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Review

Biosignificance of bacterial cyanogenesis in the CF lung

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Abstract

Two recent studies have demonstrated the presence of biologically significant amounts of cyanide within the airways of cystic fibrosis (CF) patients infected with *Pseudomonas aeruginosa*. Whilst environmental strains of *P. aeruginosa* are known to synthesise cyanide, there has been a relative lack of investigation into bacterial cyanogenesis from a medical viewpoint, despite the role *P. aeruginosa* plays in many serious infection settings and especially in CF lung disease. This review discusses the implications of cyanogenesis in the CF airway in terms of bacterial ecology, host immune response, progression of lung disease and potential treatment options.

Keywords: Cystic fibrosis; Pseudomonas; Cyanogenesis; Toxicity; Lung

Contents

1. Introduction

Cystic fibrosis is the most common lethal genetic disorder in humans, affecting 70,000 individuals worldwide. The clinical spectrum of CF disease is multifaceted, however the main factor associated with morbidity and mortality is chronic respiratory

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infection with *Pseudomonas aeruginosa* and resultant lung tissue destruction [1]. Early lung disease is characterised by intermittent infection with a variety of respiratory pathogens and airway inflammation. Pulmonary infection with *P. aeruginosa*, a ubiquitous Gram-negative biofilm forming bacterium can be detected in a substantial proportion of young infants with CF using bronchoscopic sampling techniques [2,3]. Despite a robust local innate immune response and aggressive antibiotic therapy, *P. aeruginosa* persists in CF leading to a progressive decline in lung function and ultimately, respiratory failure. The factors underlying the dominance and persistence of *P. aeruginosa* in the CF lung are uncertain, although the adoption of a biofilm-dwelling phenotype is undoubtedly an important contributor that serves to increase antibiotic resistance and the ability to evade the host immune response [4].

Prior to the demonstration of high levels of cyanide in CF sputum from *P. aeruginosa* infected patients [5,6], it had been demonstrated that clinical strains of *P. aeruginosa* cultured *ex vivo* produce cyanide and that this contributed to killing of Drosophila melanogaster in a virulence model [7–9]. Hydrogen cyanide synthesis or cyanogenesis occurs in only a few groups of bacteria, including the fluorescent pseudomonads and cyanobacteria [10]. Cyanide production was first noted in human infection several decades ago, but the role of cyanogenesis in disease pathogenesis has not been pursued further [11]. In this review, we discuss the potential contribution of cyanogenesis to *P. aeruginosa*'s success within the CF lung.

1.1. Biosynthesis and regulation of cyanide production by P. aeruginosa (Fig. 1)

There remain many unknowns with respect to *P. aeruginosa* cyanogenesis and several of the proposed biosynthetic processes have not been confirmed experimentally, but it is has been established that glycine constitutes the amino acid substrate for cyanide production [12,13]. Conversion of glycine to cyanide is catalysed by cell membrane-associated hydrogen cyanide synthase through a process of oxidative decarboxylation, during which molecular oxygen functions as a terminal electron acceptor [8,14,15]. However, HCN synthase is rapidly inactivated by exposure to atmospheric oxygen concentrations [14,16]. The cyanide synthase enzyme is encoded by the *hcnABC* operon in *P. aeruginosa* [8]. The genetic control of this operon and hence of cyanogenesis is complex and cyanide biosynthesis occurs only under strict environmental conditions [12]. The main factors influencing cyanogenesis are decreased oxygen concentration and increased bacterial cell density [17]. Maximal production of cyanide occurs between 34 °C and 37 °C under microaerophilic conditions [17]. There are four main regulatory proteins involved in control of *P. aeruginosa* cyanogenesis; the anaerobic regulator ANR, global activator GacA, alginate regulator AlgR and RhlR [16,18]. Oxygen influences cyanide production via the anaerobic regulator, which is a transcriptional regulator exquisitely sensitive to environmental oxygen conditions [8,12,14]. The ANR protein promotes cyanide synthase expression at low oxygen levels, mediated by an oxygen responsive $[4Fe–4S]^{2+}$

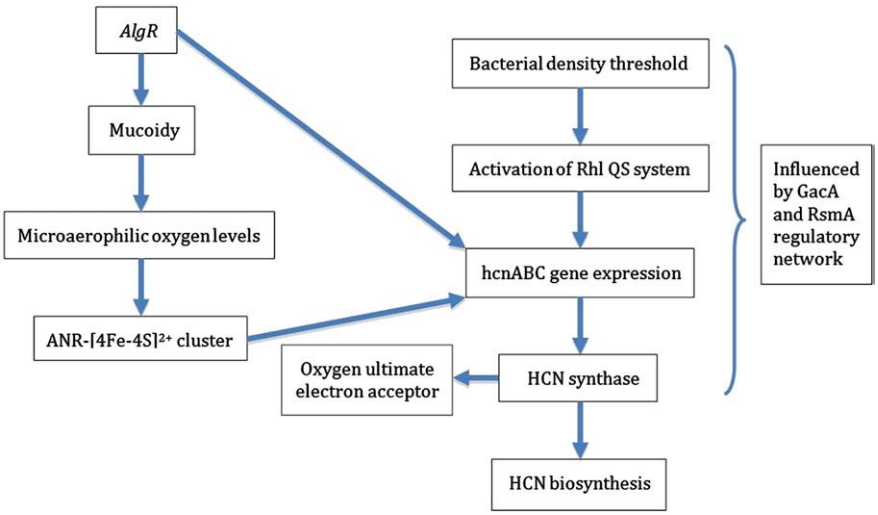

Fig. 1. Schematic representation of cyanide biosynthesis in mucoid *P. aeruginosa*. The two main control pathways are detection of an optimal oxygen-limited environment and a critical population density. The former is regulated in part by *algR*, the gene that controls alginate production (mucoidy) in *P. aeruginosa* [7]. Although not the sole determinant, alginate also limits oxygen diffusion and the low oxygen tensions experienced by bacterial cells stimulates $[4Fe–4S]^{2+}$ cluster formation and dimerization of the ANR regulator [17], which then functions to activate transcription of the genes (*hcnABC*) controlling HCN synthase. Although *P. aeruginosa* can grow under strictly anaerobic, denitrifying conditions, cyanide is not produced anaerobically as the HCN synthase requires oxygen as an electron acceptor [14]. In the other main regulatory pathway, when bacterial cell density reaches a critical threshold, Rhl autoinducers of the quorum sensing system increase in concentration and up-regulate transcription of *hcnABC* and thus cyanide production [7,8,14,15,17,28,74]. GacA — global activator involved in *hcnABC* expression at the translational and post-transcriptional levels and RsmA — repressor of secondary metabolites (mRNA binding protein that also functions at the post-transcriptional level) [10,23].

cluster, which leads to dimerization of the ANR monomers, to activate gene expression [8,14,15]. This regulatory mechanism probably reflects an evolutionary survival mechanism whereby cyanide's toxicity can be used to overcome competing microbial populations that are depleting local oxygen levels [14]. ANR also appears responsive to environmental iron conditions and increased iron may promote cyanogenesis through increased availability of Fe–S clusters and thus dimer formation with resultant up-regulated *hcnABC* expression, but these mechanisms require further investigation [10]. GacA activity appears to be regulated by cell density and influences cyanogenesis independently of ANR at both a transcriptional and post-transcriptional level through modulatory effects on the quorum sensor system (see below) and via a regulatory network involving an RNA binding protein (RsmA) respectively, but the exact mechanisms remain to be fully elucidated [10]. A comprehensive overview of the control of *P. aeruginosa* cyanogenesis is beyond the scope of this article, but there have been recent excellent reviews on this subject and we direct readers to these for further detailed information [8,15].

It has been postulated that the cyanide does not need to kill competing microbes outright, as any inhibition of their respiration will give *P. aeruginosa* a competitive advantage [14]. Although the primary action of cyanide is via impairment of cellular respiration, cyanide also binds to the iron, molybdenum and copper cofactors of metalloproteins involved in protection from oxidative stress, i.e. the superoxide dismutases (SODs). These enzyme systems are primarily expressed in aerobic bacteria, but many anaerobes also possess them, which would be advantageous within the diseased CF lung where the oxygen content varies considerably and bacteria need to rapidly respond to changing micro-environmental conditions. Cyanide production by *P. aeruginosa* in the CF lung may therefore also harm anaerobic organisms, albeit to a lesser extent than aerobic organisms [15,19,20]

The demonstration of cyanide accumulation in the CF lung has added to the ongoing debate as to whether microbes in the CF lung reside under aerobic, microaerophilic or anaerobic conditions, or perhaps as is most likely, some combination of these scenarios. The detection of sputum cyanide in reasonable concentrations suggests that a substantial proportion of the *P. aeruginosa* population in CF are living under microaerophilic conditions [6].

