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Antibiotic resistance in chronic respiratory diseases: from susceptibility testing to the resistome

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In chronic respiratory diseases, resistome analysis of respiratory samples provides a more comprehensive understanding of the mechanisms of resistance emergence, constituting a prospective complementary strategy to antibiotic susceptibility testing. <https://bit.ly/3vOSPRL>

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Abstract

The development of resistome analysis, *i.e.* the comprehensive analysis of antibiotic-resistance genes (ARGs), is enabling a better understanding of the mechanisms of antibiotic-resistance emergence. The respiratory microbiome is a dynamic and interactive network of bacteria, with a set of ARGs that could influence the response to antibiotics. Viruses such as bacteriophages, potential carriers of ARGs, may also form part of this respiratory resistome. Chronic respiratory diseases (CRDs) such as cystic fibrosis, severe asthma, chronic obstructive pulmonary disease and bronchiectasis, managed with long-term antibiotic therapies, lead to multidrug resistance. Antibiotic susceptibility testing provides a partial view of the bacterial response to antibiotics in the complex lung environment. Assessing the ARG network would allow personalised, targeted therapeutic strategies and suitable antibiotic stewardship in CRDs, depending on individual resistome and microbiome signatures. This review summarises the influence of pulmonary antibiotic protocols on the respiratory microbiome, detailing the variable consequences according to antibiotic class and duration of treatment. The different resistome-profiling methods are explained to clarify their respective place in antibiotic-resistance analysis in the lungs. Finally, this review details current knowledge on the respiratory resistome related to therapeutic strategies and provides insight into the application of resistome analysis to counter the emergence of multidrug-resistant respiratory pathogens.

Introduction

In the past years, connections have been established between disruption in the composition of the respiratory microbiome, also called dysbiosis, and the evolution of multiple chronic respiratory diseases (CRDs), strengthening the assumption of a role for the pulmonary microbiome in their pathophysiology [1]. Such new insights into the respiratory ecosystem have been revealed by next-generation sequencing (NGS) techniques, which allow an in-depth analysis of respiratory microbial communities. Among NGS approaches, shotgun metagenomics sequencing (mNGS) provides access to broader information than the sole taxonomic characterisation of the microbiome. This strategy includes analysing resistance markers, by identifying plasmid-borne or chromosome-borne antibiotic-resistance genes (ARGs), as well as virulence markers, which provides insight into the specific microenvironment. CRDs such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) and bronchiectasis (BE) are related to abnormal clearance of airway mucus, which can promote chronic infections and recurrent pulmonary exacerbations; these are



deleterious because they contribute to lung inflammation [2–5]. Patients with these CRDs face multiple and prolonged courses of antibiotics, either as prophylaxis, chronic suppressive therapy or with curative intent, in order to reduce inflammation, the frequency of pulmonary exacerbations and disease progression. In parallel, severe asthma is related to chronic inflammation of the lower respiratory tract, with episodes of airflow obstruction [2, 6], and chronic suppressive therapies can be recommended in some severe conditions [7]. However, benefits associated with repeated exposure to antibiotic therapies come at the price of increased selection pressure on the respiratory ecosystem, leading to antibiotic resistance.

Traditional phenotypic methods used in the laboratory, *e.g. in vitro* antibiotic susceptibility testing (AST), appear to have some limits for analysing the phenotypic resistance of pathogens in CRDs. Several studies have pointed out the absence of correlation between *in vitro* AST results and clinical outcomes in CRDs such as CF [8–10]. This seems to be related to the difficulty in considering the complexity of the lung environment. Physiological aspects such as mucus composition or interbacterial cooperative/competitive interactions can also be essential in treatment success and bacterial eradication [9, 11]. The resistome was defined by WRIGHT [12] as the collection of all the ARGs and their precursors in pathogenic and non-pathogenic bacteria. In contrast, AST focuses on susceptibility testing of bacteria considered as pathogens, substantially limiting the analysis. Deciphering the resistome should allow a more comprehensive understanding of the mechanisms underlying the emergence of antibiotic resistance and ARGs. Interbacterial horizontal gene transfer enables ARG dissemination in many interacting microbial communities that comprise the lung microbiome [13]; the challenge is to elucidate the respiratory reservoir of these resistance genes. Assessing the molecular network of ARGs might allow individualised, targeted therapeutic strategies and suitable prophylactic and curative antibiotic stewardship in CRDs, depending on individual resistome and microbiome signatures [14].

This review article describes the influence of antibiotic treatment recommended in CRDs on the respiratory microbiome and aims to decipher the respiratory resistome in these pathologies. First, the consequences of the antibiotic protocols prescribed in CRDs on the whole respiratory microbiome are detailed, with an analysis of the disparities between antibiotic classes and treatment durations. Second, the techniques used for respiratory resistome profiling are described and compared from the perspective of resistance analysis. Finally, this article reviews the current body of knowledge on the respiratory resistome in CRDs (figure 1) to reveal the potential of resistome analysis in future antimicrobial stewardship strategies.

Impact of antibiotic therapy on the respiratory microbiome composition

Patients with CRDs are exposed to multiple courses of antibiotic therapy (table 1). Thus, to understand the development of these diseases, it seems critical that the impact of antibiotic treatments on the respiratory microbiome is explored. