Pseudomonas aeruginosa, cyanide accumulation and lung function in CF and non-CF bronchiectasis patients

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ABSTRACT: In patients with cystic fibrosis (CF) and non-CF bronchiectasis, *Pseudomonas aeruginosa* is the most important respiratory pathogen. It is able to synthesise hydrogen cyanide, a potent inhibitor of cellular respiration.

The present study 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 out of 25 CF and non-CF bronchiectasis patients with current *P. aeruginosa* infection; however, it was not detected in any of the 10 patients without this organism. Maximum levels were 130 µM (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 sex. However, those with detectable cyanide had significantly poorer lung function than those without (forced expiratory volume in one second (% predicted) 26.8±3.8 versus 46.0±6.7%; forced vital capacity (% pred) 44.4±4.9 versus 60.1±7.7%).

Cyanide is detectable in sputum from cystic fibrosis and non-cystic fibrosis bronchiectasis patients infected with *Pseudomonas aeruginosa*, and is also associated with impaired lung function.

KEYWORDS: Airway inflammation, asthma, hyperresponsiveness, lung function, noninvasive markers, rhinovirus

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In cystic fibrosis (CF), *Pseudomonas aeruginosa* is the most important respiratory pathogen 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 non-infected patients [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 also have a high rate of infection with *P. aeruginosa*, 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 lipopolysaccharide 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 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 at low oxygen concentrations; conditions which *P. aeruginosa* are believed to inhabit in the mucus layer of the CF lung [16].

The present authors aimed to develop a method for measuring cyanide in sputum and to then determine whether cyanide was produced in the lungs of CF and bronchiectasis patients infected with *P. aeruginosa* and, if present, whether it was associated with any impairment in lung function.

MATERIALS AND METHODS

Adult patients were recruited from the Royal Brompton Hospital (London, UK). Informed consent was obtained from each subject and the study was approved by the Ethics Committee of the Royal Brompton Hospital, Harefield Hospital (Middlesex, UK) and the National Heart and Lung Institute (London). Identification of infecting organisms in patients' sputum was carried out by the Clinical Microbiology laboratory at the Royal Brompton Hospital by means of selective media, microscopy and biochemical analysis using the API system (bioMerieux, Marcy l'Etolie, France) [17, 18]. All patients with *P. aeruginosa* had been infected for ⩽2 yrs and in most cases for 5–10 yrs; thus, all patients had chronic infections. In total, 75% of patients studied had infective exacerbations. A patient was considered to be infected with *P. aeruginosa* if the bacterium had been cultured from their sputum for ⩾2 yrs and was cultured within 1 week of cyanide measurement. Patients were recruited into the non-*P. aeruginosa*-infected group if they had not grown *P. aeruginosa* from any sputum sample over the previous 2 yrs. All patients were adults with a mean (range) age of 31.5 (19–58) yrs for 27 CF patients and 58.2 (52–67) yrs for eight non-CF bronchiectasis 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 determine if there was a correlation between detection of cyanide and decreased lung function. Lung function data was only used if it was available for a period within 2 weeks of cyanide measurement (it was usually within a few days).

Fresh sputum samples were collected in 50-mL polypropylene test tubes, which were sealed immediately with a gas-tight rubber, Suba-Seal bung (Scientific Laboratory Supplies, Nottingham, UK) and placed on ice. Cyanide concentration was measured a maximum of 1 h after collection. To raise the pH and trap the cyanide, $2\times$ volume/weight 0.1 M NaOH was injected into the sputum sample through the rubber bung and the sample was vortexed. Cyanide measurements followed immediately using an IS-146 Cyanide Ion Sensing Electrode (Lazar Laboratories Inc., Los Angeles, CA, USA) connected to a Cyber Scan pH meter (Eutech Instruments, Nijerk, the Netherlands), as previously described [9]. The electrode was calibrated after every day of use in cyanide standards made up in 0.06 M NaOH. All cyanide measurements made using an NaOH calibration curve were subsequently converted using the following equation (fig. 1):

actual [cyanide]=0.6747(apparent [cyanide] – 52.643)   (1)
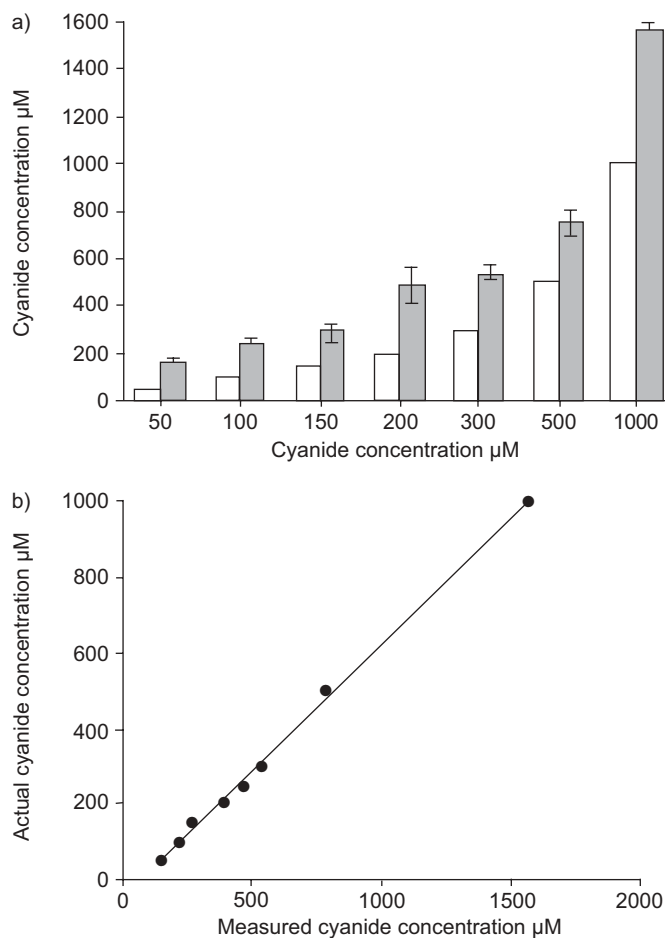
FIGURE 1. Cyanide spiking of sputum. a) Cyanide was added to a sputum sample from a non-*Pseudomonas aeruginosa*-infected patient and the actual (□) and measured (■) cyanide in the sputum was plotted. b) Linear plot of actual cyanide against measured. y=0.674x-52.643; R_2: 0.9979.

Spirometry was performed on an EasyOne spirometer (ndd Medical technologies, Zurich, Switzerland) according to American Thoracic Society/European Respiratory Society Guidelines [19]. 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. The p-values quoted are based on the weighted means.

Means were compared using an unpaired t-test or ANOVA. A p-value of <0.05 was taken to indicate statistical significance.

RESULTS
Development of a method to assay cyanide in sputum
To the authors' knowledge this was the first study to assay cyanide in sputum using an ion-selective electrode and, as such, it was necessary to develop an appropriate method. In order to test the current method, a cyanide spiking experiment was carried out to determine whether cyanide in sputum could be measured accurately. Cyanide was added to the sputum of a *P. aeruginosa*-uninfected patient and the 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 three separate spiking experiments using sputum from three 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). The equation was used to convert all sputum cyanide measurements into actual sputum cyanide concentrations.

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

Having established a method for measuring cyanide in sputum, the sputum from 25 *P. aeruginosa*-positive and 10 *P. aeruginosa*-negative patients was then assayed. Sputum from 15 of the 25 *P. aeruginosa*-positive patients tested positive for cyanide. In contrast, no sample from the any of the 10 *P. aeruginosa*-negative patients had detectable cyanide (fig. 2; $p<0.001$). The mean concentration of cyanide in the patients who tested positive 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 $\pm$ SD 35 ± 3.2 and 32 ± 3.3 yrs for cyanide-positive and -negative, respectively) or levels of cyanide measured. No correlation was found between the length of colonisation and HCN levels. In addition, while *P. aeruginosa* loads in the cyanide-positive cohort ranged 10^3–10^7 cfu·mL^{-1}, there was no correlation between bacterial load and cyanide levels (data not shown). Of patients studied in the *P. aeruginosa*-positive group, ~75% had infective exacerbations, but similar proportions of cyanide-negative and cyanide-positive samples were found in patients with or without infective exacerbations. Patients in the present study were prescribed a range of anti-pseudomonal antibiotics, based on

sensitivity testing and clinical considerations, but there were no patterns to distinguish between cyanide-positive and cyanide-negative patient groups.
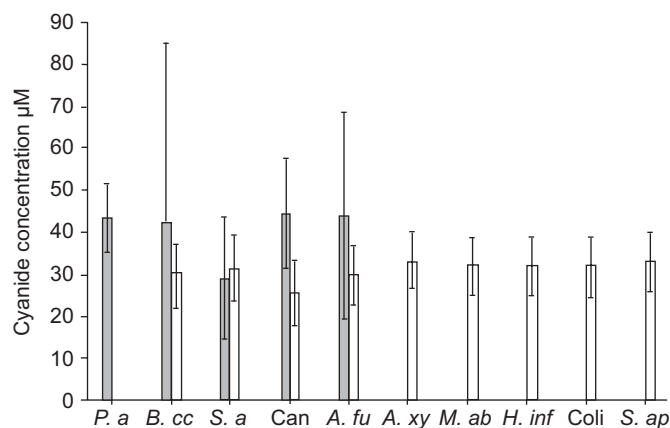
A wide range of other microbes were detected in addition to *P. aeruginosa* in sputum samples. There was no significant difference between the mean microbial loads for the sputum cyanide-positive and the sputum cyanide-negative groups (10^6 cfu·mL^{-1} for both groups). It is possible that the presence or absence of co-infecting microbes influenced the cyanide levels in sputum, but no evidence of this was found. The data presented in figure 3, showing how the mean sputum cyanide concentrations were affected by the presence or absence of a specific infecting microbe, demonstrate that: 1) the presence of *P. aeruginosa* is an absolute requirement for detecting sputum cyanide, *i.e.* if *P. aeruginosa* is absent no sputum cyanide was detected; and 2) 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.

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 the present authors to examine if cyanide in sputum *per se* had any affect on lung function. Lung function data were available retrospectively for all the CF patients in this study. In order to minimise differences in other factors that could affect lung function, lung function data were analysed from the CF-*P. aeruginosa*-positive cohort only. The cyanide-positive group (n=11) had significantly lower mean lung function indices than the cyanide-negative group subjects (FEV1 % predicted: 26.8 ± 3.8 *versus* 46.0 ± 6.7%, $p<0.01$; FVC % pred: 44.4 ± 4.9 *versus* 60.1 ± 7.7, $p<0.05$; fig. 4a and b). This could not be explained by differences in age (fig. 4d), sex or total microbial pathogen counts between the two groups (fig. 4c). When the lung function data of these patients from 6 months
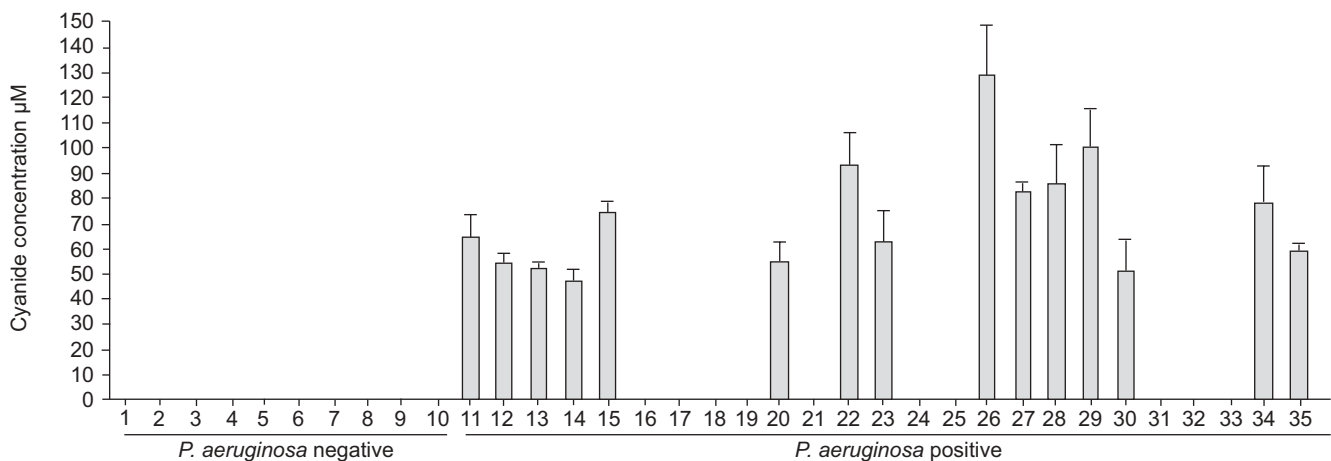
FIGURE 2. *Pseudomonas aeruginosa* infection was associated with cyanide in sputum. Cyanide concentrations in sputum samples from cystic fibrosis (CF; 1–4 and 11–33) and non-CF bronchiectasis (5–10, 34 and 35) patients with and without *P. aeruginosa* lung infection. Error bars show the SE of three measurements. In the *P. aeruginosa*-positive group, 15 out of 25 patients had quantifiable levels of cyanide in their sputum (mean $\pm$ SD 72 ± 24 µM). However, cyanide was not detected in any of the *P. aeruginosa*-negative group patients.

