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Challenges and opportunities in the development of novel antimicrobial therapeutics for cystic fibrosis

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Abstract

Chronic respiratory infection is the primary driver of mortality in individuals with cystic fibrosis (CF). Existing drug screening models utilised in preclinical antimicrobial development are unable to mimic the complex CF respiratory environment. Consequently, antimicrobials showing promising activity in preclinical models often fail to translate through to clinical efficacy in people with CF. Model systems used in CF anti-infective drug discovery and development range from antimicrobial susceptibility testing in nutrient broth, through to 2D and 3D *in vitro* tissue culture systems and *in vivo* models. No single model fully recapitulates every key aspect of the CF lung. To improve the outcomes of people with CF (PwCF) it is necessary to develop a set of preclinical models that collectively recapitulate the CF respiratory environment to a high degree of accuracy. Models must be validated for their ability to mimic aspects of the CF lung and associated lung infection, through evaluation of biomarkers that can also be assessed following treatment in the clinic. This will give preclinical models greater predictive power for identification of antimicrobials with clinical efficacy. The landscape of CF is changing, with the advent of modulator therapies that correct the function of the CFTR protein, while antivirulence drugs and phage therapy are emerging alternative treatments to chronic infection. This review discusses the challenges faced in current antimicrobial development pipelines, including the advantages and disadvantages of current preclinical models and the impact of emerging treatments.

INTRODUCTION

Cystic fibrosis (CF) is a hereditary disease affecting one in 2500 individuals of European descent [1]. It is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which results in defective production, trafficking or function of the CFTR ion channel expressed on the epithelial cell surface. CF is a multisystem disease but particularly impacts respiratory function, impairing transport of water, chloride and sodium. This leads to increased airway mucous viscosity, hindering mucociliary clearance and facilitating colonisation of the airways by opportunistic pathogens [2]. People with CF (PwCF) are significantly more susceptible to acute and chronic respiratory infections compared to healthy individuals, with progressive lung disease and subsequent respiratory failure accounting for 95% of morbidity and mortality [3]. Considering this burden, significant effort is given to novel antimicrobial development, as currently available treatment options are insufficient for controlling established infections and are unable to combat emerging antibiotic resistance amongst respiratory pathogens [4].

Although a narrow range of antibiotic therapeutic options are available, efficacy against chronic infection is poor. This is partially attributable to the complexity of the CF environment, as host immunity, bacterial virulence factors, sputum composition and other factors contribute to treatment failure and the emergence of antimicrobial resistance. As such, PwCF are subject to intense

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Abbreviations: AA, amino acids; AQ, 2-alkyl-4-quinolones; ASM, artificial sputum media; Bcc, burkholderia cepacia complex; BSA, bovine serum albumin; CA, casamino acids; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DMEM, dulbecco's modified eagle medium; DOPC, dioleoylphosphatidylcholine; DTPA, diethylenetriamine pentaacetate; ECM, extracellular matrix; GlcNAc, N-acetylglucosamine; HBE, human bronchial epithelial cells; LB, Luria Bertani media; MEM AA, minimum essential media amino acids; MH, Mueller Hinton media; MI, Meconium ileus; MRSA, methicillin-resistant *Staphylococcus aureus*; NETs, neutrophil extracellular traps; NTM, nontuberculous mycobacterium; NTM-PD, nontuberculous mycobacterium-associated pulmonary disease; OAC, organ-on-a-chip; PABA, p-aminobenzoic acid; PDSM, polydimethylsiloxane; PKPD, pharmacokinetic-pharmacodynamic; PwCF, people with cystic fibrosis; QS, quorum sensing; QSSM, quorum sensing signalling molecules; RPMI, Roswell Park Memorial Institute medium; RWV, rotating wall vessel bioreactor; SCFM, synthetic cystic fibrosis media.

Impact Statement

Chronic respiratory tract infection is a major driver of morbidity and mortality in individuals with cystic fibrosis (CF). Existing antimicrobials are often ineffective in the treatment of chronic infection, due to the complex nature of the CF lung environment. A variety of preclinical models exist to assess the efficacy of novel antimicrobial compounds, though there is no consensus as to which best capture the unique features of the CF lung and which are most appropriate for use as part of drug development pipelines. This review evaluates existing preclinical models for CF antimicrobial development, considering their strengths and limitations alongside opportunities for further optimisation. Appraisal of the challenges and opportunities in preclinical antimicrobial development is a necessary first step towards standardisation and refinement of preclinical pipelines. This will contribute to the acceleration of antimicrobial development, to deliver more effective therapeutics to resolve bacterial infection in people with CF.

and complex antibiotic regimes, often necessitating extended hospital visits, and which are associated with adverse reactions and long-term complications [5]. Treatment complexity also increases with age, following the establishment of chronic infection. One study reported that patients were prescribed a median of ten treatments, including long-term nebulised antibiotics and airway clearance techniques [6]. This high burden has sparked a growing focus on the development of more effective, less burdensome therapeutics.

There is a considerable challenge in translating from preclinical to clinical endpoints. Potential antimicrobial compounds that are efficacious against bacterial species *in vitro* often have little impact *in vivo* [7]. This disparity arises due to the complexity of the CF lung environment. To address this, both *in vitro* and *in vivo* models have been developed that aim to mimic different aspects of the CF lung, such that evidence of drug activity at the preclinical stage has greater predictive power for efficacy in a clinical setting. The recent proliferation of such models, whilst undoubtedly of benefit to both basic and translational research, has created a further problem, in that it is often unclear which model is most appropriate for any given research application [8]. As such, the preclinical pathways for CF anti-infective drug development lack standardisation between academic researchers, SMEs, and pharmaceutical companies.

This review aims to highlight the strengths and limitations of current antibiotic development pipelines, with a focus on the preclinical models used. We aim to identify models of utility to future pipelines and describe current and future opportunities in the development of CF antimicrobials. Standardisation of antimicrobial development is essential to accelerate drug discovery and increase investment in the sector.

THE CF LUNG ENVIRONMENT

Defects in CFTR protein function or trafficking result in impaired water transport across the airway epithelium. This causes thickening and acidification of secreted mucous, inhibiting mucociliary clearance and immune function. This makes the respiratory tract conducive to chronic infection from numerous pathogens, which can colonise and grow aerobically or anaerobically [9].

CF sputum provides a nutrient-rich environment for microbial growth. Protein, lipid, carbohydrate, and free-DNA concentration are all increased in PwCF, alongside iron, which is sequestered by lactoferrin, transferrin and ferritin [10, 11]. CF sputum has a decreased pH, which inhibits the function of secreted antimicrobials and may similarly impair target-site activity of prescribed antibiotics [11]. Neutrophil abundance is chronically elevated, though their function is impaired. Neutrophils undergo necrosis, releasing toxic cell contents into the extracellular environment [12]. Increased extracellular DNA concentration is also attributable to neutrophils, which release their DNA in the form of neutrophil extracellular traps (NETs).

This chemical and immunological complexity is poorly reflected in standard antimicrobial testing pipelines, impeding product development and highlighting the requirement for use of bespoke and well-validated CF models [13]. The leading microbial pathogens of the CF lung are described below, with reference to the contribution that the environment makes to antimicrobial resistance through induction of adaptive resistance mechanisms.

Pseudomonas aeruginosa

The most prevalent species isolated from the CF lung is *Pseudomonas aeruginosa*, a Gram-negative bacterium that causes chronic infection in 31.9% of individuals over 16 years old [14]. It is the largest contributor to mortality, increasing risk of death twofold, or eightfold for antibiotic resistance infections [15].

P. aeruginosa has an extensive arsenal contributing to intrinsic antibiotic resistance. Intrinsic resistance mechanisms include reduced membrane permeability, expression of efflux pumps, and synthesis of antibiotic-inactivating enzymes [16]. These serve to reduce entry, increase removal, and increase the breakdown of the antibiotic, respectively. *P. aeruginosa* also exhibits acquired

resistance through positive selection of *de novo* mutations or horizontal acquisition of resistance genes. Within the CF lung, environmental conditions stimulate adaptive resistance, which can drive the formation of biofilms [17].

Biofilm formation is the primary mode of growth during chronic infection and it confers resistance to multiple classes of antibiotic [16, 18]. A significant obstacle to antimicrobial therapy are persister cells, dormant bacteria that constitute up to 1% of the biofilm mass. These cells are metabolically inactive, which reduces the number of antibiotic targets, subsequently allowing the bacteria to survive under considerable antibiotic pressure. Once this pressure is removed, persister cells can repopulate the biofilm [19]. Differential expression of resistance genes also occurs within biofilms, with cells reducing expression of flagella to evade host immune activation and increasing expression of resistance genes to inhibit host derived antimicrobials and antibiotics [16]. Carbohydrates associated with biofilm growth inhibit binding of immune lectins, further disrupting the host immune response [20]. These combined factors make *P. aeruginosa* an extremely successful coloniser, with a vast arsenal of resistance mechanisms affording it comprehensive protection against both the host immune system and administered therapeutics [17]. Crucially, many of these resistance mechanisms are environmentally induced. The cues to form biofilms, to alter efflux pump expression or to secrete toxins are often host-derived. Therefore, robust testing of candidate antimicrobial drugs requires testing in conditions where these chemical cues are available.

Burkholderia cepacia complex

Burkholderia cepacia complex (Bcc) is a group of 22 Gram-negative species ubiquitous throughout the environment [21, 22]. In PwCF, infection can cause severe decline in lung function that can develop into a life-threatening systemic infection known as cepacia syndrome [21, 23]. Prevalence of species isolated from PwCF varies geographically: *B. cenocepacia* and *B. multivorans* are most commonly isolated in Australia, New Zealand, Canada, and European countries; *B. multivorans* is the most common isolate in the United States; and *B. gladioli*, a non-Bcc species, has become third most common in the United States, though it is uncommon in the European population [21, 24].

Bcc species possess the same innate and acquired resistance mechanisms previously described for *P. aeruginosa*. They also carry intrinsic polymyxin resistance, as they express an atypical lipopolysaccharide structure [25]. Bcc resistance to chloramphenicol, co-trimoxazole, ciprofloxacin, tetracycline, rifampin, avibactam, and co-amoxiclav has been identified in over 50% of isolates [25]. Additionally, Bcc impacts growth of *P. aeruginosa* during coinfection, with one study observing an increase in *P. aeruginosa* biofilm formation and host inflammation in response to Bcc coinfection [26]. The impact of Bcc on both other respiratory pathogens and on the host highlights the requirement for more optimised polymicrobial models for the study and treatment of CF lung infection.

Nontuberculous mycobacteria

Nontuberculous mycobacterium (NTM) are ubiquitous environmental organisms, persisting in both aerobic and microaerobic conditions [27]. There are over 150 species that can be categorised phenotypically as fast-growing (3–7 days to observe colonies) or slow growing (>7 days). NTM live an extracellular lifestyle, though become intracellular following phagocytosis by macrophages during pulmonary infection [28].

Prevalence among PwCF has varied across studies, from 4.2–40.9%, with the disparity between studies likely due to differences in healthcare provision or geographical location. Of individuals who grow a positive NTM culture, 50% go on to develop pulmonary disease believed to be attributable to NTM (NTM-PD) [29]. This is difficult to diagnose in the CF context, as infection may be misidentified due to considerable symptomatic overlap between NTM-PD and other aspects of CF lung disease.

In PwCF, NTM infection has been associated with a greater decline of FEV_1 when compared to both *P. aeruginosa* and Bcc, which highlights the need to improve both identification of NTM infection and the development of treatments [30]. This requires preclinical models that accurately reflect NTM-specific factors. Such models should involve incorporation of host cells, to replicate an intracellular lifestyle, and optimisation for greater longitudinal resolution to account for their slower growth rate.

Other colonisers

As of 2016, ~60% of PwCF in the US were infected with *Staphylococcus aureus*, though infection appears to decrease throughout life [31]. The polymicrobial nature of the CF lung makes it difficult to discern the contribution that *S. aureus* makes to declining lung function, though infection with methicillin-resistant strains (MRSA) has risen significantly over the last decade. As of 2014, over 25% of PwCF in the US had positive MRSA cultures, though European and Canadian CF populations had a prevalence of 3–11% [32]. A large range of Streptococci and Rothia species are also commonly identified in microbiome studies, along with anaerobic bacteria such as *Prevotella* and *Veilonella* spp. [33]. Emerging pathogens such as *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* can develop into the dominant species in the lungs of PwCF, although the prevalence is much lower than that of *P. aeruginosa*, the dominant Gram-negative pathogen [33, 34].

Fungal species such as *Candida albicans* and *Aspergillus* spp. are also common in the CF lung. The impact of *C. albicans* is unclear, though *Aspergillus* spp., namely *A. fumigatus*, can cause allergic bronchopulmonary aspergillosis and acute infection of the lung parenchyma [35].

In an ideal scenario, development of a robust preclinical framework that is capable of tackling CF infection would include all CF-associated microbes. Microbiome community members may impact on respiratory health, but also on the survival and behaviour of other members of the community. A focus on polymicrobial models is necessary, such that the specific implications of these interactions can be determined. However, increasing complexity has associated challenges, particularly with regards to model tractability and throughput.

CHALLENGES IN ANTIMICROBIAL DEVELOPMENT

The spatial structure of the respiratory tract, sputum composition and a biofilm mode of growth that is characteristic of chronically infecting bacteria are all factors that influence the pharmacokinetics of antimicrobial therapy, while polymicrobial and host-pathogen interactions contribute to antimicrobial pharmacodynamics. No single model has the capability to accurately recapitulate the multitude of factors influencing the CF respiratory environment. To develop more representative models, it is essential that factors intrinsic to the environment can be mimicked, and that preclinical models are assessed against benchmarks that capture the influence of those factors on both pathogen and host.

It is also of paramount importance to test against representative microbial populations and isolates. Antibiotic susceptibility testing against a single strain or isolate of *P. aeruginosa* may yield promising results, but diversification of the microbial population is a feature of chronic infection in CF, with potential for population-level benefits deriving from factors produced by single genotypes within the community. This population diversification is facilitated by the biofilm mode of growth. Accurate antimicrobial testing must therefore mimic not only the abiotic environment of the CF lung – structure, sputum composition and other host factors, but must also represent the microbial landscape accurately. Achieving this would augment translation of preclinical findings into clinical efficacy endpoints, but the challenge is not trivial, given the complexity of those polymicrobial communities and the diversity that exists even within a single species. In the sections hereafter, currently available preclinical models are discussed, including their advantages and limitations.

High throughput screening tools for CF antimicrobials

High throughput models range from widely available bacterial culture media, through to chemically defined media that attempt to replicate the chemical composition or rheology of CF sputum [36]. Using high throughput screening tools, CF pathogens can be rapidly assessed against libraries of antimicrobial compounds, in a multiplex fashion. Generally, the more chemically-complex the media, or the more viscous, the less suitable it is for high-throughput screens.

Simple media formulations such as nutrient-rich Luria Bertani (LB) and Mueller Hinton (MH) have the highest throughput due to their defined, nutrient-rich formulation, though they lack CF-specific factors. The absence of relevant environmental cues in these media results in a misleading assessment of bacterial virulence or antimicrobial resistance, giving them limited utility in antimicrobial susceptibility testing [37].

The two most widely accessible types of CF specific media, designed to mimic sputum composition, are artificial sputum media (ASM) and synthetic cystic fibrosis media (SCFM) [37]. There are nine variations of ASM which have been iteratively altered to recapitulate the CF sputum with increasing accuracy (Fig. 1). Variation between formulations is described in a publication by Neve *et al.* [38]. Soothill ASM was formulated in 1997 from then-known constituents of CF sputum [39]. It is comprised of mucin, DNA, sodium, potassium, chloride, lipids, DTPA as an iron chelator and egg yolk emulsion as a source of lecithin. This was modified to create Romling ASM in 2005, with the addition of 250 mg per litre of the 20 canonical amino acids [40]. Winstanley ASM, published in 2012, is compositionally identical to Romling ASM, though has a reduced buffering capacity [41]. Romling ASM was also adapted with the addition of bovine serum albumin, reduction of mucin concentration and increase in DNA concentration culminating in the creation of ASMDM ASM [42]. Further research into CF sputum composition resulted in creation of Cordwell ASM, derived from ASMDM ASM, which has a reduced buffering capacity, replacement of amino acids with casamino acids, and replacement of DTPA with ferritin [43]. Finally, SDSU ASM was derived from Cordwell ASM, with the removal of bovine serum albumin, replacement of casamino acids with minimal essential media amino acids and increased mucin concentration [44].

There are three generations of SCFM, defined using average concentrations of compounds found across CF sputum samples (Fig. 1). SCFM1 is formulated using average concentrations of ions, including sodium, chloride, potassium, calcium, ammonium, and magnesium, amino acids, glucose, and lactate [37]. This was developed further to create SCFM2, which contains DNA, mucin, *N*-acetylglucosamine and dioleoylphosphatidylcholine, once again at concentrations representing those in CF sputum samples [45]. Added to SCFM2 were *p*-aminobenzoic acid, NAD^+, adenine, guanine, xanthine and hypoxanthine, to create SCFM3 [45].

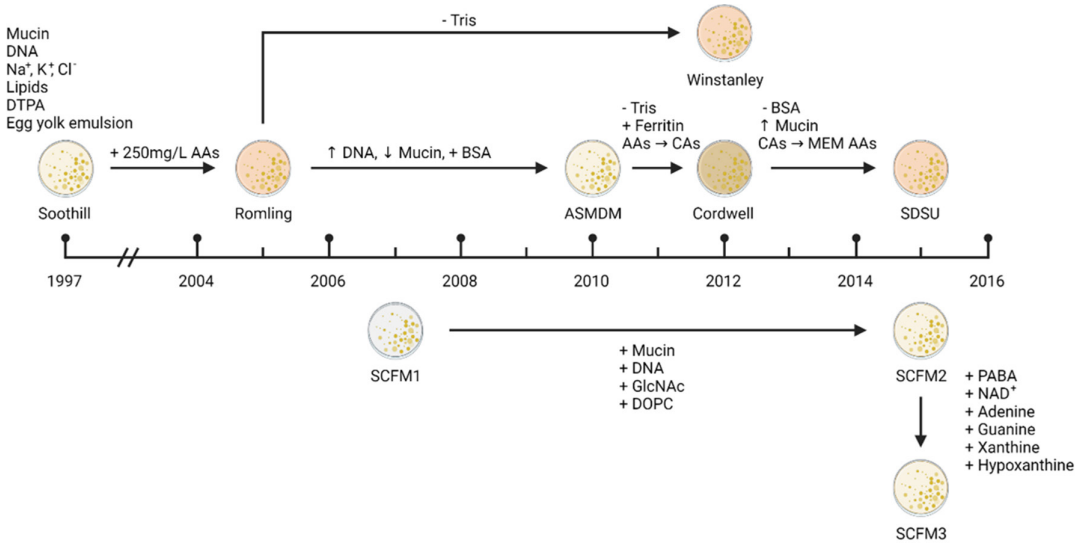

Fig. 1. Artificial Sputum Media formulations through time [38]. DTPA, diethylenetriamine pentaacetate; AA, amino acids; BSA, bovine serum albumin; CA, casamino acids; MEM AAs, minimum essential media amino acids; DOPC, dioleoylphosphatidylcholine; GlcNAc, Nacetylglucosamine; PABA, *p*-aminobenzoic acid. Created with BioRender.com.

Sputum mimics recapitulate important features of CF sputum, though are limited in their representation of host factors, which have been shown to impact *P. aeruginosa* adaptation and colonisation. Host-derived antimicrobials, such as β-defensins, lysozyme and cathelicidins, confer a selective pressure on microbial communities, driving adaptation. Bricio-Moreno *et al.* exemplified this, detailing adaptation of *P. aeruginosa* to the airways in a mouse infection model, in which a missense mutation in the *P. aeruginosa pmrB* gene, acquired during long term colonisation, conferred resistance to clearance of infection from the lungs [46]. The identified *pmrB* mutation conferred resistance to lysozyme but, in *in vitro* antimicrobial susceptibility assays, an increase in susceptibility to multiple antibiotic classes was observed and was attributed to an increased negative charge of the outer membrane, aiding uptake of cationic antimicrobials. When investigated *in vivo* however, this apparent trade-off was negated [47]. Positively charged host-derived molecules, such as the polyamine spermidine were found to bind to the outer membrane of *pmrB*-deficient *P. aeruginosa*, reducing permeability to cationic antimicrobials. In this example, the lack of relevant host-derived factors in *in vitro* antimicrobial susceptibility testing media resulted in misleading conclusions being drawn as to the impact of the mutation on antimicrobial resistance. This highlights the need to capture the chemical complexity of the airway environment in drug testing models. Recent efforts have been made to address this, through the development of CF mimicking media that include host-derived chemicals, such as polyamines and antimicrobial agents, such as lactoferrin and lysozyme [36]. Different formulations of these media can be used to capture conditions of the lung or of the upper airways. The latter allows for study of early infection processes and eradication therapies, as the paranasal sinuses are thought to act as a portal of entry and early colonisation site for CF airway pathogens.

Sputum mimics are generally lower throughput than standard nutrient rich media, as they have higher viscosity [36]. While incorporating more components may create a more representative environment, it makes the models less economical and less suitable for automated screening [48]. Sputum mimics are also unable to capture the three-dimensional structural environment of the lung, which may limit their ability to reproduce the biofilm mode of growth that occurs on biological substrates. Accordingly, sputum mimics, when used in isolation, may be more suitable for screening out antimicrobial compounds that would pass primary screens in nutrient broth, but which may be less efficacious in sputum-like conditions. This would reduce the number of compounds making it through to testing in the more time consuming and costly models described below.

Screening in biofilms and polymicrobial models

Bacterial populations growing in biofilms show enhanced antibiotic resistance through secretion of extracellular polymers that reduce antibiotic diffusion, and formation of persister cells which have greater capacity to tolerate antimicrobials [21]. Polymicrobial infection is also common, with interspecies interaction considerably altering bacterial virulence [9, 49]. Jean-Pierre *et al.* identified at least ten genera within the lung microbiome of 25 PwCF [50]. They also detailed simple cross-feeding relationships between the four most common genera (*Prevotella, Pseudomonas, Staphylococcus and Streptococcus*), showing that polymicrobial interactions can significantly impact metabolite availability. Various models have been developed to model both the polymicrobial nature of infection as well as the biofilm mode of growth common to chronic infection.

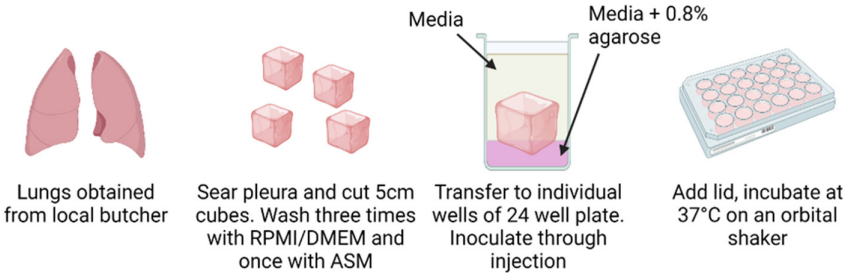

Fig. 2. The *ex vivo* porcine lung model [51]. Cubes of porcine lung provide a solid substrate upon which bacterial species, namely *Pseudomonas aeruginosa*, can grow. RPMI, Roswell Park Memorial Institute medium; DMEM, Dulbecco's modified eagle medium; ASM, artificial sputum medium. Created with BioRender.com.

Porcine lung model

The porcine lung model is an *in vitro* model used for investigation of polymicrobial infection. It involves use of dead lung tissue as a substrate for microbial growth [51] (Fig. 2). The approach utilises ASM as a medium, while providing similar spatial structure to the human lung. By modelling the gross structure of the lung, growth rate, virulence factor production and cell-cell interaction can be directly investigated, while reducing the use of protected species in research. The porcine lung model does not recapitulate host immune factors, which make a significant contribution to host-pathogen interactions. Regardless, it has proven an invaluable resource, being utilised in comparisons of the *Pseudomonas aeruginosa* biofilm transcriptome against SCFM, modelling of polymicrobial biofilms between *P. aeruginosa and S. aureus* and recapitulation of clinical *S. aureus* infection [52–54].

Continuous flow polymicrobial biofilm model

O'Brien *et al.* outlines a model derived from the porcine lung model that uses a continuous flow of media, allowing establishment of steady-state polymicrobial biofilms and planktonic cultures (Fig. 3) [55]. Cubes of agar or porcine bronchiole inoculated with bacteria are suspended in a beaker with continuous ASM inflow and waste outflow. Biofilms and planktonic populations can be easily sampled throughout the experiment without impeding microbial growth. The continuous-flow model has been used to model polymicrobial infection with *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans*, in which the polymicrobial influence on genotypic and phenotypic characteristics that impact therapeutic efficacy were investigated [56]. The model enables real-time study of the CF microbiome in a relevant environment.

The primary limitation in this *in vitro* model is the lack of live host cells. Moreover, host factors that contribute to clearance of infection in health or promote chronicity of infection in CF are not present. Immune cells, such as neutrophils, contribute towards the disease state and drive virulence phenotypes in pathogens. Dynorphins, a class of host-derived opioids, are produced by the host in response to stress, and can increase virulence of *P. aeruginosa*, stimulating the production of pyocyanin [57]. It is

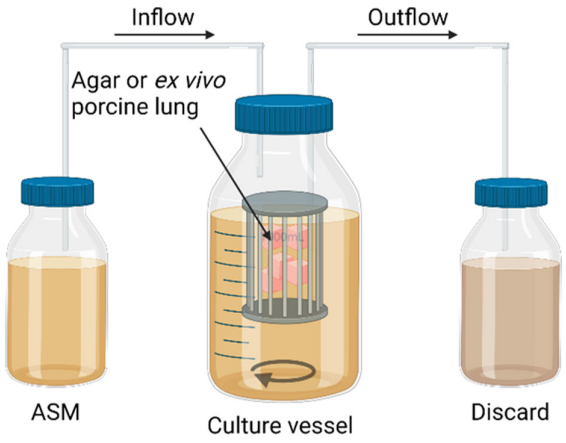

Fig. 3. A continuous flow model of polymicrobial infection [55]. Artificial sputum medium (ASM) is pumped into a culture vessel containing 5cm^3 cubes of agar or *ex vivo* porcine lung, which serve as a solid substratum for bacteria to grow. An outflow of ASM is maintained such that nutrient availability is constant. Created with BioRender.com.

Table 1. Common cell lines used in cystic fibrosis pharmaceutical development

Name	Description	Reference
Fisher rat thyroid epithelia	Used extensively in CF research and drug discovery as the cells lack cAMP-regulated chloride channels, those defective in CF.	[112]
CF bronchial epithelial cells	Involved in neutrophil recruitment in bronchial mucosa. Infection of *P. aeruginosa* results in differential expression of many genes.	[113, 114]
Gene-edited human bronchial epithelial cells	Immortalised 16HBE14o- cells expressing wild-type CFTR. Used to study the function of the CFTR gene, especially in the creation of CFTR mutants.	[115]
Human bronchial epithelial (HBE) cells	Isolated from biopsy samples and lung explants. Cells can be cultured to resemble pseudostratified airway epithelia and are used to study the lower respiratory tract.	[116]
Human nasal epithelial cells	Similar to HBE cells. Benefits from the lack of a need for invasive procurement.	[116]
Induced pluripotent stem cells	Pluripotent stem cells derived from adult somatic cells. Can be differentiated into different cell types, such as pancreatic ductal epithelial cells, that express CFTR.	[117]

imperative to investigate the influence of these and other host-derived factors, to determine the impact they have on microbial communities. While the continuous-flow model has limitations in this regard, it is nevertheless useful in modelling growth of polymicrobial cultures on a similar substrate to that found in humans and has potential to reduce the use of vertebrate species for infection studies.

Free-floating and surface-attached biofilms

Bacterial growth has been previously assumed to fall into two categories - surface attached biofilm formation or planktonic growth. Current CF infection models primarily recapitulate these growth modalities. However, free-floating aggregates of tightly linked cells that bear structural similarity to surface-attached biofilms, without being attached to a surface substrate, are increasingly understood to be relevant to CF infection [58]. When *P. aeruginosa* samples were taken from PwCF and compared with free-floating aggregates grown *in vitro*, the two populations were transcriptionally and phenotypically similar. This indicates aggregate formation as a significant mode of *in vivo P. aeruginosa* growth. Sriramulu *et al.* detailed a mechanism of aggregate formation, where *P. aeruginosa* cultured with ASM formed aggregates bound to sputum proteins [40]. Further study revealed a sputum component, neutrophil elastase, responsible for promoting aggregate formation [59].

Free-floating aggregates and surface attached biofilms of *P. aeruginosa* express similarity in growth rate, aggregate matrix components, antibiotic resistance, and phagocyte resistance [60]. Despite this, isolates taken from chronic CF infection are often unable to form surface attached biofilms [58]. More investigation into the phenotypic differences between the two modes of growth is necessary. Nonetheless, it is important to ensure that future models are optimised for growth that is appropriate to the bacteria and infection scenario being studied.

Screening in host-pathogen *in vitro* models

Interactions between the host and pathogen drive virulence, pathogenesis, and antimicrobial resistance. Such interactions include both sensing of secreted host factors by microbes, or direct physical interactions between pathogen and host cells. While some host-derived factors are represented in sputum mimics, there is a need for complementary models that mimic cellular aspects of the host airways. Tissue culture models provide a reliable means to study host-pathogen interactions, affording insight into host responses to antimicrobial compounds and allowing the microbial resistance to treatment to be assessed in the presence of host-induced bacterial signalling responses. These models are, to a varying degree, capable of reproducing the architecture, multicellular complexity, commensal microbiota, gas exchange and nutrient gradients observed in the *in vivo* environment [61].

Various CF and non-CF cell lines provide a means to assess impacts of antimicrobial compounds on host cells, while mimicking relevant host-pathogen interactions (Table 1). These are used extensively in pharmaceutical development. Normal human bronchial epithelial cells were used in validation of cysteamine, a mucoactive antimicrobial and antibiofilm agent [62]. The cell line was used to provide a physiologically complex environment to confirm mucoactivity. Treatment of cysteamine decreased cell-derived mucus production. In an investigation of phage therapy, both CF and non-CF cell lines were used to identify changes in apoptosis and cytokine production [63]. While the cell-specific response is recapitulated well in these studies, cell lines fall short of capturing the spatial organisation of the airways. Establishment of diffusion gradients in nutrients and cytokines are also unfeasible. Moreover, immortalised cell lines often display aberrant expression of cell-specific proteins, and maintenance of their viability necessitates use of nutrient-rich tissue culture media that is not a relevant chemical environment for the study of CF pathogens.

More recently, three-dimensional models have been developed, which allow representation of multicellular organisational complexity, gas exchange, commensal microbiota, and physiological biomechanical factors [61]. The rotating wall vessel (RWV)

Table 2. Characteristics of existing animal species used in cystic fibrosis research

Organism	CFTR amino acid homology	Average lifespan	Sexual maturity	Days of gestation	Average litter size	Ethical approval	Reference
Galleria mellonella	Not present	eight to 12 weeks, 5–6 for larvae	9–11 weeks	2 weeks	~1000 eggs	None	[118]
Zebrafish	55%	three to 5 years	3 months	3–4 days	174.1±89.8 eggs	>120 h post-fertilisation.	[119, 120]
Mice	78%	2 years	six to 8 weeks	21	6	Required	[121]
Ferret	91%	eight to 10 years	four to 6 months	42 days	8	Required	[121]

bioreactor, consisting of a rotating drum that allows self-organisation of cellular aggregates on the interior of the walls, creates a low-shear-stress environment, where the liquid medium moves with little velocity compared to the vessel wall. This allows for the development of multicellular complexity, representative of the host environment. The RWV model was employed in the study of *P. aeruginosa* anti-biofilm antimicrobials, accurately replicating phenotypic traits of lung tissue and the biofilm phenotype of *P. aeruginosa* [64]. RWV models are unable to develop vascular and immune complexity and require costly volumes of media to be maintained.

Three-dimensional organoids are built around an extracellular matrix (ECM), providing a scaffold around which cells can proliferate. Factors such as spontaneous cell proliferation, cytokine production, gene expression, mucus production and antimicrobial responses are all mimicked with accuracy [61]. One such model was used in the study of mucociliary cell function, in which the inhibitor CFTRinh-172 was tested for its ability to inhibit the CFTR protein [65]. It shows potential for the use of organoids with specific inhibitors, to enable recapitulation of the CF phenotype. In this relatively nascent field, limitations include a lack of standardisation in the ECMs used, and variability in the 3D structures achieved by the cells formulating the organoid. In general, establishment of organoid systems is time consuming and requires considerable troubleshooting.

Organ-on-a-chip (OAC) models involve the engineering of micrometre scale 3D architecture of a specific organ [61]. They can mimic both tissue- and organ-level functions, such as vascular perfusion, while allowing control of forces across the tissues, modelling fluid shear and peristalsis. OAC models are structured with fluidic channels separated by porous membranes, which allow tissue development in adjacent chambers that can interact. OAC models have been used to mimic the lung environment, with one study observing that the system can partially recapitulate the innate immune response to pulmonary infection by *E. coli* [66]. More investigation is necessary to determine whether this applies to *P. aeruginosa* infection, and whether OAC models can be developed with cells bearing *CFTR* mutations. Another challenge is mimicry of the array of cell types found *in vivo* [67]. Finally, and perhaps the most significant limitation of OAC models for antimicrobial screening, is that the polydimethylsiloxane (PDSM) scaffold that is used can absorb small hydrophobic molecules, including antimicrobials [61]. This can impact cell signalling and drug screening studies, though if the rate of absorbance is calculated, the issue could be minimalised.

In vivo screening

In vivo screening involves antimicrobial susceptibility testing in animal Cmodels of infection. These may be wild-type species, or transgenics that mimic the CF phenotype. Each model has unique advantages and limitations.

Galleria mellonella

The larvae of *Galleria mellonella*, the greater wax moth, have gained popularity for use as an infection model [68]. The invertebrate is easy to establish and maintain, has a short lifespan, and requires no ethical approval (Table 2). *Galleria* have remarkable similarities in their immune response to the innate response of vertebrates, with cellular and humoral components serving similar functions, including the production of phagocytes, opsonins, antimicrobial peptides and extracellular nucleic acid traps (analogous to vertebrate NETs) [68]. The model is also capable of hosting polymicrobial infection, so that interspecies interactions and their implications for drug efficacy may be modelled [69].

The short lifespan of *Galleria* makes it ideal for high throughput *in vivo* screening, providing an environment more representative of the human immune environment than existing high throughput *in vitro* models. Lead antimicrobials that passed *in vitro* testing could be screened out using a *Galleria* screen, reducing the burden of preclinical testing in vertebrate models. *Galleria* has also been utilised in acute toxicity testing of novel pharmaceutical compounds. In one study, 11 compounds were injected in increasing doses, until the concentration at which 50% of the larvae died (LC_{50}) was determined [70]. LD_{50} values correlated with those recorded in mammalian models. As with all organisms used in toxicity testing, it is challenging to correlate with LD_{50} in humans, as no such data is collected. Regardless, *Galleria* has proven a valuable model for such investigations, and could be utilised in the context of CF antimicrobials.

The genome of *Galleria mellonella* is yet to be sequenced in its entirety, and there is no current means to generate mutants. Additionally, while there are ABC transporter proteins with some similarity to CFTR, the *CFTR* gene itself is not present in invertebrate species [71]. These factors make mimicry of the CF phenotype currently unfeasible. Given the gross anatomical differences between *Galleria* and humans, and the systemic impacts of *CFTR* mutation, one would expect a lower degree of translation between antimicrobial success in the *Galleria* model and clinical outcomes, as compared to that of vertebrate species with lungs and availability of *CFTR* mutants. There is also a high degree of genotypic variance within populations of *Galleria* which results in lower consistency between experimental outcomes. One example of this variability is between two experimental procedures investigating virulence of a strain of *Streptococcus pyogenes*. One study infected with 10^6 bacteria and observed *Galleria* survival of 45% after 24 h and 25% after 96 h, while another infected with 8×10^6 and observed survival of ~90% after 24 h and 70% after 96 h [68]. This disparity is also consequence of variation in breeding and handling. Despite these limitations, the *Galleria* model remains of considerable utility for CF antimicrobial development as it allows high-throughput screening, and accurately mimics aspects of vertebrate innate immunity.

Zebrafish

Zebrafish are non-mammalian vertebrates used primarily to study host-pathogen interactions during infection (Table 2). They are relatively inexpensive, easy to maintain and carry the unique characteristic that their embryonic phase is transparent, enabling real-time live imaging of infection. During embryonic development, the innate immune system is active, with macrophages and neutrophils among the first to develop [72]. Additionally, expression of proinflammatory cytokines, complement proteins, toll-like receptors and acute-phase proteins closely resemble those in humans [73]. The zebrafish CFTR protein has 55% homology with human CFTR [72, 74]. It is possible to knock-down the *CFTR* gene transiently, through the injection of translation-blocking oligonucleotides, or to produce *CFTR* knockout fish lines. Zebrafish are readily amenable to genetic manipulation and mutant CF lines show evidence of pancreatic dysfunction and haematopoietic defects [72]. Infection of CF zebrafish with *P. aeruginosa* strain PA14 yielded bacterial populations in early stage of infection that were at 3.5-fold times the density of that seen in wild-type controls.

In an example of utility for therapeutic testing, zebrafish embryos infected with *P. aeruginosa* PA14 were injected with ciprofloxacin or imipenem, which rescued them from lethality [73]. Meanwhile, in *CFTR* loss-of-function zebrafish infected with PAO1, phage therapy was tested using a cocktail of phages. The treatment decreased lethality, bacterial burden, and the pro-inflammatory response [75].

Zebrafish are an attractive model for research into host-pathogen interactions during infection with CF-associated pathogens. Embryos within the first 120 h of development require no ethical approval, so study within these models reduces the number of protected animals used in CF research [76]. In recapitulating the CF respiratory environment, the major limitation of the zebrafish is their lack of lungs, though in mimicking the disease state in other faculties, the model provides an attractive solution for study of pathogenesis and development of treatments.

Rodents

Rodent models are the most extensively used animal models in biomedical research [77]. Their application in CF research is as ubiquitous, with many CF models and infection models being developed. As a mammalian vertebrate model, it is well suited for modelling the human environment, while the mouse CFTR protein bears 78% amino acid homology with human CFTR (Table 2) [3, 78].

CF models

Since the discovery of the *CFTR* gene, 16 CF murine models have been created through mutation of the *CFTR* gene (Table 3). These are grouped into two categories. In the former, replacement mutation generates knockout mutants through disruption of the gene, while insertion mutation results in the creation of knockdown mutants with ~10% residual function. The second category involves CF mutants created using known human mutations.

CF mutants can recapitulate the CF environment with varying accuracy. *Cftr*^{tm1Unc} develops spontaneous, progressive lung disease in the absence of pathogenic infection, characterised by loss of mucociliary transport, alveolar hyperinflation, parenchymal interstitial thickening, and inflammatory cell recruitment [3]. Further studies have since increased knowledge of these models, and the CF environment. *Cftr*^{tmr1Hgu} mimics inflammation; mucociliary clearance dysfunction is observed in *Cftr*^{tm1Hgu} and *Cftr*^{tm1Unc} and nasal epithelium airway surface liquid depletion in *Cftr*^{tm1Unc}. In the *Cftr*^{tm1G551D} mouse, defective pulmonary clearance and high *P. aeruginosa* sensitivity is observed [3].

While the described CF mutants mimic the lung environment with accuracy, they also exhibit severe intestinal disease, with the neonatal death rate from meconium ileus (MI) at ~60% in knockout mice, and 35% in F508del mice [3]. This is significantly higher than the 10% death rate from MI observed in human patients. The defect occurs due to a lack of cAMP-stimulated Cl^- transport in intestinal epithelial cells. A gut-corrected mutant has since been developed, which contains the FABP-h*CFTR* transgene [79]. h*CFTR* is expressed in the intestine, though remains undetectable in both lung and nasal epithelia. Talniflumate has also

Table 3. Existing cystic fibrosis transgenic murine models [3]. Superscript notation relates to the type of mutation and the model origin

Model	Cftr mRNA vs wild-type	Survival to maturity
Knockout models (replacement method)		
Cftr$^{tm/Unc}$	None detectable	<5%
Cftr$^{tm/Cam}$		<5%
Cftr$^{tm/Hsc}$		25%
Cftr^{tm3Bay}		40%
Cftr^{tm3Uth}		25%
Residual function models (Insertional method)		
Cftr$^{tm/Hgu}$	10%	90%
Cftr$^{tm/Bay}$	<2%	40%
F508del models (Replacement-gene targeting method)		
Cftr^{tm2Cam}	30%	<5%
Cftr$^{tm/Kth}$	Decreased levels in intestine	40%
F508del models (Hit and run method)		
Cftr$^{tm/Eur}$	Normal	90%
Other models		
Cftr^{tm2Hgu}	50%	65%
Cftr^{tm3Hgu}	Normal	90%
Cftr^{tm2Uth}	5–20%	95%

been used to correct the phenotype. The drug had three modes of action: inhibiting mucous synthesis, exerting non-steroidal anti-inflammatory effects, and inhibiting exchanges implicated in intestinal NaCl absorption. Treatment of CF mice increased survival from 26–77% [80].

Though these methods have high accuracy in their mimicry of the upper respiratory tract, the lower respiratory tract of PwCF is not well modelled. Thus far, no CF mice develop spontaneous infection, potentially because living conditions are relatively aseptic compared to the varying pathogenic challenge faced by humans [3]. Physiological alterations also contribute. Non-CFTR calcium-activated Cl^- channels are expressed in some mouse tissues, lowering the ion imbalance that causes CF lung disease, while low expression of ATP12A, a proton-pump, reduces pH acidification which allows normal function of host defences [3, 78]. Consequent to the lack of spontaneous infection, novel methods have been formulated to mimic natural colonisation of common pathogens (Fig. 4) [81].

Agar bead model

The agar bead model was developed to mimic advanced chronic *P. aeruginosa* infection [82, 83]. It involves the surgical implantation of *P. aeruginosa* enmeshed in agar beads into the trachea (Fig. 4). While initial stages of infection are not replicated, chronic infection in the CF lung is modelled well, with increased inflammation, neutrophil influx, and increased cytokine production. Infection mimics natural aggregate formation, with a small degree of migration that is also observed in natural infection. Fixation to the agar beads also affords protection from immune clearance, and provides a microaerobic environment for growth, which is also observed in biofilms. *P. aeruginosa* is well adapted to microaerobic respiration, so mimicking this aspect could provide accurate insight into the transcriptomic changes associated with growth in this environment [84].

Though the model is of significant benefit to research into chronic infection, its utility is limited to late, established infection. The model also requires surgical procedures, introducing additional sources of variability and confounding factors that may influence experimental outcomes. Finally, it is difficult to accurately distribute the agar beads across the multiple lobes of the rodent lungs. High density, late-stage chronic infection does, however, make the model attractive in antimicrobial testing.

