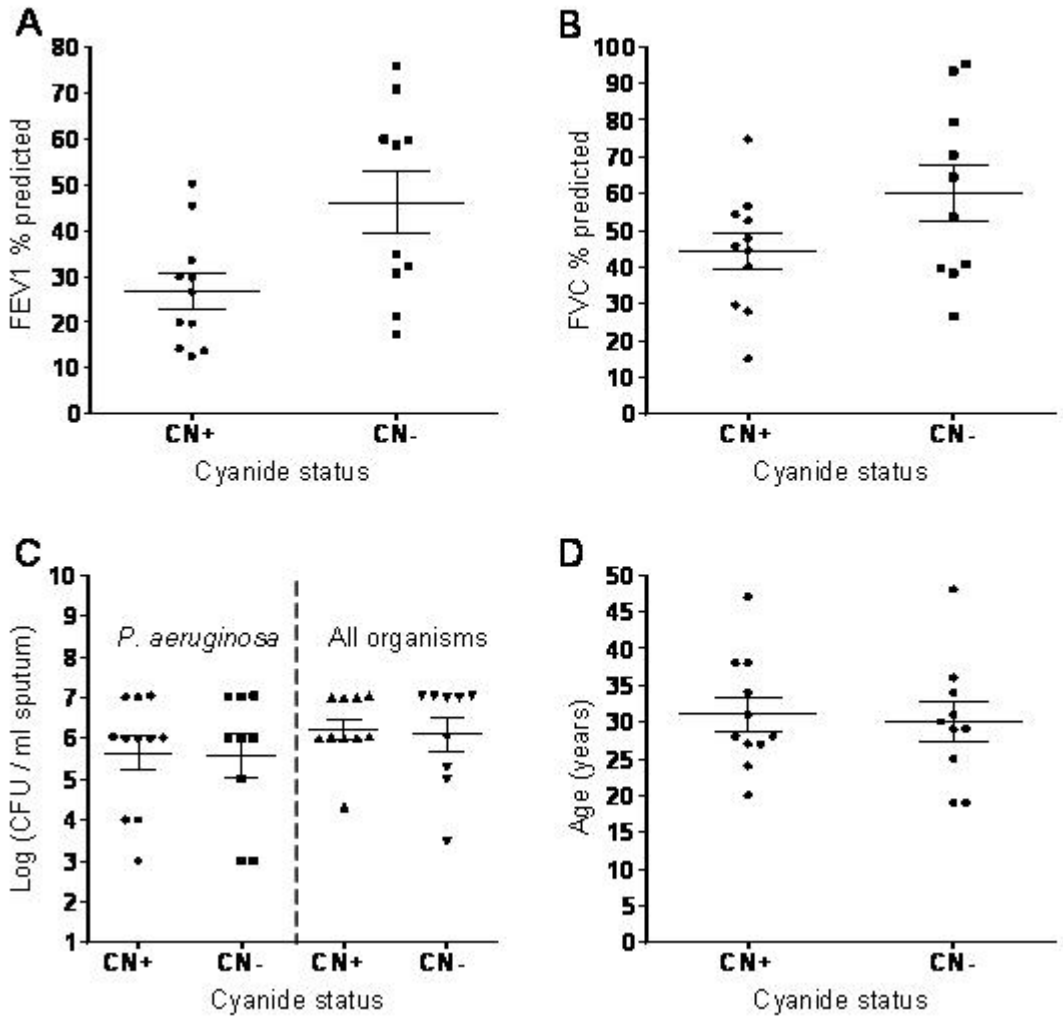
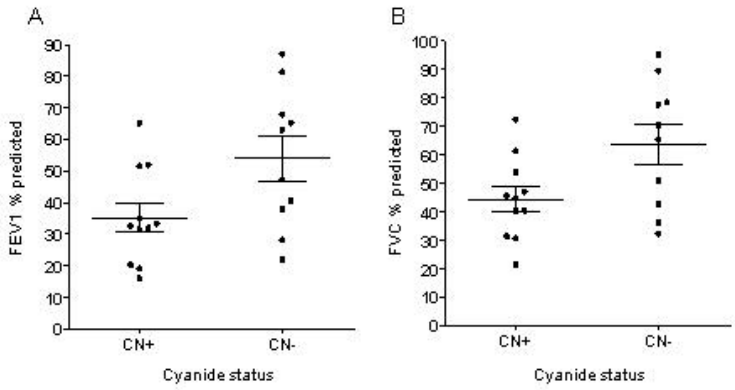


FIGURE 5.

Lung function is impaired in sputum-cyanide positive CF patients over an extended time period. Average FEV1 % predicted (A) and FVC% predicted (B) scores for CN+ and CN-, *P. aeruginosa* positive, CF patients. Each data point is an average of lung function scores taken over a period of 6 months prior to 6 months post cyanide reading. The number of readings making up the average varied for each patient and the data was weighted to reflect this when performing statistical analysis using SPSS software.



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