FIGURE 3. Cyanide concentration in sputum was not affected by the presence of any organism other than *Pseudomonas aeruginosa* (*P. a*). A wide range of microbes was detected in addition to *P. a* 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. a* was the sole or co-infecting organism and the presence of other organisms had no significant affect on the cyanide concentration detected. In total, 25 patients had *P. a* infection and 10 did not. Error bars represent SD. *B. cc*: *Burkholderia cenocepacia*; *S. a*: *Staphylococcus aureus*; Can: Candida; *A. fu*; *Aspergillus fumigatus*; *A. xy*: *Acromobacter xyloxidans*; *M. ab*: *Mycobacterium abscescus*; *H. in*: *Haemophilus influenza*; Coli: Coliform; *S. ap*: *Scedosporium apiospermum*. For *B. cc*, *A. xy* and *S. ap*, two patients were infected by each organism and 33 were not infected. For *M. ab*, *H. inf* and Coli, one patient was infected by each organism and 34 were not infected. For *S. a*, seven patients were infected by the organism and 28 were not. Nine patients were infected by Can and 26 were not. Three patients were infected by *A. fu* and 32 were not.

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 (FEV1 % pred: 35 ± 4.6 *versus* $54.0 \pm 7.0\%$, p<0.05; FVC % pred: 44 ± 3.08 *versus* 64 ± 7.05, p<0.05; fig. 5).

DISCUSSION

The present study has 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 130 µM cyanide was measured in sputum from CF and bronchiectasis patients with an average concentration of 72 µM. This concentration is of potential clinical significance in comparison 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 understood 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 the current findings is that *P. aeruginosa* synthesises cyanide in the lungs of CF and bronchiectasis 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* colony forming unit 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]. With regard to cyanide, a study of 167 clinical isolates of *P. aeruginosa* from CF patients found that 74% of strains could produce HCN and out of 103 patients, 83% were infected with at least one strain capable of producing cyanide *in vitro* [26]; but clearly cyanide nonproducing 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], with its synthesis being induced at high population densities. However, although the *P. aeruginosa* load ranged $10^3–10^7$ cfu·mL^{-1} 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. First, 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 the present authors 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 patients' lung rather than simply the total *P. aeruginosa* load. In this context it is interesting to note that some isolates of the Liverpool CF 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. There is a great difference between the 10^3 bacterial cfu·mL^{-1} homogeneously distributed in liquid culture and the heterogeneous environment of the CF lung that yields the sputum samples with the same net population density. Contributing to this heterogeneity is 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 (anaerobic regulator of arginine deaminase and nitrate reductase). 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, the possibility that cyanide in the sputum results from nonmicrobial sources cannot be entirely ruled out. Hydrogen cyanide can be produced by neuronal tissue in
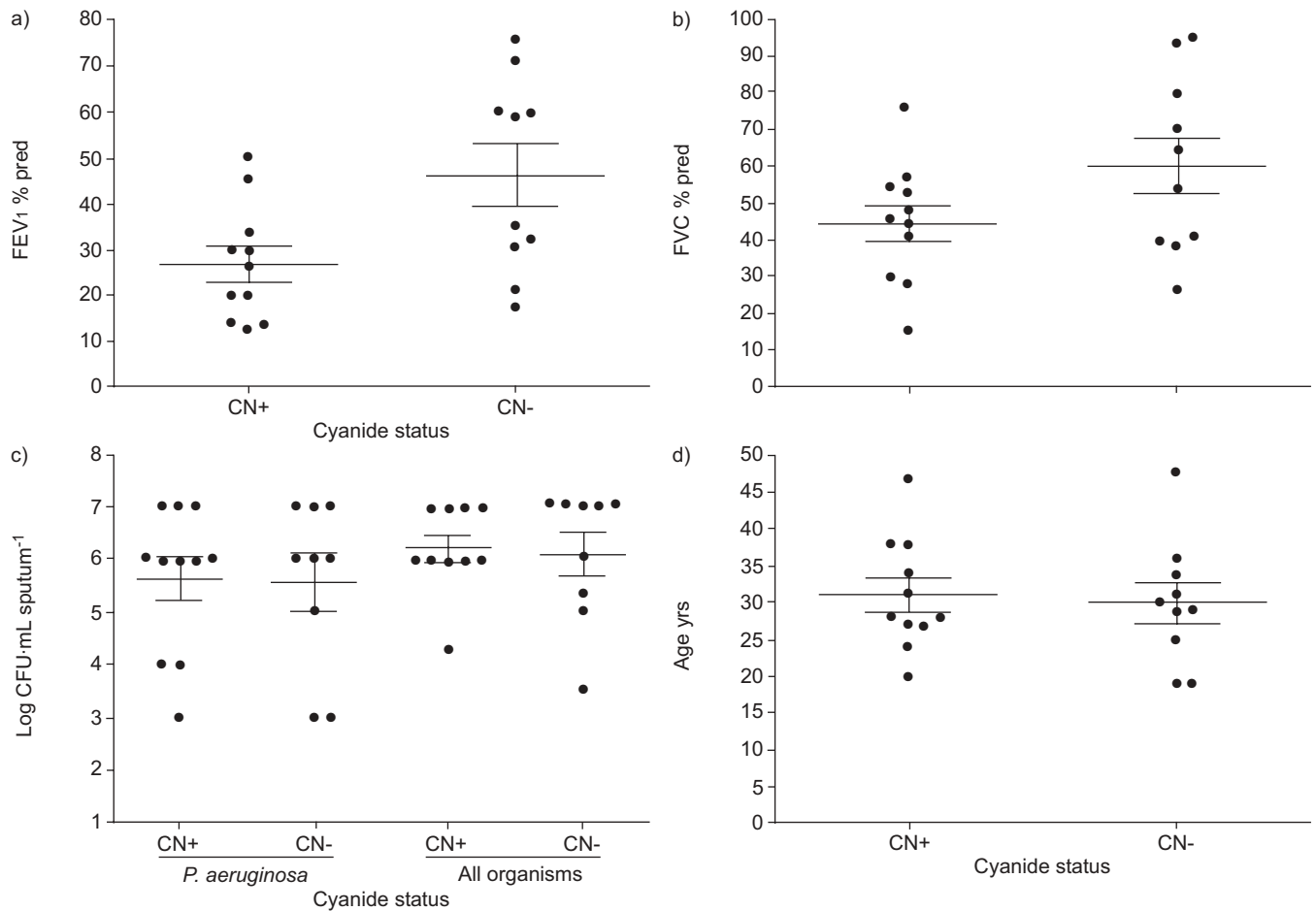
FIGURE 4. Cyanide in the sputum of *Pseudomonas aeruginosa*-infected cystic fibrosis patients was associated with impaired lung function. Cyanide was associated with a significant decrease in a) forced expiratory volume in one second (FEV1) % predicted and b) forced vital capacity (FVC) % pred. This could not be explained by differences in bacterial load of either *P. aeruginosa* alone or total pathogens (c) or by difference in patient age (d). Horizontal bars show mean value (central bars) and SD (upper and lower bars). CN+: cyanide-positive group; CN-: cyanide-negative group.