Inhalation model

To study early infection processes and the transition to chronic infection, a number of infection models have been created that utilise a more natural route of infection. Fothergill *et al.* created a model *P. aeruginosa* infection that mimics chronic infection

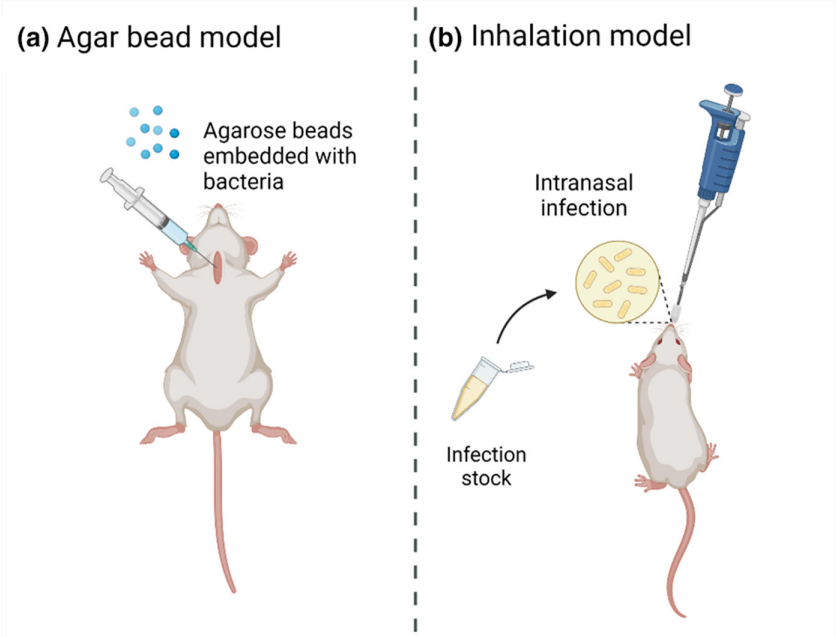

Fig. 4. Methods of inoculation utilised in the modelling of cystic fibrosis infection. (a). The agar bead model involves the injection of agarose beads laden with *Pseudomonas aeruginosa*, which establishes chronic infection. (b). Intranasal infection via the inhalation model involves infection with a liquid stock of cultured bacteria. The model accurately mimics early stages of infection. Created with BioRender.com.

over a minimum 28 day period (Fig. 4) [85]. The model involves intranasal infection of BALB/cOlaHsd mice, using a CF-adapted isolate of *P. aeruginosa*. Over the course of infection, genotypic and phenotypic changes observed in the bacteria were found to mirror those observed in PwCF. Genes associated with biofilm formation were upregulated, antibiotic resistance to tobramycin and ciprofloxacin was increased, and adaptation to the *in vivo* environment at the expense of the *in vitro* environment was observed.

The inhalation model presents an opportunity to study the early stages of infection, which is challenging in humans, given the difficulty of obtaining early-infection samples from PwCF. The method of inhalation is more natural than the agar bead model, though could be improved upon through nebulised administration, whereby the pathogen is administered in a fine spray. Nevertheless, the model accurately mimics the proposed progression of *P. aeruginosa* infection from the upper airways (sinuses and nasopharynx) into the lung. The primary limitation of the model, particularly with regard to antimicrobial development, is the low density of *P. aeruginosa* infection within the lung that is achieved. Development of methods to increase the achievable density of lung infection would significantly improve the utility of this model for such studies.

Although murine models have utility in CF research, their extensive use in drug development pipelines runs contrary to the principles of reduction, replacement, and refinement that underpin ethical use of animals in research. Murine models would be best utilised in later-stage preclinical trials, where only a handful of the most promising agents are being considered. Developing more representative non-vertebrate models would reduce burden on murine models, with fewer false positives reaching that stage.

Ferrets

Ferrets are mammalian vertebrates with long lifespans and CFTR amino acid homology of 91.9% compared to humans (Table 2) [78, 86]. Similarity of the anatomy and lung cell biology to humans means the ferret has been widely used in lung infection studies, including of severe acute respiratory syndrome and influenza virus [87, 88].

As in humans, CF ferrets develop spontaneous lung infection shortly following birth, which establishes itself as chronic infection. Bacterial genera including *Streptococcus*, *Staphylococcus* and *Enterococcus* were found to infect newborns, with lower burdens of three *Pseudomonas* species (*P. fluorescents, P. putida* and *P. fulva*) also present [3]. Immunity is also well modelled in ferrets, with macrophages producing high levels of cytokines following infection [89]. As with humans, newborn CF ferrets exhibited lower IL-1β levels but higher IL-8 and TNF-α. Postnatally, mucociliary clearance reduces sevenfold in adults compared to juvenile ferrets, while airway surface liquid thickness also decreases throughout development [3].

Newborn *Cftr*$^{-/-}$ ferrets also develop mild pancreatic pathology, though to a lesser extent than humans [3]. One drawback to the use of CF ferrets is mortality associated with intestinal disease. Between 50–100% of newborn CF ferrets develop MI, a bowel obstruction, and die within 36 h due to obstruction and sepsis. Regardless, ferret models are useful in the study of spontaneous

infection, which murine models do not develop. If the burden of MI can be alleviated, ferrets may prove valuable for evaluation of antimicrobials during early-stage, spontaneous infection.

CHALLENGES IN MODEL VALIDATION

While it is important to create preclinical models that accurately mimic the CF respiratory environment, models are of little use if the preclinical endpoints do not correspond with those observed in the clinic. Models that mimic clinically-relevant endpoints to greater accuracy will result in fewer failures in the transition between positive preclinical results and clinical trials.

Development of preclinical and clinical endpoints

Currently, microbiological parameters, such as eradication of infection or reduction in colony forming units are the primary endpoints observed in preclinical models. However, resolution of chronic infection is rarely achieved in PwCF [90]. In the clinic, patient-defined assessments are made, including lung function measured by spirometry, quality of life questionnaires and time between pulmonary exacerbations [91]. Measures such as these cannot be made in preclinical models. It would be desirable, therefore, to develop a biomarker panel that links preclinical and clinical evaluation. That is, a set of analytes that can be measured in testing models and for which increases or decreases in PwCF, post-antimicrobial therapy, are correlated with improvements in lung function, quality of life or time between exacerbation.

Bacterial load is used as a measure of therapeutic efficacy in preclinical models, although it does not consistently correlate with resolution of exacerbation [92, 93]. Virulence factors that contribute to a reduction in frequency and severity of exacerbation may instead be a relevant starting point for a biomarker panel. In *P. aeruginosa*, pyocyanin production has been correlated with pulmonary exacerbation, with the proportion of pyocyanin-overproducing isolates being significantly increased during periods of exacerbation compared to periods of stability [94].

Expression of quorum sensing (QS) molecules has also been correlated with positive clinical outcomes. QS regulates swarming motility, biofilm maturation, and antimicrobial resistance, as well as virulence determinants including pyocyanin, elastases, cyanide, and exotoxins [95]. QS signalling molecules (QSSM) including 2-heptyl-4-hydroxyquinoline, 2-nonyl-4-hydroxyquinoline and 2-heptyl-4-hydroxyquinoline-*N*-oxide were positively correlated with *P. aeruginosa* load at the start of exacerbation [95]. During antibiotic treatment, only two QSSMs decreased significantly, while bacterial load remained relatively constant between the start and end of exacerbation. The biomarkers investigated in the study are present in sputum, plasma, and urine, their ease of sampling increasing their utility. Another study [96] outlines a positive correlation between three 2-alkyl-4-quinolones (AQs) produced by *P. aeruginosa* and bacterial load in sputum, plasma, and urine. This was detected using culture-independent methods that alleviated some variation in culture-based approaches. This research is supported by another study, that detected AQs in PwCF with chronic *P. aeruginosa* infection and found a positive correlation between saliva AQs, sputum AQs and quantitative bacterial load [97]. These biomarkers serve as potential non-invasive correlates of *P. aeruginosa* infection.

There has been increasing interest in QS-targeting anti-virulence treatments, as QSSMs regulate numerous virulence determinants without impacting growth. This limits the capacity for selection of resistance [98]. Development of a biomarker panel that includes virulence factors of not only *P. aeruginosa* but those of other CF microbes would provide comprehensive evaluation as to the efficacy of potential anti-virulence treatments. Combined use of both pathogen and host biomarkers, such as inflammatory markers, immune signalling molecules or tissue remodelling factors, would enable development of both antimicrobial therapeutics, anti-virulence treatments or immune modulating treatments within the same preclinical framework.

Refining treatment regimens for people with CF

PwCF face a considerable therapeutic burden, being prescribed a median of ten simultaneous treatments [6]. Sawicki, Sellers and Robinson [99] assessed the burden of treatment for 204 individuals, in which the median number of treatments was seven, and the mean time taken to complete therapy, 108 min. Overall adherence to treatment varies between route of administration, though generally decreases with age [100]. As of 2011, 39% of adults and 67% of children adhered to nebulised therapy, while only 30% of adults adhered to chest physiotherapy [101]. The opportunity to alleviate the burden of treatment, and by extension increase adherence of PwCF to treatment, is enabled through development of antimicrobials with high efficacy and multiple mechanisms of action. This requires the creation of accurate, high throughput preclinical models, only achievable if a comprehensive model validation system is in place.

Optimising therapeutics for nebulised and inhaled delivery is also necessary to reduce burden of treatment. Nebulisation of antibiotics offers the advantage of achieving a higher concentration within the respiratory tract, without the systemic impacts that other modes of administration such as intravenous have [102]. Aerosolised aztreonam lysine is one example, significantly reducing frequency of exacerbation and bacterial load, while improving pulmonary function and quality of life [92]. To optimise development of nebulised and inhaled treatments, preclinical models must allow for their mode of delivery. Mammalian models are advantageous in this regard, whilst some *in vitro* models may be suitable for aerosolised or nebulised exposure, with optimisation.

OPPORTUNITIES

There are opportunities to streamline antimicrobial development and improve outcomes for PwCF. Existing models can be benchmarked and refined, and pharmacokinetic-pharmacodynamic (PKPD) modelling can provide valuable insight into antimicrobial deliverability and efficacy. Meanwhile, modulator therapies are altering the CF respiratory environment, impacting the efficacy of existing antimicrobials, and research into emerging alternative treatments is providing novel means of tackling infection.

Benchmarking of preclinical models

New and existing preclinical models require benchmarking in order to evaluate how well they recapitulate core components of the CF environment. To do this, a quantitative framework has been developed which analyses transcriptomic changes in *P. aeruginosa* from preclinical models and compares the data to those obtained from *P. aeruginosa* directly isolated from CF sputum [13]. The framework considers the proportion of genes for which expression in a model environment is within two standard deviations of the expression observed in CF sputum. SCFM2 and an airway epithelial cell model were found to be the most representative models of those tested, with the number of genes for which expression fell between two standard deviations at 86 and 84%, respectively [13]. Meanwhile, LB had a value of only 80%. Expression of functional categories of genes was also assessed. The murine infection model more accurately represented porin expression than SCFM2, while the epithelial cell model had more representative protein synthesis. Use of the quantitative framework will be vital in not only the benchmarking of existing models, but in the formulation of models tailored to specific phenotypic traits of CF microbes.

Pharmacokinetic-pharmacodynamic models

PKPD modelling provides valuable insight into the delivery, metabolism, and efficacy of potential antimicrobials. It is an essential part of therapeutic development packages. While pharmacokinetics of therapeutics administered orally and parenterally are well studied, pulmonary administration of inhaled antimicrobials is less well characterised [103]. Analysis of PKPD characteristics of novel drugs affords researchers an understanding of delivery, activity, clearance, and therapeutic index. This is particularly important in the context of *P. aeruginosa* biofilm formation, as drugs with higher penetration into the aggregate are more desirable. Sou *et al.* investigated the PKPD of aerosolized tobramycin in a preclinical rat infection model, where plasma samples were collected following pulmonary delivery of varying doses [103]. Growth and efficacy data were used alongside dosage information to generate a PKPD model that provided a quantitative description of the exposure-response relationship for the drug. Evaluating other preclinical models in this way will allow better insight into how well antimicrobial delivery, metabolism and efficacy are recapitulated in preclinical *in vivo* models, leading to greater refinement of the infection systems we use.

CF modulator therapies

Modulator drugs are aimed to correct the CFTR protein defect, restoring normal function. To date, two types of modulators have been approved to treat CF, potentiators and correctors [104]. As of 2020, highly effective CFTR modulators are available for PwCF carrying specific mutations and have been associated with remarkable improvements in clinical outcomes [105, 106]. CFTR function restoration has been associated with significant changes in lung microbiology and may also impact mucous composition, host immunity and antimicrobial exposure [107]. This is an area that will require further investigation, as antimicrobials will need to be created that are efficacious in the altered respiratory environment and screening models will need to be updated to reflect those changes.

Collaboration within the CF community

The development of preclinical models, benchmarking frameworks, CF modulator therapies and antimicrobials is only possible through collaboration of all stakeholders implicated in development, licensure and use. The level of engagement of PwCF with research is high. In the UK, The Cystic Fibrosis Trust supports groups of lay reviewers to liaise with researchers, allowing input into trial design and review of their outcomes [108]. Individuals can also participate in focus groups to share their views and experiences in surveys, or become peer advocates, who share their experiences and communicate the value of research to others. The James Lind Alliance has also established a priority setting partnership for CF, in which PwCF and researchers can align on priority research topics [4]. This collaboration can be leveraged to ensure the most suitable therapeutics for PwCF are prioritised, with a long term goal of reducing the burden of treatment and streamlining administration regimes.

Alongside collaboration with PwCF, it is vital to have strong integration between those directly involved in research and development. Initiatives that bring together academics, industry and regulators are vital in increasing both the efficiency at which we develop solutions, and in most effectively leveraging the funding available in the sector.

Emerging treatments

The emergence of CF modulators is changing the landscape of CF lung disease and is likely to impact on the development and utility of antimicrobial therapeutics. It is too early to accurately assess the long term outcomes of modulator therapy and given the

high morbidity of chronic lung infection it is important to continually develop antimicrobials that can tackle current pathogens. Nebulised antibiotics and anti-virulence therapies are at the core of this future development. Currently, there is only a limited selection of aerosolized antimicrobials, including colistin, tobramycin, aztreonam lysine and levofloxacin [4]. Research into anti-virulence drugs is ongoing, with one study identifying two compounds, nitrofurazone and erythromycin estolate, that reduce the expression of PqsE, a QSSM implicated in virulence factor production and biofilm formation [109]. Antibiotic potentiators are also in development, with the potential to increase the efficacy of antibiotics against Bcc infections in PwCF [110]. Meanwhile, phage therapy is a rapidly emerging area of therapeutic interest with clinical success against some CF pathogens already reported [111]. Therapeutic interventions such as these may prove an important part of our antimicrobial toolkit in the continuing fight against chronic infection in PwCF.

CONCLUSION

Preclinical drug development frameworks that are standardised, and that make use of refined and comprehensively validated models would accelerate antimicrobial development. Increasing the proportion of new drugs that make the successful transition from promising preclinical findings to demonstration of efficacy in clinical trials would represent a more efficient use of limited financial resources available for antimicrobial drug discovery. In the unique case of treatment of chronic lung infection in PwCF, it is of paramount importance to focus on capturing the environmental conditions of the drug target site in preclinical model systems. Only through collaboration between funders, researchers, industry, people with CF, regulators and policy makers will the necessary innovation and acceleration in this area be possible.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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