The cystic fibrosis lung microenvironment alters antibiotic activity: causes and effects

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Shareable abstract (@ERSpublications)
Bacterial and host mediators in the CF lung milieu influence antibiotic susceptibility of Pseudomonas aeruginosa, and vary between patients. This could explain why current antibiotic susceptibility tests poorly predict activity in patients. https://bit.ly/3vtYsRU

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Abstract
Chronic airway colonisation by Pseudomonas aeruginosa, a hallmark of cystic fibrosis (CF) lung disease, is associated with increased morbidity and mortality and despite aggressive antibiotic treatment, P. aeruginosa is able to persist in CF airways. In vitro antibiotic susceptibility assays are poor predictors of antibiotic efficacy to treat respiratory tract infections in the CF patient population and the selection of the antibiotic(s) is often made on an empirical base. In the current review, we discuss the factors that are responsible for the discrepancies between antibiotic activity in vitro and clinical efficacy in vivo. We describe how the CF lung microenvironment, shaped by host factors (such as iron, mucus, immune mediators and oxygen availability) and the microbiota, influences antibiotic activity and varies widely between patients. A better understanding of the CF microenvironment and population diversity may thus help improve in vitro antibiotic susceptibility testing and clinical decision making, in turn increasing the success rate of antibiotic treatment.

Introduction
Cystic fibrosis (CF) is the most common life-threatening inheritable disease in the Caucasian population, as one in 3000–4000 newborns is affected [1]. Due to mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR), which results in mucus dehydration and stasis, the airways of patients with CF are particularly susceptible to chronic bacterial infections. These infections are followed by an aggressive inflammatory response, resulting in progressive airway tissue damage, lung function decline and premature death [1].

While a decreasing prevalence of the opportunistic pathogen Pseudomonas aeruginosa has been observed in patients with CF in recent years [2], it is still one of the most important causes of morbidity and mortality in the CF population [3]. The use of CFTR modulator drugs, which restore at least in part the underlying genetic defect, have shown promise in delaying the initial acquisition of P. aeruginosa, yet the long-term effects on chronic colonisation of this pathogen remain to be determined [4]. Annual reports of various national CF registries show that the percentage of the CF population with a positive culture for P. aeruginosa increases with older age, with a stabilisation at around 50–70% after the age of 20–25 years [2, 3]. The mean age of the first P. aeruginosa acquisition ranges between 6.5 and 7.1 years [5]. Although eradication of these early infections is feasible with antibiotic therapy, intermittent and chronic infections emerge more often as patients grow older [3]. The switch from intermittent to chronic infections is mediated by the ability of P. aeruginosa to form biofilms, which are communities of aggregated bacteria present in a self- or host-produced matrix that provide shelter from the immune system and antimicrobials [6, 7]. Antibiotic therapy is used both for eradication of the initial infection and for control of chronic...
infections, but also in periods of pulmonary exacerbations. For early eradication therapy, regimens differ between CF centres, with no clear consensus on the most effective one [8]. A study comparing the combination of oral ciprofloxacin and inhaled tobramycin with the combination of oral ciprofloxacin and inhaled colistin showed both treatments were equally effective (successful eradication in 66% of the patients) [9]. Chronic P. aeruginosa infections are typically managed (i.e. slowing down disease progression, as eradication is often no longer possible) by inhalation therapy with nebulised tobramycin, colistin, aztreonam or levofloxacin or with dry powder inhalators of ciprofloxacin or tobramycin [10–13]. The treatment of pulmonary exacerbations, however, is primarily based on the administration of oral (ciprofloxacin) or intravenous antibiotics (β-lactam antibiotics combined with aminoglycosides or colistin) with within- and between-centre differences in antibiotic choice [14, 15]. Although the decision on which antibiotic to administer conventionally depends on the results of standardised antibiotic susceptibility testing (AST), a poor correlation exists between the outcome of AST and the effectiveness of antibiotics to control chronic infections and treat pulmonary exacerbations in vivo [16, 17]. Therefore, antibiotic choice is mostly empirically based in these cases and driven by the experience of physician and patient, including previous toxicity or intolerance, rather than on in vitro test results [18]. Other factors determining the antibiotic choice are practical reasons in the case of outpatient parenteral antibiotic therapy, or previous or current colonisation with other pathogens such as Staphylococcus aureus [19]. This discordance between in vitro activity and in vivo efficacy has, to our knowledge, not yet been described for early eradication therapy. Nevertheless, the success of antibiotic treatment for early eradication varies between patients, which further challenges the current one-treatment-fits-all approach [9, 18]. The reasons for this lack of predictability of treatment success, and the discrepancy between in vitro activity and in vivo antibiotic efficacy, are poorly understood. A few reports have linked specific phenotypic traits of P. aeruginosa isolates, such as mucoidy or diminished motility, to unsuccessful eradication (reviewed in Jackson and Waters [20]), while others did not find any association between phenotype and persistence. Therefore, there is an increasing interest in understanding the role of the CF lung microenvironment, which comprises host factors (e.g. nutrients, metabolites, immune cells and their mediators) and bacterial factors (lung microbiota) in the bacterial response to antibiotics.