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, nonsmoking subjects with most of the cyanide being formed by saliva in the oropharynx [34]. Most interestingly, human leukocytes 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*. It cannot be ruled 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 leukocytes. However, if the cyanide the present authors detected in sputum resulted from leukocyte activation then it would be expected that cyanide accumulate, irrespective of the nature of the infecting microbe, would be seen and was 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 the present 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 as inducible nitric oxide synthase 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 polymorphonucleates 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
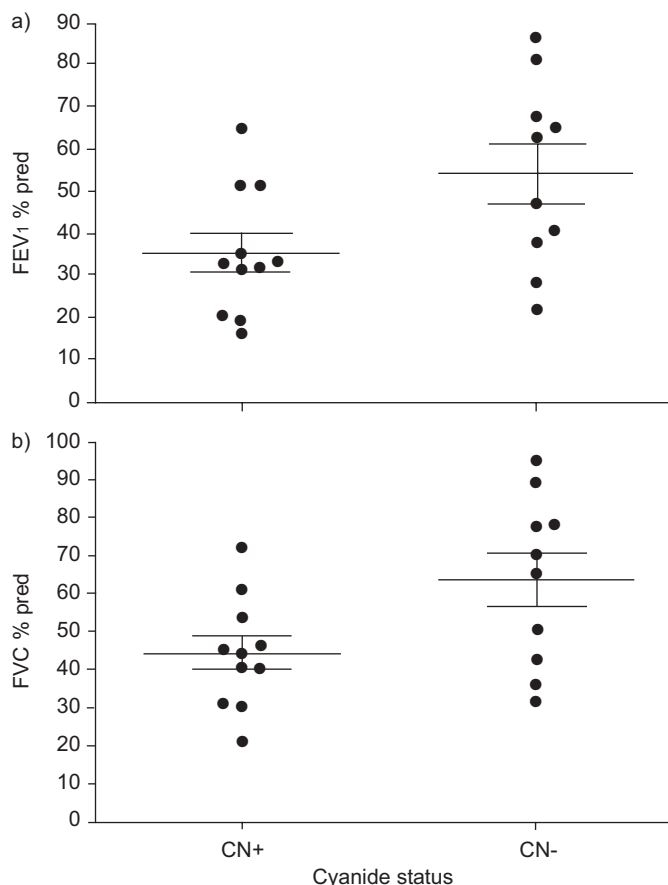
FIGURE 5. Lung function was impaired in sputum cyanide-positive cystic fibrosis (CF) patients over an extended time period. a) Average forced expiratory volume in one second (FEV$_1$) % predicted and b) forced vital capacity (FVC) % pred scores for cyanide-positive and -negative groups (CN+ and CN-, respectively) of *P. aeruginosa*-positive CF patients. Each data point is an average of the lung function scores taken over a 6-month period 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. Horizontal bars show mean value (central bars) and SD (upper and lower bars).

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 analysed were significantly reduced in *P. aeruginosa*-infected CF patients with cyanide-positive sputum compared with similarly infected patients with cyanide-negative sputum. The greatest effect was on FEV$_1$. 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, five of the study patients died and four of those 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 in 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.

The present study, though preliminary, has produced interesting and significant data. To further investigate these findings, future work will need to use longitudinal measurements to observe how cyanide levels change over time and how, in turn, this affects lung function. Following patients through a cycle from no *P. aeruginosa* noninfected 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 *Pseudomonas aeruginosa* in the lungs of cystic fibrosis and bronchiectasis patients is associated with the accumulation of cyanide in sputum. In addition, the presence of cyanide is associated with a decrease in lung function.

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