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Review

New concepts in antimicrobial resistance in cystic fibrosis respiratory infections

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ABSTRACT

In this review, we summarize the main points that were raised and highlighted during the pre-conference meeting to the 17th European Cystic Fibrosis Society Basic Science Conference, held from 30 March to 2 April, 2022 in Albufeira, Portugal. Keynote lectures provided an update on the latest information regarding the phenomenon of antimicrobial resistance (AMR) in cystic fibrosis (CF). Traditional themes such as *in vitro* antibiotic susceptibility testing and its clinical value, AMR evolution in persistent *Pseudomonas aeruginosa* infection and the impact of biofilm on AMR were discussed. In addition, the report gives an overview on very recent AMR-related topics that include an ecological view of AMR in CF lung, referred to as resistome, and novel anti-infective approaches in preclinical or early clinical research such as antibiofilm drugs and bacteriophages.

1. Introduction

Never has there been a more exciting time to be working in the science behind cystic fibrosis (CF). The progress over the last few years in CFTR modulator therapy and the energy this has catalysed in drug development is genuinely game-changing. Improvements in diagnosis and standards of care over the last few decades have led to health and survival benefits, with a huge proportion of the CF population now reaching adulthood. However, the majority of these people have recurrent or chronic pulmonary infections and, at least to date, there is little evidence that even transformational therapies will have a major impact on these. We are still completely reliant on antimicrobials that are administered to many people with CF (pwCF) on a daily basis as a means for eradication of newly acquired infection, treatment of pulmonary exacerbations or suppressive maintenance therapy in cases of chronic infections [1]. Both healthcare providers and pwCF express their concerns about the inevitable increase in antimicrobial resistance (AMR), mostly perceived in an association with frequent use of inpatient (i.e., intravenous) as well as outpatient (i.e., inhaled or oral) antibiotics against traditional CF pathogens such as *Pseudomonas aeruginosa, Burkholderia cepacia* complex or nontuberculous mycobacteria [2]. Thus, AMR is a well-recognised and worsening problem in CF. This report aims to summarize the latest knowledge and the key aspects of the AMR in CF at the research, clinical laboratory and healthcare levels, presented by opinion leaders at the pre-conference meeting on this topic at the 2022 European CF Society Basic Science conference.

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2. Caveats of antimicrobial susceptibility testing in CF isolates

The ultimate goal of antimicrobial susceptibility testing (AST) from a clinical standpoint is to predict the success or failure of therapy with an antimicrobial drug, based on categorization of isolates as "susceptible" or "resistant". While the performance of AST is subject to microbiological standards of care, its results, subsequent correlation with clinical outcome and ultimately, indication and frequency of its repeated performance in chronic CF infections continue to be a matter of debate with available evidence demonstrating utility is poor [3–6]. This most likely relates to the fact that standard AST is designed to be applied to single bacterial species, cultured in the context of acute infection, not to a community of microorganisms causing a chronic polymicrobial infection.

A growing body of evidence raised doubts about the reliability of current microbiological tests for identifying clinically-effective antibiotics in CF. For example, multiple studies [5,7-9] found no relationship between *in vitro* susceptibilities of *P. aeruginosa* isolates and patients' clinical responses to consequent antibiotic choices. These studies primarily focused on *P. aeruginosa;* however, similarly poor predictive capacities of susceptibility tests have been found for nontuberculous mycobacteria in CF [10], as well as for a range of non-CF chronic infections [11]. Diverse potential contributors and explanations for this problem have been suggested. For example, Foweraker et al. [12] demonstrated that *in vitro* susceptibilities of *P. aeruginosa* isolates in individual CF sputa vary dramatically even within a sample, raising doubts about the accuracy to be expected from testing a single isolate.

The concept of resistance of CF lung pathogens, the potential usefulness of AST in the selection of appropriate antimicrobial therapy and the need for appropriate clinical breakpoints for the interpretation of the antibiogram have recently been reviewed [13–15]. Neither the U.S. Clinical and Laboratory Standards Institute (CLSI) nor the European Antimicrobial Susceptibility Testing (EUCAST) Committee have included inhaled antibiotics in their proposals of defining clinical breakpoints and standardisation of AST. This decision was related to the differences between those microorganisms isolated from patients with chronic lung infection and those that cause sepsis or any other acute infection such as community or hospital-acquired pneumonia. Also, it has been challenging to apply current standards for performing AST to CF pathogens due to their characteristic growth (reduced growth rate and often in biofilms rather than in planktonic mode), great diversification to multiple morphotypes, their ability to exhibit tolerance, persistence and heteroresistance, and the high frequency of hypermutator phenotypes [15–19].