Here we provide an overview of microenvironmental factors in the CF lung that are encountered during the infection process of P. aeruginosa and have been documented to influence the activity of antibiotics in vitro. These microenvironmental factors influence the susceptibility of P. aeruginosa to antibiotics directly, and on top of that, intra- and interpatient variability in the microenvironment can lead to diversification in the bacterial population, affecting antibiotic susceptibility. In the first part of the present review, we will provide an overview of host and microbial factors influencing antibiotic activity towards P. aeruginosa, both in early and chronic infections. In the second part, we highlight intra- and interpatient variability in the microenvironment, and its downstream effect on P. aeruginosa susceptibility. These collective insights may offer explanations for the difficulties in eradicating P. aeruginosa chronic infections and the high interpatient variability in therapeutic success, and identify knowledge gaps that may guide us towards optimisation of antibiotic use in patients with CF.

**Host factors in the lung microenvironment influencing antibiotic activity**

Microenvironmental factors can influence the activity of antibiotics against P. aeruginosa through different mechanisms: 1) by altering bacterial physiology in a way that impacts tolerance; 2) by interfering with biofilm formation; and/or 3) by influencing mucus composition (figure 1). Host microenvironmental factors that influence antibiotic activity and their mode of action are discussed below and summarised in table 1.

**High iron availability**

CF airway secretions contain high concentrations of iron (on average 242 ng·mg⁻¹ in CF sputum while it is nondetectable in non-CF sputum) [64]. Firstly, airway epithelial cells, homozygous for the most common CFTR mutation (F508del), release more iron than healthy epithelial cells in vitro due to a lower expression of haemeoxygenase-1 resulting in higher intracellular iron levels [65]. Secondly, inflammatory and epithelial cell death is induced by bacterial pathogens and an aggressive immune response, which releases intracellular ferritin [66]. Ferritin, an innate defence molecule able to sequester ferric ions (Fe³⁺), can readily release these ions in the presence of siderophores produced by P. aeruginosa [66]. P. aeruginosa produces two types of siderophores, pyoverdine and pyochelin; both contribute to the pathogen’s virulence by depriving host cells of iron, providing iron for bacterial growth, stimulating the production of other virulence factors and promoting biofilm formation [67]. Interestingly, the production of siderophores is typically diminished in isolates that are adapted to the CF airways [68]. In most biological environments, ferric iron is the most common ionic state of iron. In the CF lung, however, the high levels of neutrophilic superoxides, low oxygen levels and acidic pH are believed to contribute to the high
percentage (56%) of ferrous ions (Fe$^{2+}$) being present [69]. *P. aeruginosa* is able to take up both ferric and ferrous iron [69]. High iron levels can influence the activity of antibiotics in multiple ways. Firstly, higher iron levels influence *P. aeruginosa* biofilm formation and thus antibiotic activity. Indeed, airway epithelial cells, homozygous for F508del, increase biofilm formation in comparison to airway epithelial cells with functioning CFTR, leading to higher tolerance to tobramycin [21]. Furthermore, iron availability and the transport of ferric iron by the siderophore pyoverdine is necessary for biofilm formation, with higher iron concentrations required in anoxic conditions [70]. High iron levels were also reported to modify biofilm structure (i.e. biofilm thickness and biomass), whereas iron sequestration, a possible treatment strategy, resulted in a lower biomass of the lab strain *P. aeruginosa* PAO1 and some clinical isolates [71]. This biofilm promoting effect of high iron levels was found to be the result of Psl production, an important exopolysaccharide [22]. Psl decreases antibiotic activity towards *P. aeruginosa* not only in biofilms, but also in planktonic cells by electrostatic interaction between Psl and antibiotics (tobramycin, colistin, polymyxin B and ciprofloxacin) [23]. The possible therapeutic use of iron chelators is, however, challenged by the finding that iron limitation can induce production of the exopolysaccharide alginate and result in mucoidy [72], which in turn impacts antibiotic penetration [73].

Secondly, iron can influence antibiotic activity through its role in the production of reactive oxygen species (ROS), mediated by the Fenton reaction. High levels of intracellular ferrous iron (Fe$^{2+}$) shift the balance of the Fenton reaction in favour of ROS production. Downstream effects thereof on antibiotic activity are discussed in the section “Mediators of the immune response”.

**Viscous mucus**

Mucus in CF airways is characterised by a higher concentration of mucins, such as MUC5AC and MUC5B, due to volume depletion caused by the CFTR defect and increased mucin production as a consequence of bacterial infection and inflammation [74]. CF lung mucus also contains large amounts of extracellular DNA (eDNA, 3–14 mg·mL$^{-1}$) and actin [75, 76]. Variable sources of eDNA in CF sputum have been described, including living bacterial cells that actively secrete DNA [77], DNA released from bacterial membrane vesicles [78], lysed bacterial cells [79] and neutrophil NETosis and/or necrosis. The latter can be induced by *P. aeruginosa* virulence factors (e.g. rhamnolipids) or by the combination of bacterial ingestion and airway surface liquid acidification [80, 81]. The high levels of actin in CF sputum are also a consequence of excessive neutrophilic inflammation, since neutrophils induce actin polymerisation in response to chemotactic stimulation [82]. In turn, actin filaments improve eDNA fibre formation in CF mucus [83].

The specific composition of CF mucus (high mucin content, eDNA and actin) leads to a high viscosity, which lowers the diffusion rate and impedes the efficient penetration of antibiotics [27, 31, 34]. In addition to increasing viscosity, mucins and eDNA – which have a net negative charge [84] – interact with the positively charged aminoglycosides, colistin and polymyxin B [25, 30, 34]. Mucins can also interact with antibiotics (aztreonam, ceftazidime and levofoxacin) through hydrophobic interactions [26]. Additionally,
| Host factor         | Antibiotic(s)$^a$                                      | Antibiotic activity$^a$ | Mechanism                                                                 | References |
|---------------------|--------------------------------------------------------|-------------------------|---------------------------------------------------------------------------|------------|
| High iron availability | Tobramycin | ↓ | Increased biofilm formation | [21] |
|                     | Tobramycin, colistin, polymyxin B, ciprofloxacin      | ↓ | Increased PsI production, changed biofilm structure and enhanced antibiotic tolerance | [22, 23] |
| Mucus               |                                                        |                         |                                                                           |            |
| Mucins              | Tobramycin, colistin, polymyxin B, levofloxacin, aztreonam, ceftazidim | ↓ | Antibiotic binding through charge and/or hydrophobic interactions | [24–26] |
|                     | Gentamicin, β-lactam antibiotics                       | ↓ | Higher viscosity                                                        | [27] |
|                     |                                                        |                         | Lower diffusion rate                                                     | [22, 23] |
|                     | Aminoglycosides, colistin, polymyxin B, norfloxacin, ciprofloxacin, β-lactam antibiotics | ↓ | Induction of expression of resistance genes | [28] |
|                     | Tobramycin                                            | ↓ | Increased biofilm formation                                              | [29] |
| Extracellular DNA   | Tobramycin                                            | ↓ | Charge interaction                                                      | [30, 31] |
|                     | Aminoglycosides, colistin, polymyxin B                | ↓ | Mg$^{2+}$ sequestration and acidification, leading to LPS modification | [32, 33] |
| Actin               | Tobramycin, colistin                                  | ↓ | Higher viscosity, leading to lower antibiotic diffusion                 | [34] |
| Anoxia              | Aminoglycosides                                       | ↓ | Low metabolism                                                         | [35–37] |
|                     | β-lactam antibiotics                                   | ↓ | Higher expression of efflux pumps                                      | [38, 39] |
|                     | Tobramycin                                            | ↓ | Increased alginate production                                          | [40–43] |
|                     | Colistin                                              | ↑ | Low metabolism leads to inactivation of adaptive resistance mechanisms | [38, 39] |
| Nutritional environment | Low glucose levels, high amino acid levels           | Tobramycin, ciprofloxacin | Development of auxotrophy and co-occurrence LasR mutations                | [44–46] |
|                     | High glucose levels                                   | Ofloxacin               | Promotion of biofilm formation by inducing PsI production                 | [47, 48] |
| NaCl content and pH |                                                       |                         |                                                                           |            |
| High NaCl content   | Tobramycin, streptomycin                              | ↑ | Reduced alginate binding                                                | [49, 50] |
|                     | Colistin, ciprofloxacin                               | ↑ | Synergism                                                              | [51, 52] |
| Acidic pH           | Tobramycin, ceftazidim, ciprofloxacin                 | ↓ | Induction of biofilm formation                                          | [53] |
| Immune response     |                                                        |                         |                                                                           |            |
| LL-37               | Gentamicin, ciprofloxacin                             | ↓ | Upregulation of multidrug efflux pumps                                 | [54] |
| Lactoferrin         | Tobramycin                                            | ↓ | Depolarization of bacterial membrane, leading to less antibiotic uptake | [55] |
| IL-1 β, IL-6, IL-17 and neutrophil elastase | Aspecific     | ↓ | Induction of mucin production                                           | [56] |
| Itaconaat           | Aspecific                                             | ↓ | Induction of biofilm formation through EPS formation                    | [57] |
| ROS                 | Aspecific                                             | ↓ | Increased alginate production                                          | [58] |
|                     | Aminoglycosides                                       | ↓ | Higher oxidation level of mucins, leading to less antibiotic diffusion  | [59, 60] |
|                     |                                                       |                         | Higher occurrence of hypermutators, which is linked to higher occurrence of persisters | [61] |
|                     |                                                       |                         | Induction of efflux pump gene expression                                | [62] |
|                     |                                                       |                         | Reduced membrane potential and modification of LPS                      | [63] |