The quorum sensing (QS) system of *P. aeruginosa* is also involved in regulation of expression of the *hcnABC* gene cluster responsible for cyanogenesis, as well as the expression of multiple other virulence factors [7,21,22]. The QS system, particularly the Rhl system (see below), may in turn be regulated by key genes involved in biofilm formation such as *algR*, which controls the synthesis of alginate, a major constituent of bacterial biofilms and *algR* may also partly regulate transcription of the *hcnABC* operon [7]. These potential links between alginate synthesis and cyanide production, probably explain the increased cyanide production observed in mucoid strains of *P. aeruginosa* [7,22]. *Pseudomonas aeruginosa* possesses three interdependent quorum-signalling systems (Las, Rhl and PQS) [21]. Expression of the *hcnABC* synthase operon is mainly controlled by the Rhl system. Once a critical bacterial density is achieved, the Rhl QS system is activated and autoinducer signalling molecules increase in concentration and up-regulate genes, including *hcnABC*, explaining why cyanide production peaks during transition from the late exponential to stationary phase of bacterial replication [17]. There are also suggestions that the global activator GacA (see above) may increase cyanide synthesis at a transcriptional level via effects on Rhl [23]. Intracellular glycine concentrations also rise during the phase of rapid bacterial replication, but this by itself is insufficient to activate cyanogenesis [14]. The trigger for cyanogenesis is generation of local low oxygen concentrations because of high bacterial consumption during exponential growth. However, cyanide production then diminishes as bacterial cells enter the stationary phase and oxygen levels once again rise [12]. Interestingly, if anaerobic conditions are deliberately imposed during stationary phase, cyanide synthase activity will continue [12,14]. Representative of bacterial numbers in the CF lung, CF sputum may contain $>10^9$ *P. aeruginosa* per mL [24], numbers that exceed the critical cell density threshold for cyanogenesis. Cyanogenesis is likely to be encouraged by the presence of microaerophilic niches within the CF lung and regions that are frankly anaerobic may permit cyanogenesis, even during the stationary phase [14,25].

1.2. Magnitude of cyanide production by P. aeruginosa

Pseudomonas aeruginosa cultures can produce levels up to 300–500 μM cyanide when grown under cyanogenic conditions [8,17,26]. The amount of cyanide production achieved in these culture models would be sufficient to inhibit aerobic metabolism of competing organisms living in close proximity due to the ease of diffusion by this small non-ionic molecule. Microarray analyses of the transcriptome of a *P. aeruginosa* CF isolate demonstrated a fourfold increase in expression of *hcnB* when assessed directly in sputum samples compared to *hcnB* expression in the same strain when grown *in vitro* [27], suggesting robust cyanide production *in vivo*. The alginate biosynthesis transcriptional regulator (AlgR) favours both mucoidy and induction of *hcnABC* transcription during the chronic phase of *P. aeruginosa* infection, which heralds relentless clinical decline [7,28,29]. Alginate production itself may promote cyanogenesis by providing a physical barrier that limits oxygen diffusion [14,25].

1.3. P. aeruginosa protects itself against cyanide toxicity

Cyanide inhibits aerobic respiration of prokaryote and eukaryote cells by binding to the ferric (Fe^{3+}) iron of the cytochrome oxidase enzyme component of the respiratory chain. *Pseudomonas aeruginosa* avoids cyanide toxicity through a number of adaptations, including possession of an additional terminal oxidase (cyanide insensitive oxidase, CIO), which allows *P. aeruginosa* to tolerate concentrations of cyanide in the millimolar concentration range under low oxygen tensions [8,30–32]. Cyanide is also actively detoxified by *P. aeruginosa* to thiocyanate via the enzyme rhodanese (thiosulphate sulfurtransferase) [32,33].

1.4. Cyanogenesis may contribute to dominance and persistence of P. aeruginosa in the CF lung

As *P. aeruginosa* is a common soil and water inhabitant, the majority of the research has focused on the biocontrol role of cyanide in interspecies competition within these ecological niches, but whether cyanogenesis is employed in a similar role in the CF lung has not been considered.

Although *P. aeruginosa* becomes the dominant pathogen, the CF lung supports a complex community of different microbes. Interest has recently focused on the role of anaerobic bacteria, as anaerobes are frequently present in numbers that match those of *P. aeruginosa* within the CF lung [34]. *Pseudomonas aeruginosa* cyanogenesis may be partly driven by this polymicrobial infection, as a response to both population pressure and the low oxygen tensions encountered within mucus-occluded airways in the CF lung. *In vitro* experiments have demonstrated that cyanide production by *P. aeruginosa* kills *Staphylococcus aureus* suggesting that cyanide's biocontrol function may also be active in the CF lung. Cyanide may also indirectly kill bacteria within the CF lung by increasing their susceptibility to conventional antibiotics via inhibition of cytochrome oxidase-dependent drug efflux pumps [35], but these potential interactions require further investigation in what is obviously a very dynamic system.

1.5. Cyanide toxicity to host cells

Cyanide is toxic to mammalian cells, acting as a cellular asphyxiant. The chemical structural similarity to oxygen [36], explains cyanide's strong binding affinity to heme iron (Fe^{3+}), which is a key component of cytochrome-*c* oxidase (and other flavoproteins). This enzyme is involved in the respiratory chain, as part of aerobic respiration in mitochondria [37]. Cyanide is primarily a mitochondrial poison, but it affects other biochemical systems including activity of antioxidant enzymes [20]. Cyanide forces cells into anaerobic respiration causing a toxic build up of lactic acid [38]. Organs, tissues and immune cells that are metabolically active with a high oxygen demand are therefore particularly vulnerable to cyanide.

Cyanide levels between 20 and 40 μM in the systemic circulation result in cardiac toxicity and hypertension and levels between 40 and 100 μM cause neurotoxicity, coma and ultimately death [39]. Fatal blood cyanide concentrations are variably reported to be >25 μM to >100 μM [40,41]. The levels reported in CF sputa (up to 130 μM) are thus well within the toxic range for cell aerobic respiration [5]. Levels may relate to the clinical status of the patient, being higher in patients who are acutely unwell and they are significantly lower following a course of intravenous antibiotics [6]. Ryall et al. demonstrated that sputum cyanide was negatively correlated with lung function, perhaps indicating that more advanced lung disease may facilitate cyanide production or alternatively, that cyanide itself is contributing to disease severity, but prospective studies are needed to adequately address this possibility. However, regardless of the cause and effect question in this context, the mean sputum cyanide concentrations reported in these two recent studies (34 μM; Sanderson et al., and 72 μM; Ryall et al., respectively) are well within the range reported to be cytotoxic [5,6].

Interestingly, cyanogenesis may not be limited to *P. aeruginosa* in the CF lung as strains of the *Burkholderia cepacia* complex (Bcc), which also infect the CF lung, have recently been shown to synthesise cyanide *ex vivo* in concentrations comparable to *P. aeruginosa* [42]. These observations raise the potential for cyanide to contribute to the development of necrotising pneumonia and the "cepacia syndrome," that is usually fatal in Bcc infected patients but this requires further investigation [43].

1.6. Detoxification of cyanide by host cells

The body has the ability to detoxify cyanide through conversion to thiocyanate, which is then excreted in urine. This conversion requires thiosulfate and the related enzymes rhodanese and mercaptopyruvate sulfurtransferase (MST), which both occur widely in mammalian tissues, especially the liver and kidney, including circulating red blood cells [44–47]. There are also minor non-rhodanese pathways for detoxification of cyanide, for example complex formation with hydroxycobalamin (a vitamin B_{12} precursor) [48]. The effects of cyanide are exacerbated by poor nutrition by reducing the amount of available thiosulphate, an important component in endogenous cyanide detoxification [49]. Poor nutritional status is common in CF and has been related to lung disease severity [50]. It is worth speculating that CF-related malnutrition may impair the body's endogenous detoxification of cyanide, contributing to the long term deleterious effects of *P. aeruginosa* on disease progression.