The CLSI does offer two brief technical recommendations: that AST of *P. aeruginosa* isolates from CF patients can be performed by both disc diffusion and dilution methods, and that the incubation of the tests should be extended up to 24 hours to facilitate their reading [20]. The EUCAST includes epidemiological cutoff (ECOFF) values for topical use, but explicitly excludes their use for inhaled antibiotics [21]. ECOFF refers to inhibitory concentration values that discriminate wild-type bacterial populations from those with acquired resistance mechanisms [22,23]. However, they are not applicable to scenarios where much higher concentrations of antibiotic are reached at the infection site when compared with those obtained with the oral or intravenous route of administration [13].

Consensus documents recommend the performance of AST for isolates from pwCF for varying reasons; specifically, for understanding the potential impact of antimicrobial use on pathogens and their evolution of AMR, selection of treatment for current or next exacerbation, and to explain treatment failure [6,15,24,25]. It is routinely recommended to study different isolate morphotypes separately, avoiding the pooling of multiple colony types, and to incubate with antimicrobials for 24 hours. Diffusion techniques, either with discs or gradient strips, also allow the phenotypic detection of potential hypermutator strains, which can lead to a closer monitoring of the possible failure due to selection of populations with lower antimicrobial sensitivity [26]. The study of CF isolates' susceptibilities as biofilms has also been proposed, in general applying a methodology similar to the determination of minimal inhibitory concentration (MIC) (by using the Calgary biofilm device), in which the proposed value is the biofilm inhibitory concentration (BIC), i.e. the lowest concentration preventing the growth in biofilms [27] (Table 1). In this case, the inoculum used is an already formed biofilm. Arguably more representative AST value would be the concentration that eliminates biofilm (biofilm bactericidal concentration; BBC) or the concentration that prevents biofilm formation (BPC) [27,28] where the antimicrobial interacts with the biofilm at the time as it is formed (Figure 1).

However, current evidence does not support the use of biofilm AST to guide antimicrobial treatment of *P. aeruginosa* pulmonary infections in pwCF. Neither microbiological (e.g., a change in *P. aeruginosa* density in sputum), nor clinical outcomes (see below) demonstrated that biofilm AST was superior to conventional AST [29,30].

In addition to the inherent methodological problems with AST mentioned above, there are also problems of defining microbiological or clinical endpoints to evaluate the efficacy of the therapy, either empirical or driven by the AST results [31]. Eradication, once chronic infection is established, is difficult to achieve, so other parameters such as the decrease in bacterial load, reduced antibody responses, reduction in exacerbation frequency, time to the next exacerbation, improvement in lung function or even improvement in the quality of life have to be assessed [13,32-34]. As a consequence, efforts to define the clinical breakpoints for inhaled antibiotics and both microbiological and clinical outcomes should continue, to better understand benefits of antimicrobial treatment. Without overcoming technical issues and finding meaningful clinical correlates, reservations about utility and clinical value of AST are appropriate and shared by the authors of this review.

3. Is measuring CF lung resistome clinically useful?

The term resistome was coined in 2006 by Gerry Wright at the University of Michigan [35,36], referring to an ecological, rather than a clinical, concept [37]. His definition was "a collection of all the antibiotic resistance genes and their precursors in pathogenic and non-pathogenic bacteria", i.e., specifically including bacteria that are both identified and not identified as pathogens, and also "precursor" genes that could confer resistance only if adapted or upregulated. This focus of the term resistome on the presence or absence of genes within an entire, diverse population of microbes highlights a key difference from what the clinical microbiology laboratory usually measures from CF respiratory samples: the expression of resistance in individual microbial isolates during *in vitro* monoculture.

Current methods used in clinical CF microbiology are intentionally selective [38]. Respiratory samples are cultured using a battery of media formulated to identify pathogens most associated with CF lung disease, while selecting against common microbes without a known role in disease. Cultured isolates are then individually tested for AST without defining mechanisms of resistance. These features of clinical laboratory results - defining phenotypes of specific pathogens - contrast sharply with those of genomics-based resistome analyses that focus on the presence or absence (but not activity) of canonical resistance mechanisms among all bacteria in a sample without considering whether those bacteria are pathogens [37,39]. For these reasons, CF resistome results can be expected to differ substantially from conventional CF labo-

Table 1
Different antimicrobial susceptibility testing parameters and inoculum used.

Parameter	Definition	Inoculum
Minimal inhibitory concentration (MIC)	Lowest antibiotic concentration that inhibits the visible bacterial (planktonic) growth after overnight incubation	Planktonic (10^5 CFU/ml)
Minimal bactericidal concentration (MBC)	Lowest antibiotic concentration that reduces an initial bacterial (planktonic) inoculum with 99.9% (≥3 log)	Planktonic (10^5 CFU/ml)
Biofilm inhibitory concentration (BIC)	Lowest antibiotic concentration that results in an OD650 nm difference of ≤10% (1 log difference in growth after 6 h of incubation) of the mean of two positive control well readings when a biofilm is used as inoculum	Sessile (biofilm previously developed)
Biofilm prevention concentration (BPC)	Same definition as the BIC, but bacterial (planktonic) inoculation and antibiotic exposure occur simultaneously to avoid biofilm development	Planktonic (10^5 CFU/ml)
Biofilm bactericidal concentration (BBC)	Lowest antibiotic concentration that reduces an initial biofilm inoculum with 99.9% (≥3 log) as compared to the growth control	Sessile (biofilm)

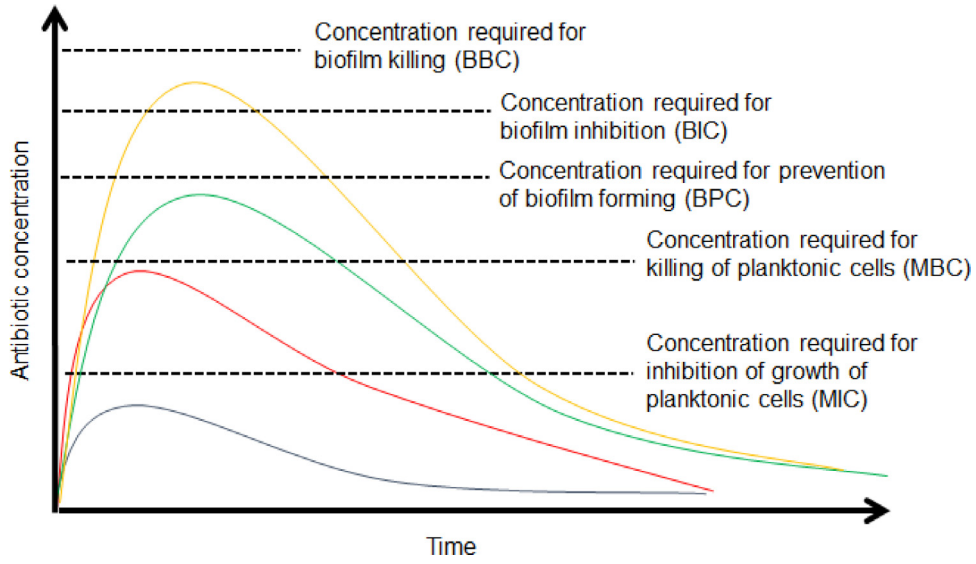

Fig. 1. Concentration over time at the site of infection of four hypothetical antibiotics. The dashed lines indicate the concentrations required for various effects against planktonic cells and biofilms as defined by MIC, MBC, BPC, BIC, and BBC (Table 1).

ratory phenotypic test results. In addition, sequencing-based (microbiome) methods often identify many bacteria in CF respiratory samples at abundances resembling those of conventional, cultured CF pathogens [40,41]. While the roles of these nonconventional bacteria in pathogenesis or response to therapy remain unknown, it has been suggested that interspecies interactions and other influences common in the CF airway [42] can alter the effects of antibiotics on pathogens [43].

Recent studies demonstrated the power of genomic methods for identifying the dominant contributors to *in vitro* susceptibilities for individual pathogens, such as *Mycobacterium tuberculosis* and *Staphylococcus aureus* [44], with the capacity to be faster and cheaper than culture-based methods. Notably, these pathogens are well-represented in genomic databases and are therefore ideal test organisms for molecular methods. By comparison, many CF pathogens have relatively few complete genomes available for computational comparison; for example, genomics seem more likely to predict resistance for *P. aeruginosa,* given the numerous genomes available for computational comparison, than for *Achromobacter* spp. [45]. In addition, given the limited clinical utility of *in vitro* AST [7,9,10,42], and because genomic methods are generally optimized to predict *in vitro* resistance of individual isolates of specific, well-studied species, it is unclear whether a pathogen-focused genomic predictor will be any more useful for clinical care than culture-based predictors.

Published reviews have detailed the enormous promise resistomics holds for improving cost and efficiency of predicting resistance among pathogens such as *M. tuberculosis* [46]. However, CF respiratory infections are often polymicrobial, with additional genomic diversity among populations of traditional pathogens such as *P. aeruginosa.* The roles in clinical responses to antibiotics of each microbe identified using untargeted genomics of CF respiratory samples is a topic of controversy and the focus of ongoing studies [47–49]. Resistome analyses would not easily determine which specific species carries a given resistance determinant, whether that species is important for clinical response, or whether

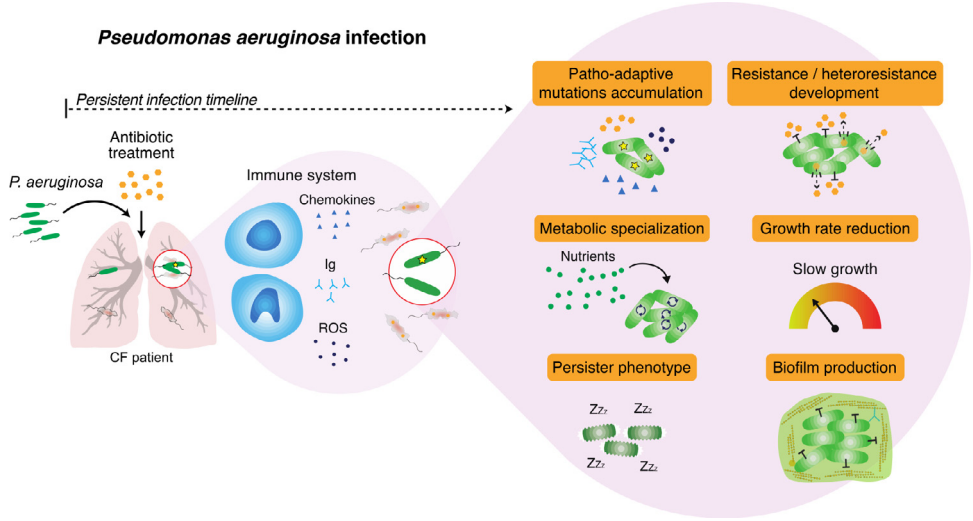

Fig. 2. *P. aeruginosa* infection timeline. Environmental *P. aeruginosa* strains colonize the airways of people with cystic fibrosis persisting for decades. To escape the immune system and resist antibiotic treatment, bacteria have to survive natural selection due to their pre-existing variants of resistant phenotype; furthermore, they modify their phenotype and adapt their physiology through accumulation of adaptive mutations and changes in gene expression profiles. Unconventional mechanisms such as heteroresistance development, metabolic specialization, growth rate reduction, persister phenotype and biofilm associated lifestyle strengthen further their persistence in the host.

that gene is active *in vivo*. Therefore, there are many challenges inherent in developing genomics-based CF resistomics measures for clinical use, including therapy guidance. However, the growing efficiency and power of genomic methodology provide hope for a future role for these approaches in directing CF care. This future will require a great deal of research, data validation and methodologic refinement.

4. Development AMR in bacteria: *P. aeruginosa* as an exemplar

Pathoadaptation to the environment of CF airways has been most extensively studied in *P. aeruginosa*, the pathogen that still causes chronic infections in over 40% of the European adult CF population [50]. The following section focuses specifically on findings in *P. aeruginosa*, recovered from young pwCF. Almost half of these pwCF were persistently infected with a single *P. aeruginosa* clone type [51,52] and the AST on a collection of early and subsequent *P. aeruginosa* isolates showed that during the first 5 to 10 years of infection, most of them remained susceptible to all anti-pseudomonas antibiotics, except for quinolones towards which resistance had developed in about 10 to 20% of the isolates. If we assume that AST provides clinically meaningful information (despite all the concerns related to the AST mentioned earlier), then these *P. aeruginosa* isolates should remain susceptible to antimicrobial therapy. However, they survive antibiotic exposure *in vivo*. Thus, their ability to establish chronic infection is likely a consequence of several other mechanisms beyond those conventionally involved in the development of resistance [53].

P. aeruginosa is known to develop various tolerance traits during infection in CF lungs (Figure 2). Slow growth is one of its adaptive phenotypes, and the metabolic footprint for amino acids, organic acids, and sugars also changes over time. In association with slow growth, antibiotic resistance towards ceftazidime, carbapenems, quinolones and aminoglycosides has been observed [54]. Persister cells, tolerant to antibiotics, are found in all bacterial populations. Although the persister phenotype *per se* is not associated with genetic changes, a fraction as high as 20% with a high-persister phenotype has been found among early CF isolates [55].

As an example of a less expected AMR mechanism, it was found that *P. aeruginosa* isolates from patients receiving tobramycin therapy developed L6 ribosomal protein mutations and associated with aminoglycoside resistance. The L6 mutations had additional impacts on the bacterial phenotype such as decreased growth rate, and development of collateral sensitivity to chloramphenicol. The L6 mutants were eliminated from the patient airways after cessation of tobramycin treatment [56]. Another common mechanism of aminoglycoside resistance in *P. aeruginosa* CF isolates is associated with mutations in the *mexZ* gene encoding a negative regulator protein, resulting in over-expression of the efflux pump proteins MexY and MexX. In the collection of nearly 500 whole genome sequenced *P. aeruginosa* clinical isolates, almost 40% carried a mutation in *mexZ*. However, only a minority showed clinical aminoglycoside resistance. Instead, they showed subtle, no more than 2-fold increased aminoglycoside and fluoroquinolone resistance relative to the wild type [57]. The link between *mexZ* mutations and the level of AMR as well as the reasons for the high frequency of these mutations among *P. aeruginosa* isolates needs to be further studied.

The use of azithromycin was adopted for treatment of pwCF in the 1990s to take advantage of its immunomodulatory and anti-alginate effects [58]. It was assumed that, as *P. aeruginosa* is inherently resistant to macrolides (high MIC values in standard AST), azithromycin resistance should not be induced in *P. aeruginosa* infecting CF airways. However, macrolide therapy in fact does select for AMR development in *P. aeruginosa,* related to mutations in the ribosomal protein gene L4 when assessed in alternative substrates [58]. When azithromycin resistance did develop, both the immunomodulatory effect and the inhibition of mucoidy were severely impaired. It is therefore important to reconsider this long-term therapy in pwCF; specifically, that azithromycin may lose efficacy after one to two years from the start of therapy [58].

As stated above, the successful survival of *P. aeruginosa* exposed to frequent intensive antibiotic treatment in CF airways arises from a combination of bacterial features. Initial infection usually involves antibiotic-sensitive and fast-growing environmental *P. aeruginosa* isolates. Over the course of infection, the bacteria adapt to the CF lung environment via the accumulation of pathoadaptive mutations and changes in their metabolism. A substantial fraction of the bacterial population enters a state of dormancy, becoming persisters. Eventual development of a reduced growth rate markedly contributes to the development of phenotypic resistance [53,59].

5. Biofilm, the inherent defence mechanism of CF pathogens against antibiotics

In vivo, microorganisms behave in a very different way from how they behave under laboratory conditions, forming multicellular aggregates embedded in a host-derived and/or self-produced extracellular matrix. These aggregates are designated as biofilms, and can be surface-attached (e.g. on the surface of a medical device), suspended (e.g. in synovial fluid) or embedded in host tissue (e.g. in a chronically infected wound). Cells in a biofilm are much less susceptible to antimicrobial agents compared with planktonic cells, and treatment of biofilm-related infections is often difficult (Figure 1). There is convincing evidence that important pathogens like *P. aeruginosa* occur as biofilms in the lungs of pwCF, which might help to explain (along with other bacterial adaptation processes mentioned earlier) why eradication of these infections is so difficult [60–63]. Multiple mechanisms are involved in reduced antibiotic susceptibility of bacteria in biofilms [64,65] and the microenvironmental conditions in the lung play an important role in this [42,66]. Indeed, changes in microbial metabolism, at least partly related to gradients in oxygen and nutrient levels, can have a profound effect on antimicrobial susceptibility [67–69]. The exact metabolomic adaptations vary between different microorganisms [69], and much remains to be learned about microbial metabolism in the infectious micro-environment (e.g. how the presence of multiple species affects metabolism and susceptibility [70]), but a common theme nevertheless starts to emerge. Biofilm-associated bacteria typically downregulate their central metabolism (e.g. the tricarboxylic acid, TCA cycle) with a concomitant upregulation of alternative pathways (e.g. the glyoxylate shunt); by doing so they produce fewer reducing equivalents (NADH, $FADH_2$) which slows down the electron transport chain and reduces the production of toxic reactive oxygen species [67,71,72]. The important contribution of the microenvironment and metabolism to biofilm susceptibility also has implications for *in vitro* evaluation of antimicrobial strategies and strongly suggests that the model systems to be used should closely mimic the *in vivo* micro-environmental conditions.

The observation that microbial metabolism plays a crucial role in reduced susceptibility during biofilm-associated infections also opens the door for novel treatment approaches: what if we could counteract the metabolic changes *in vivo*? Would this allow us to overcome bacterial defence mechanisms and increase antibiotic susceptibility? In this context, carbon sources can likely work as potentiators of antimicrobial activity. While their use to increase activity of antibiotics is not new (see for example [73,74]), this strategy has not been systematically explored for biofilms. A recent study using *P. aeruginosa* biofilms formed in an artificial CF sputum medium [75] demonstrated that, by activating the TCA cycle, it is feasible to potentiate the anti-biofilm activity of ciprofloxacin (using D,L-malic acid) and ceftazidime (using sodium acetate). While the observed anti-biofilm effects appeared to be antibiotic and strain dependent, and while much more work is needed (including validation in *in vivo* models), this study can be considered as a proof-of-concept that direct interference with biofilm metabolism can increase antibiotic susceptibility. Another intriguing alternative approach to overcome the biofilm barrier is hyperbaric oxygen therapy (HBOT). In CF, infected endobronchial mucus quickly becomes anoxic due to O_2 consumption by activated polymorphonuclear leukocytes that are recruited to kill the infecting bacteria. The resulting very low levels of oxygen force *P. aeruginosa* to generate energy in a different way (e.g. using nitrate as terminal electron acceptor), but this switch results in lower metabolic activity and growth, which in turn reduces the susceptibility to antibiotics [76]. The idea behind using HBOT is that reoxygenation of the anoxic zones (by exposure to 100% O_2 at 2.8 bar for 90 min) will activate microbial aerobic metabolism and will increase antibiotic susceptibility. Indeed, *in vitro* it has been shown that HBOT dramatically increased killing of *P. aeruginosa* biofilms by ciprofloxacin [77,78] and tobramycin [79]. In addition, HBOT lowered the tobramycin concentration required to achieve a 3-log (99.9%) reduction in the number of colony forming units by over 50% (i.e., the same killing could be achieved with much lower antibiotic concentrations) [79]. While HBOT has been used to treat various infections, mostly wounds with anaerobic bacteria, more evidence is needed that it will be clinically beneficial as adjuvant therapy for antibiotics in the treatment of respiratory tract infections in CF [76].

Finally, while development of resistance against these alternative (combination) treatments seems less likely than with current strategies, it cannot be ruled out. For example, while several quorum sensing inhibitors drastically increased the antimicrobial activity of conventional antibiotics against different bacterial biofilms [80,81], resistance towards this potentiating activity rapidly develops *in vitro* [82,83]. Moreover, resistance against these antibiotic-potentiating quorum sensing inhibitors was observed in clinical *P. aeruginosa* isolates that were never exposed to them before, illustrating the difficulties of finding anti-biofilm therapies that are "evolution-proof" [84,85].

6. Expanding the therapeutic arsenal against CF pathogens?

The emergence of AMR and the increased prevalence of difficult-to-treat pathogens highlight the need for novel antimicrobial molecules and/or strategies in pwCF. The novel molecules currently under evaluation as anti-infective drugs in the U.S. CF Foundation and the European CF Society drug development pipelines are mostly in early (phase 1 and 2) stages of development (Figure 3). These investigational products include gallium, nitric oxide and other antimicrobial substances (e.g., lactoferrin-hypothiocyanite or substances active against biofilm), and bacteriophages.

Gallium is a metal, nearly identical to iron, that disrupts iron metabolism in bacteria and exhibits therapeutic effects in mice and humans with lung infections [86]. Intravenous gallium is approved by the Food and Drug Administration for intravenous use in humans and is being studied in phase 1 or 2 trials in pwCF using intravenous or inhaled formulations for targeting *P. aeruginosa* or *Mycobacterium abscessus* infections. Novel formulations of gallium are being studied and may show improved antimicrobial effects against Gram-positive and Gram-negative bacteria, and nontuberculous mycobacteria [87].

Nitric oxide is a gas that exerts natural antimicrobial effects. One hypothesis that has been suggested for many years regarding severe infections is that increasing levels of nitric oxide could help kill bacteria and eliminate their biofilms in the lungs of pwCF [88]. Phase 1 and 2 studies are being conducted in pwCF. A new inhaled glycopolymer SNSP113 that may disrupt bacterial biofilms and target the mucus layer in the lung has been recently developed and will be tested in pwCF.

A combination of lactoferrin and hypothiocyanite, two natural substances with antimicrobial activities, has been proposed to be a potentially useful strategy for treating bacterial infection in pwCF. *In vitro* studies have revealed promising antibacterial effects on CF pathogens, including *P. aeruginosa* [89]. However, the first in man clinical study has been ongoing for several years and has been terminated due to financial issues; it is unknown whether this compound will be further developed in pwCF.

Great hope has emerged with the revival of research into bacteriophages, which had been put on hold or overlooked for many years during past periods of full confidence in success of antibiotics. Bacteriophages are viruses that exclusively infect bacteria and can act as potent bactericidal agents [90] thanks to their ad-

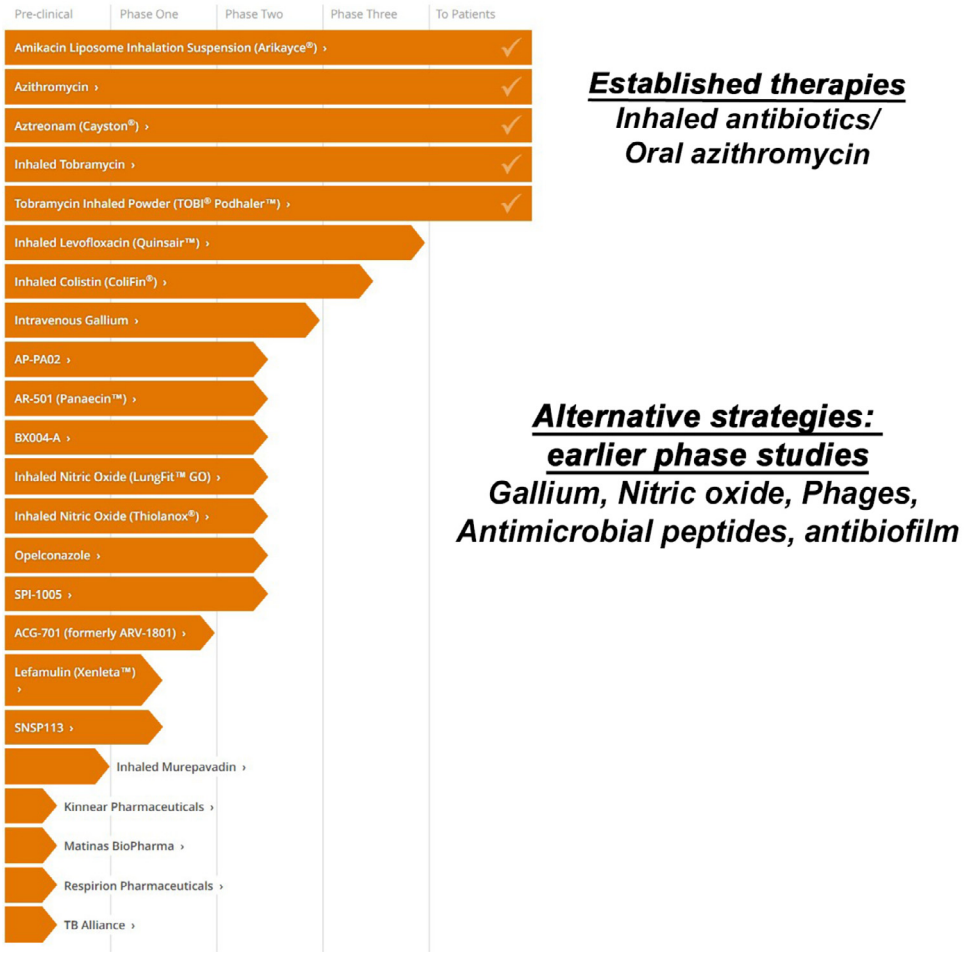

Fig.3. The antimicrobial compounds in the CF therapeutic development pipeline as of October 2022 (adapted from CF Foundation website).

vantageous features such as self-amplification at the site of infection or the capacity to disrupt biofilm matrix. Their high host specificity makes them very promising tools for targeted and personalised anti-infective therapy. Anecdotal reports described interesting effects of inhaled or intravenous bacteriophages in pwCF who developed infections with untreatable *M. abscessus* [91,92], pan-drug resistant *A. xylosoxidans*[93] or *P. aeruginosa*[94]. Phage cocktails (ready-to-use, or "magistral", customized preparations [95]) have been produced by several laboratories worldwide, and early phase clinical trials on the phage therapy of *P. aeruginosa* in CF have been designed. Compassionate use of bacteriophages is also ongoing in multiple countries. Yet many questions remain unanswered, including how to test the efficacy of phage therapy for pwCF both *in vitro* and *in vivo*, how to select phage cocktails, how to combine phages with antibiotics, and how best to deliver phages to CF airways [96,97].

Of note, the CF pipelines mentioned above are not the only routes for approval of new antimicrobial compounds for pwCF. Additional novel antibiotics, applicable also to CF infections, have been commercialized in the past few years, although they have not been subject to clinical trials in pwCF. These novel broad spectrum antibiotics, mostly beta-lactams in combination with beta-lactamase inhibitor (including ceftolozane-tazobactam, ceftazidime-avibactam, imipenem-cilastatin-relebactam, meropenem-vaborbactam and cefidorocol) may be useful in the treatment of Gram-negative bacteria (e.g., *P. aeruginosa, A. xylosoxidans, Stenotrophomonas maltophilia*, and *B. cepacia* complex) and nontuberculous mycobacteria and could therefore be considered in pwCF with difficult-to-treat airway infections [98,99]. These novel antibiotics have shown interesting *in vitro* activity in several studies using Gram-negative bacterial strains isolated from CF sputum [100–102] and are being increasingly used in pwCF, as reported in short case series [103,104]. Imamovic et al. have further suggested novel strategies of cycling approaches using available antibiotics, as the evolution of AMR to *P. aeruginosa* confers predictable sensitivities to other classes of antibiotics [105]. To the best of our knowledge, this recently-presented approach is not currently being tested in clinical trials.

At this time, antibiotics remain the main approach to fight airway infection in pwCF and their wise use, with the aim to maximize therapeutic effect and to minimize adverse events, should be guided by professionals from antimicrobial stewardship teams who are knowledgeable of specifics of CF microbiology [106]. Other approaches are still in early stages of drug development and there will be major challenges in designing clinical trials, especially at the upcoming time when highly effective CFTR modulators reduce both exacerbation rates and sputum expectoration in pwCF. Nonetheless, current CFTR modulators have limited effects on established bacterial infection [107], and developing novel anti-infective strategies for pwCF is of utmost importance.

7. Conclusions

Adaptation of pathogens to the CF lung environment results in the development of persistent infections. One, but not the only, adaptive mechanism is the evolution towards the AMR phenotype,

which is not a simple correlate of mutational changes in their known resistance genes. Bacteria tend to switch to a metabolically less active state with slower growth rate, characteristic of the biofilm associated mode of growth; existing subpopulations of persisters also survive exposure to antibiotics. Standard AST is not designed to consider these bacterial properties, and for these reasons, a broader concept of resistome testing may currently be also of limited clinical value. For more reliable AST, concentration values related to biofilm may be further investigated and clinical breakpoints for antibiotics when administered via inhalation need to be defined. The drug development pipeline for anti-infective therapeutics is rather limited but includes a number of relatively unconventional approaches, such as the use of bacteriophages and antibiotic potentiating drugs.

Declaration of Competing Interest

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