LPS: lipopolysaccharide; EPS: exopolysaccharide; ROS: reactive oxygen species; $^a$: if the effects are described for more than two antibiotics of an antibiotic class, the class is mentioned; if a host factor has a speculative, indirect effect on antibiotic activity in general, the term “aspecific” is used; $^b$: the definition of antibiotic activity differs between studies; in some studies activity is measured by minimal inhibitory concentration and in other studies by performing biofilm experiments, ranging from biofilms formed on plastic to biofilms in the presence of in vivo-like epithelial cell models.
eDNA and mucins influence expression of genes involved in antibiotic resistance. Specifically, eDNA interferes with the PA3552-PA3559 cationic antimicrobial peptide (CAP) resistance operon, resulting in a lipopolysaccharide (LPS) modification and thus reduced cell wall permeability to aminoglycosides and polymyxins [32]. This effect is due to Mg\(^{2+}\) sequestration and acidification by eDNA [32, 33]. Similarly, Sun et al. [28] described that mucins influence the expression of multiple resistance genes leading to broad spectrum adaptive antibiotic resistance. This altered gene expression also results in enhanced surfing motility, a form of surface adaptation, in which mucins act as a surfactant allowing rapid surface motility of \(P.\) aeruginosa [28].

In addition, interactions of \(P.\) aeruginosa with mucins are reported to result in the formation of larger cellular macrocolonies with increased antibiotic tolerance compared with biofilms formed on uncoated surfaces or surfaces coated with DNA or actin [29].

**Hypoxia**

As a consequence of mucus stasis, \(P.\) aeruginosa is exposed to low oxygen levels in the CF airways. Indeed, the thick mucus layer impedes oxygen diffusion, leading to steep hypoxic gradients in thick, mucopurulent masses [40]. Additionally, higher oxygen consumption in CF epithelial cells and neutrophils, a side-effect of the higher sodium uptake due to CFTR malfunction, further contributes to hypoxia [85]. Moreover, \(P.\) aeruginosa penetrates in mucopurulent masses shortly after infection and consumes the remaining oxygen, leading to complete anoxia locally [40]. \(P.\) aeruginosa is able to grow in anaerobic conditions in the presence of an alternative terminal electron acceptor (e.g. nitrate) [35] or by arginine fermentation [86]. Nitrate is present in higher concentrations in CF sputum [35, 87], which is a consequence of the chemical reaction between the immune effectors nitric oxide and superoxide [87]. High levels of nitrate stimulate anaerobic respiration and thus enable bacterial metabolism in anoxia.

Reduced antimicrobial activity in anoxia has been reported for aminoglycosides (tobramycin, amikacin and gentamicin), aztreonam, ceftazidim and the combination of penicillins with a β-lactamase inhibitor (piperacilline-tazobactam and ticarcillin-clavulanic acid) [75, 89–92]. Anaerobic conditions have a minimal influence on the antibiotic activity of fluoroquinolones (ciprofloxacin and levofloxacin) [75, 89–92]. In contrast, an enhanced antimicrobial activity of tetracyclins and colistin in anoxia has been described [38, 90, 93]. Remarkably, although the general trend is clear, variability in outcomes between studies describing the influence of anoxia on antibiotic activity is observed. This variability can possibly be explained by the different strains of \(P.\) aeruginosa tested, with a profound difference between lab strains [38, 90, 91] (e.g. PAO1, PA14) and CF clinical isolates [89, 93, 94] and the use of different growth media (especially differences in arginine or NO\(^3\)\(^–\) levels could influence antibiotic activity in anoxia) [36].

The reduced activity of aminoglycoside antibiotics in anoxic conditions can be attributed to a lower bacterial metabolic rate. Many studies demonstrated an increased antibiotic susceptibility in anoxia upon addition of nitrate, nitrite or arginine, which stimulated metabolism [35–37]. The opposite has been reported for colistin, which has higher activity towards metabolically inactive cells [38, 39]. Indeed, metabolically inactive cells are incapable of activating energy-consuming adaptive resistance mechanisms, such as expression of efflux pumps and/or alteration of LPS (the target of colistin) [39]. Alternatively, inhibition of efflux pumps in anoxia led to a susceptibility profile similar to that in normoxia for ceftazidim, aztreonam, piperacilline-tazobactam and ticarcilline-clavulanic acid, indicating that efflux pumps (MexEF-OprN) are responsible for the low activity of these antibiotics in anoxic conditions [92].

Finally, anoxia leads to increased alginate production by \(P.\) aeruginosa, even in nonmucoid isolates, resulting in the formation of a more robust biofilm. As a consequence, this mechanism can contribute to higher tolerance towards antibiotics [40–43].

**Nutritional environment**

Wetmore et al. [95] reported an altered glucose metabolism in primary human airway epithelial cells from CF patients compared with non-CF cells, leading to lower levels of glucose in CF airways. Lower levels of carbon sources such as glucose may result in decreased bacterial production of electron donors (e.g. NADH), a lower proton motive force (PMF), reduced levels of ROS (essential for the activity of some antibiotics) and thus higher tolerance to antibiotics which uptake depends on the PMF, such as aminoglycosides [7]. However, 29% of the adult CF population has CF-related diabetes, and acute hyperglycaemia has been linked to high airway glucose concentrations [2, 47]. These high glucose levels have not only been associated with a higher bacterial load but also promote biofilm formation, through induction of Psl production [47, 48].
In addition, the increased neutrophilic protease activity in CF airways leads to a higher level of amino acids in CF airways (5–25 mM) compared with healthy airways [96]. *P. aeruginosa* adaptation to this specific nutritional environment (low glucose levels and high amino acid levels) often involves the development of auxotrophy and co-occurring LasR mutations, which are linked to fluoroquinolone and aminoglycoside tolerance [44–46]. Finally, the elevated levels of amino acids have been shown to be important to form tight biofilm-like macrocolonies, similar to those found in the CF airways [97].

**NaCl content and pH**

Sputum NaCl concentrations in patients with CF are typically higher (10.5 g·L$^{-1}$) in comparison with healthy controls (7.4 g·L$^{-1}$) [88] as a consequence of CFTR malfunction. This high NaCl concentration can contribute to the vulnerability of CF airways to bacterial infections, by inhibiting the effect of endogenous antibacterial defences [98]. However, high NaCl concentrations can also lead to higher antibiotic activity by reducing alginate binding to antibiotics (aminoglycosides, tobramycin, streptomycin) [49, 50]. Furthermore, a synergistic effect of NaCl on the antibiotic activity of colistin and ciprofloxacin was observed [51, 52].

Another secondary effect of CFTR malfunction is the acidification of the airway surface liquid (ASL) which has been reported both *in vitro* and *in vivo* [99, 100]. Recently, Liu et al. [53] have shown that low pH does not only lead to higher MIC (minimal inhibitory concentration) values, but also promotes biofilm formation and lowers the antibiofilm activity of tobramycin, ciprofloxacin and ceftazidime. Low pH also impacts the activity of antimicrobial peptides that are present in the ASL, such as β-defensins and LL-37, which exhibit synergistic activity with antibiotics under normal pH conditions [101].

**Mediators of the immune response**

As a result of chronic airway inflammation, CF lung mucus contains high levels of cytokines, proteases, oxidants and host antimicrobial compounds [102]. Although the excessive immune response aims at eradicating the infection, *P. aeruginosa* often persists, and several components of the immune response actually contribute to bacterial survival.

LL-37, an antimicrobial peptide, is produced by airway epithelial cells and neutrophils upon pathogen-recognition and thus is a part of the innate immune defence [103]. The levels of LL-37 in CF sputum correlate well with inflammation state [103]. LL-37 is capable of both inhibiting biofilm formation and eradicating preformed biofilms [104]. However, at physiological concentrations LL-37 leads to an upregulation of multidrug efflux pump genes (encoding MexCD-OprJ and MexGHI-OpmD) [54]. This leads to a decreased susceptibility of *P. aeruginosa* to ciprofloxacin and gentamicin.

The host-produced iron-chelator lactoferrin prevents *P. aeruginosa* biofilm formation *in vitro* [71, 105], but it seems unlikely this is relevant *in vivo*, as iron is abundantly present in CF sputum (see above). However, lactoferrin promotes antibiotic tolerance towards tobramycin, by depolarising the bacterial membrane and decreasing antibiotic uptake [55]. Recent results in a mouse model of chronic *P. aeruginosa* infection showed lactoferrin reduced excess iron levels, bacterial load and inflammation suggesting it could have potential as a new therapeutic [106]. However, the observed induction of antibiotic tolerance *in vitro* [55] suggests possible undesired side-effects.

Pro-inflammatory cytokines are soluble proteins, secreted by immune cells and epithelial cells after pathogen-recognition, with initiation and stimulation of the immune response as a consequence [102]. Besides being essential mediators of the immune response, some pro-inflammatory cytokines (*i.e.* IL-1β, IL-6 and IL-17) stimulate the production of MUC5AC and MUC5B [56, 107], hereby further impeding mucociliary clearance [108] and promoting biofilm formation [29]. Similarly, neutrophil elastase, the major neutrophilic protease which is highly abundant in CF lungs [109], induces MUC5AC production [110].

Secretion of metabolites, such as succinate and itaconate, as a result of pathogen-induced immunometabolic changes in immune cells, has been reported to be altered in CF [111]. Excessive succinate secretion in CFTR mutant macrophages has been shown to be used by *P. aeruginosa* for growth, while secreted itaconate induces biofilm formation through the production of exopolysaccharides (EPS) [57]. In addition, succinate and other metabolites have been demonstrated to alter antibiotic activity against *P. aeruginosa* [7, 112, 113].