1.7. Cyanide production and the host innate immune response

Chronic *P. aeruginosa* colonisation of the CF lung is characterised by a neutrophil dominated inflammatory response. In normal lungs, neutrophils account for approximately 1% of all cells within the airway lumen, whereas in the CF airway they account for up to 90% of the inflammatory cell population and increased neutrophilic inflammation is observed even in very young infants [51]. This florid neutrophilia is ineffective at clearing infection and damages local tissues by the production of excessive amounts of reactive oxygen species (ROS) and proteases. It is generally agreed that the respiratory failure associated with CF morbidity is primarily due to ongoing and excessive activation of neutrophils within the airways. The apparent failure of the innate immune system in CF has been the subject of many studies and an excellent review of this subject was recently published and we refer readers to this article for further in-depth background information [52].

1.8. Potential effects of cyanide on neutrophil function in CF

The microbicidal function of neutrophils is a multi-step process involving chemotaxis, phagocytosis, degranulation and the respiratory burst (see Fig. 2). Review of the available

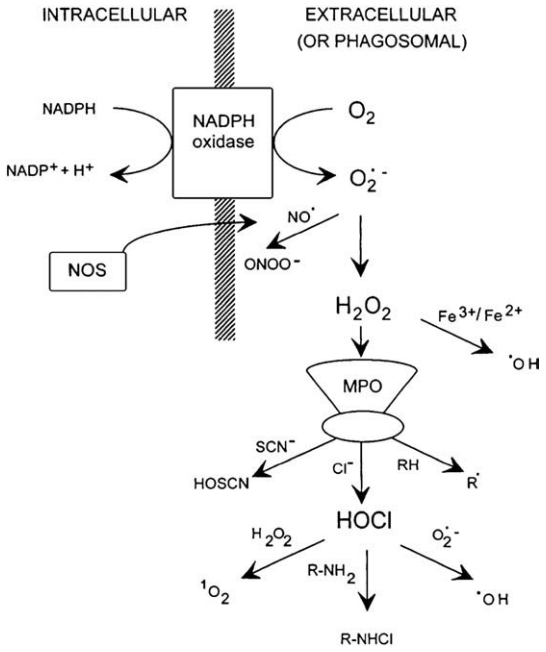

Fig. 2. Enzymatic reactions and products involved in the oxidative burst in neutrophils. Phagocytosis is followed by the "respiratory oxidative burst" to enable bacterial killing. This is a sequential process that involves the nicotinamide adenine dinucleotide phosphate (NADPH) system oxidising molecular oxygen to ROS including superoxide (O_2^-) and production of hydrogen peroxide (H_20_2). These oxidants are further catalysed to hypochlorous acid (HOCl) by myeloperoxidase (MPO). Reproduced by kind permission from Hampton et al. [75,76].

literature on the effects of cyanide on components of neutrophil function provides conflicting reports. Classically, neutrophil migration and phagocytosis have been considered to be glycolysis-dependent, which is a non-mitochondrial energy generating process and therefore theoretically relatively immune to the effects of cyanide [53,54]. However, neutrophils possess a mitochondrial network that is necessary for maintenance of the integrity of the neutrophil cytoskeleton, which is a prerequisite for normal migration and phagocytosis [55–57]. Disruption of the neutrophil cytoskeleton as a consequence of mitochondrial poisoning by cyanide has been shown to cause morphological distortions, with cell flattening and enlargement of nuclear structures [54]. The functional consequences of these shape alterations have not been adequately investigated, but a study by Fossati et al. has demonstrated impaired chemotaxis in these abnormal neutrophils [54]. Review of the available literature on the effects of cyanide on other components of neutrophil function provides conflicting reports, with some studies demonstrating inhibitory effects on the oxidative burst, or specific components that are required for bacterial killing, i.e. chlorination of bacterial cell membranes [58], whereas some investigators have demonstrated an enhanced oxidative burst [59,60] and others no discernible effect. Whether stimulatory or inhibitory, the effects of cyanide on the oxidative burst of neutrophils are probably mediated via modulation of NADPH oxidase activity (see Fig. 2). Cyanide may also inhibit the activity of neutrophil catalase, which normally scavenges H_2O_2 to limit its toxicity, thus allowing oxidative stress to continue unabated, which is a characteristic of CF lung disease [61–63]. The results of studies of cyanide effects on neutrophil phagocytosis are also somewhat conflicting, although the majority suggest that there is no significant inhibition [54,64]. The potentially important effects of cyanide on neutrophil function in the CF lung need further investigation, to determine whether cyanide may contribute to impaired bacterial clearance and increased production of damaging pro-oxidants.

2. Targeting cyanide therapeutically

2.1. The use of cyanide antidotes

The binding reaction between cyanide and cytochrome-*c* oxidase can be reversed by administration of nitrites, sodium thiosulphate and hydroxocobalamin and these compounds are used as antidotes to systemic cyanide poisoning [65]. Nitrites alter the redox state of iron in haemoglobin, reducing a small proportion of ferrous (Fe^{2+}) haemoglobin to methaemoglobin (Fe^{3+} haemoglobin), which has a higher binding affinity to cyanide than cytochrome oxidase. Cyanide bound to methaemoglobin is non-toxic, but excessive methaemoglobin will compromise oxygen carriage and this would be a limiting factor to consideration of the use of nitrites in the long term [39].

Sodium thiosulphate acts as a sulphur donor for rhodanese enzymes, catalysing the conversion of cyanide to thiocyanate, which is then excreted in the urine. This is the body's natural cyanide detoxification mechanism, but its use as a substrate is limited by the availability of rhodanese. Although, sodium thiosulphate acts more slowly than alternative antidotes, there are no serious side effects associated with short-term use at the recommended dose [37] and so it may well present a viable therapeutic approach to minimise cyanide toxicity in the CF lung, although whether systemic administration would be effective as an antidote in the lung would need assessment.

The vitamin B_{12} precursor, hydroxocobalamin chelates cyanide to form the non-toxic cyanocobalamin, which is excreted in the urine. Hydroxocobalamin (alone or in combination with sodium thiosulfate) has been successfully used in patients receiving prolonged intravenous sodium nitroprusside to prevent cyanide toxicity and hydroxocobalamin treatment improves survival following cyanide poisoning [48,66]. Cobinamide is a precursor of hydroxocobalamin, which is effective as a cyanide antidote at much lower doses [67]. Cobinamide is attractive in CF as it can be delivered by inhalation, which has previously led to discussion of its use as a possible cyanide antidote in the CF lung [67]. However, to date there are no data available on the use of any of these antidotes in CF patients.

2.2. Manipulating the CF micro-environment; therapeutic potential of supplementary oxygen

An alternative strategy is to consider whether cyanide production by *P. aeruginosa* can be reduced within the CF lung. Hyperbaric oxygen therapy (HBOT) may be a potential means by which the CF lung micro-environment could be altered to switch off *P. aeruginosa* cyanogenesis. The benefits of HBOT

in the setting of chronic soft tissue infections may be partly explained by effects on biofilm-dwelling bacteria, although there are very few data on this subject [68]. The main consideration in CF would be whether HBOT would have adverse effects on lung mechanics and such therapy would only be suitable for use in short pulses and reserved for acutely unwell patients [69].

2.3. Cyanide as a potential biomarker for Pseudomonas infection status

The use of selected ion flow tube mass spectrometry (SIFT-MS) has been studied as a non-invasive method for detecting volatile compounds from cultured sputa or direct breath analysis in a variety of airway diseases, including CF [70]. Measurement of cyanide as a biomarker of *Pseudomonas* infection has been undertaken in CF patient saliva and exhaled breath [71]. Cyanide detection as an indicator of *P. aeruginosa* infection would be especially useful as a non-invasive marker in paediatric patients and allow the effectiveness of eradication therapy to be accurately assessed, as well as provide early warning of impending exacerbation in both children and adults. An alternative would be to assess the presence of stable cyanide metabolites, such as thiocyanate in biological fluids. The existence of methods for detecting thiocyanate levels in plasma and urine lend rationale to this approach and thiocyanate has recently been detected in the saliva of children with CF [72].

3. Conclusion

In summary, cyanide is an important metabolic product of *P. aeruginosa* that has potential implications in terms of exacerbating lung damage, promoting disease progression and reducing the effectiveness of the host response in CF. Further research is needed, but confirming the disease-modifying role of cyanogenesis in CF lung disease may lead to new therapies to directly reduce toxicity, as well as the development of biomarkers that could be used to optimise therapy options. The recent successful detection of cyanide and metabolites in biological samples from *P. aeruginosa* infected individuals, including exhaled breath condensates, opens the door to improved monitoring of targeted anti-*P. aeruginosa* interventions and possibly screening for early infection and impending exacerbation [71,73].

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