Excessive neutrophilic inflammation, together with the previously discussed iron imbalance in CF airways, leads to high levels of ROS [114]. Additionally, ROS can not be inactivated efficiently as glutathion, a host antioxidant, is produced at lower levels in the CF airways [115]. The resulting high ROS levels have variable (indirect) effects on antibiotic activity. Firstly, *P. aeruginosa* produces more alginate in the
presence of ROS [58], contributing to antibiotic tolerance. Secondly, high ROS levels lead to increased oxidation of mucins, promoting mucin crosslinking by which the rheological characteristics change, further impeding mucociliary clearance and antibiotic diffusion [59, 60]. Thirdly, the occurrence of “hypermutators” is associated with oxidative stress [61]. Hypermutators exhibit increased spontaneous mutation rates compared with wild type strains due to, among others, defects in DNA repair genes such as mutS and mutL [116]. Hypermutable strains can readily adapt, which is advantageous in stressful environments [61]. In addition, the hypermutator phenotype has been linked to the occurrence of increased levels of persister cells, which are dormant bacteria with low metabolic activity that are able to survive high doses of antibiotics [62], and comprise up to 1% of the total bacterial population in biofilms [117]. Another phenotype that has recently been linked to high antibiotic tolerance is the Phoenix phenotype in which metabolically active cells in the presence of high antibiotic concentrations become susceptible once subcultured [118]. Nevertheless, the influence of the microenvironment on the occurrence of this particular phenotype has not yet been explored. Fourthly, oxidative stress induces efflux pump gene expression, promoting aminoglycoside tolerance [62]. Excessive ROS production has also been reported to lead to a reduced membrane potential and modification of LPS, which potentiates tobramycin activity [63].

**Microbial factors in the lung microenvironment influence antibiotic activity**

CF lungs host a polymicrobial community, consisting of both bacteria and fungi. The fungal community is dominated by Candida albicans and Aspergillus fumigatus [119, 120]. The most common bacterial genera found in the CF airways, besides the conventional pathogens, are Streptococcus, Prevotella, Veillonella, Rothia and Actinomyces [121, 122]. The CF lung microbiome is highly variable, not only between patients, but also within patients [121, 122], and microbiome diversity differs with age, with a high diversity typically observed in young patients (<10 years) and a lower diversity in older patients (i.e. lowest level of diversity is observed at an age of approximately 25 years, with minimal changes in diversity at older age) [122]. Lower microbial diversity is often correlated with a decline in lung function, and occurrence of chronic P. aeruginosa infections [122]. Interestingly, plenty of studies report interspecies interactions in which P. aeruginosa influences antibiotic activity towards other CF microbiome members (e.g. S. aureus, Streptococcus anginosus), while the susceptibility of P. aeruginosa is typically not affected [123]. Nevertheless, certain interspecies interactions have been reported to influence antibiotic activity against P. aeruginosa.

Most reported interspecies interactions influencing P. aeruginosa susceptibility to antibiotics involve CF pathogens. Mixed-species biofilms of P. aeruginosa with Stenotrophomonas maltophilia or Burkholderia cenocepacia resulted in a higher biomass of P. aeruginosa compared with a single-species biofilm [124, 125]. For co-cultures with S. maltophilia, this also led to lower activity of ciprofloxacin against P. aeruginosa [124]. In contrast, coexistence of mucoid P. aeruginosa and S. anginosus induces a conversion from the mucoid to the nonmucoid phenotype [126], a transformation possibly associated with higher susceptibility towards antibiotics. A recent evolution study of P. aeruginosa in the presence of S. aureus reported mutations in LPS biosynthesis pathways of P. aeruginosa, which in turn resulted in altered outer membrane properties, antibiotic penetration and thus reduced activity of β-lactam antibiotics [127, 128]. In most studies on interspecies interactions, co-cultures of two bacterial species are used, which is obviously an oversimplification compared with the complex microbial communities present in the CF airways. Per spumum sample, an average number of 6 to 13 species has been reported, based on culture [119, 129]. In a recent experimental evolution study, P. aeruginosa was co-cultured with five CF microbiome members – hereby more closely mimicking the polymicrobial CF environment [130]. In this study, the development of P. aeruginosa resistance to β-lactam antibiotics occurred during evolution irrespectively of the presence of the microbiome members.

In addition to the extensively studied CF pathogens, anaerobic species are gaining more interest in CF research as they are abundant in the CF lung microbiome and might play a role in the disease process [131]. With regard to their possible influence on antibiotic activity against P. aeruginosa, approximately 76% of Prevotella isolates, one of the most encountered genera in CF airways [122], produce β-lactamases, which protect against β-lactam antibiotics [132].

Inter-kingdom interactions also contribute to P. aeruginosa persistence in the CF airways. In mixed-species biofilms with C. albicans, for example, the expression of outer membrane proteins (OMP), that are intrinsic participants in antibiotic resistance, is induced while the expression of ROS scavenging enzymes is reduced; the latter promoting mutability [133]. In addition to bacteria and fungi, viruses such as rhinovirus and respiratory syncytial virus (RSV) often reside in the CF airways [134]. Hendricks et al. [135] reported that RSV promotes the formation of P. aeruginosa biofilms on airway epithelial cells. This is
explained by a dysregulation of iron homeostasis upon viral infection, resulting in release of extracellular vesicles by airway epithelial cells, providing high iron availability to *P. aeruginosa* [135, 136].

**Intrapatient spatiotemporal variability in the microenvironm____ent drives phenotypic diversity in *P. aeruginosa***

The described host and microbial factors that compose the CF lung microenvironment (figure 1) vary within the patient, both spatially and temporally. Different niches in CF lungs can be distinguished that vary in oxygen levels, pH, antibiotic concentrations, nutrients, and inflammation state [137–140]. The steep oxygen gradient in CF mucus plugs, ranging from normoxia at the surface to almost complete anoxia in the centre [40], can separate the microbial community in niches depending on their oxygen preference (e.g. increased abundance of anaerobic species with depth) [138]. Additionally, the oxygen gradient influences the production of virulence factors (e.g. rhamnolipids) by *P. aeruginosa*, leading to a decreased production in deeper regions [138]. A 3-D microbiome analysis and metabolome cartography revealed that CF lung microbiome composition is niche-specific as well, and is influenced by spatial variations in metabolism and drug penetration [141]. For instance, an increased abundance of *Achromobacter* spp. was observed at the lower part of the lung, that rests on the diaphragm, where meropenem penetrated less. In addition, as lung disease progresses, long-term exposure to oxidative stress and proteases results in airway remodelling [142]. This affects mucociliary clearance and possibly impacts antibiotic penetration in the mucus layer [142]. Furthermore, higher salinity of CF sputum and higher levels of ferrous iron have been linked to disease progression, with no consensus on the direction of causality [69, 97]. The CF lung microbiome also varies with disease progression (i.e. microbiome diversity and abundance of anaerobes decrease with age), resulting in a significant within-patient variability [121].

*P. aeruginosa* adapts to the CF airways during chronic infections, through various genetic transformations [143–145]. The adaptation of *P. aeruginosa* not only results in different phenotypes of the same clone within one patient over time, but also in various phenotypes in one sputum sample [143, 146]. The latter is referred to as phenotypic diversity and is observed, for example, as small colony variants, mucoid variants and hypermutable variants (table 2). *In vivo* studies showed that diversification occurs soon after infection (i.e. in the first 10 years) [146]. After colonisation the infecting clone diverges into different sublineages, which subsequently evolve independently from each other. The independent evolution is attributed to localisation in different niches within the lungs, leading to complete spatial segregation between the different lineages [146, 147]. Correspondingly, the 3-D microbiome and metabolome cartography study by GARG et al. [141] revealed that the distribution of *P. aeruginosa* biofilm-specific molecules was not similar to the distribution of *P. aeruginosa* itself, indicating spatial variation in biofilm formation. The molecular process leading to diversification is reported to be recombination [148], with genes regulating expression of efflux pumps, and the quorum-sensing regulator LasR being affected most frequently [145, 149].

This phenotypic diversity has a dual impact on antibiotic treatment. Firstly, mixed mucoid and nonmucoid communities of *P. aeruginosa* show a higher tolerance to LL-37 and oxidative stress, compared with single phenotype communities [156]. Secondly, phenotypic diversity will affect the reproducibility of standardised AST, in which only one isolate is typically considered [157]. Indeed, the mean number of susceptibility profiles per sputum sample (based on disc diffusion diameters) has been reported as six [158]. Hence, sampling one isolate will often not provide a representative picture of antibiotic resistance [158]. Alternatively, performing AST on different isolates, based on morphotype, was reported not to be a promising approach as

| Phenotype                        | Description                                      | Reference |
|---------------------------------|--------------------------------------------------|-----------|
| Small colony variants           | Slow-growing                                     | [150]     |
|                                 | High antibiotic resistance                       |           |
| Mucoid variants                 | Alginate overproduction                          | [151]     |
| Auxotrophic variants            | Dependent on specific amino acids for growth     | [46]      |
| Hypermutable variants           | Very likely to develop multidrug resistance      | [152]     |
| LasR deficient variants         | Lower virulence: lower pyocyanin production, lower production of LasA protease, less efficient biofilm initiation | [153]     |
| Type III secretion system       | Deficient ExoU (effector protein) secretion: lower cytotoxicity and lower virulence | [154]     |
| deficient variants              | Pyomelanine hyperproduction                      |           |
| Pyomelanine producing variants  | Increased antibiotic resistance                  | [155]     |

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different morphotypes do not necessarily have different antibiotic susceptibility profiles [158]. Pooling isolates, another alternative approach, performed in some centres, also leads to a nonrepresentative resistance profile, and low reproducibility remains an issue [157].

Altogether, the complex and variable CF airways create a phenotypically diverse bacterial population, rendering AST and antibiotic treatment in CF even more challenging.

Interpatient variability in the lung microenvironment could explain low benefits of “one-treatment-fits-all” approach

Each CF patient has a unique genetic background and evolution of the disease [12, 159] and high interpatient variability is observed in the lung microenvironment of patients with CF [160].

Firstly, interpatient variability has a genetic base, with over 2000 CFTR mutations described that can be classified in severe mutations (i.e. no functional CFTR protein) and mild mutations (i.e. reduced CFTR activity) [161]. However, within the same CFTR genotype, the manifestation of the disease can differ tremendously, which can (at least partially) be attributed to several modifier genes (e.g. inflammatory mediators, antioxidants, airway reactivity, etc.) [162]. In addition to the direct influence of CFTR functionality on parameters such as salinity [163], the composition of the oropharyngeal bacterial community is also related to the CFTR genotype [164], with possible downstream effects on antibiotic activity. Furthermore, the prescription of CFTR modulators fully depends on the CFTR genotype of patients, and CFTR modulators have been shown to interfere with antibiotic activity. More specifically, both Orkambi and Kalydeco enhance the activity of polymyxin B in vitro [165].

Second, CF is a multisystemic disease in which extrapulmonary manifestations, such as exocrine pancreatic insufficiency, CF-related diabetes or liver disease, are only partially explained through the patient’s genotype [166]. The presence or absence of these disease manifestations and their metabolic consequences, will in turn influence the lung microenvironment [166]. Indeed, the rate of pulmonary function decline is clearly correlated to the degree of insulin insufficiency and hyperglycaemia [167].

Finally, the composition of the polymicrobial community in the CF lung differs tremendously between patients [121, 122]. Indeed, differences in community diversity, abundance of anaerobes and others have been described [168]. Similarly, most if not all of the above-described host microenvironmental factors (e.g. iron levels, eDNA concentration, nutritional composition and immune mediators) that influence antibiotic activity, vary between patients [21, 75, 76, 97] and can thus contribute to interpatient variability in response to antibiotics. A recent study by RAGHVANSHI et al. [169] (2020) also demonstrated that the metabolome of individuals with CF was highly individualised, as β-diversity was greater across than within subjects.

Finally, drug bioavailability at the infection site is another important aspect to consider when evaluating (the lack of) antibiotic efficacy. Pharmacokinetics for certain antibiotics administered to patients with CF through inhalation, orally or intravenously differ from that in healthy controls [170, 171]. Focusing on the CF lung microenvironment, the most impactful factor is most likely the altered mucus rheology, which causes diffusion-limiting interactions with antibiotics, as described earlier. In addition, salt content and the presence of plasma proteins in the mucus layer may further affect antibiotic bioavailability [170]. Indeed, a substantial proportion of patients with CF have haemoptysis [172], and plasma proteins (most commonly albumin, alpa1-acid glycoprotein (AGP) and lipoproteins) not only bind antibiotics [170], but also influence the susceptibility of P. aeruginosa in vitro [173].

Towards narrowing the gap between in vitro and in vivo antimicrobial susceptibility

There is a gap between the complex and patient-dependent nature of the CF lung microenvironment on the one hand, and the conditions in which standardised AST are carried. Indeed, standardised AST typically involves exposing a single P. aeruginosa isolate, in planktonic state, to antibiotics in bacterial growth media (Muller Hinton broth) and environmental conditions (aerobic) (EUCAST guidelines) that are by far not reflective of the in vivo microenvironment [174]. Most if not all of the described host and microbial factors are absent in conventional AST, and the biofilm phenotype and phenotypic diversity of P. aeruginosa observed in vivo, are not captured. Nevertheless, clinical studies focusing on biofilm susceptibility to guide antibiotic therapy did not show an improvement in P. aeruginosa density in sputum or lung function parameters compared with conventional AST [175]. Hence, mimicking the biofilm phenotype alone may not be sufficient to fill the gap between in vitro and in vivo antibiotic susceptibility. In this review, we highlighted individual host and microbial factors that negatively impact antibiotic activity, as well as factors that potentiate antibiotic activity. More research is necessary to reveal which
microenvironmental factors are dominant contributors to the net susceptibility of *P. aeruginosa* to antibiotics *in vivo*, as potential inclusion of these factors in routine clinical microbiology protocols needs to be feasible from a cost, time and practical point of view. Systematically studying how the different microenvironmental factors influence antibiotic activity alone and when combined, could lead to identification of key contributors to the (lack of) treatment success, and help design more predictive model systems and next generation AST.

**Conclusion**

The overall complexity of the CF microenvironment and the microenvironmental diversity within and between patients is not considered in the current treatment approach, despite its strong influence on antibiotic activity *in vitro*. This could thus (at least in part) explain the discordance between *in vitro* activity and *in vivo* efficacy, the difficulties in eradicating *P. aeruginosa* from the CF airways and the high interpatient variability in therapeutic success. Nevertheless, a major gap that remains to be addressed, is to understand which microenvironmental factor(s) drive(s) *P. aeruginosa* susceptibility *in vivo*. Therefore, more in-depth and systematic research on the influence of the lung microenvironment and interpatient variability on antibiotic efficacy could bring much-needed insights to understand and tackle the high failure rate of antibiotic therapy in CF chronic airway infections.

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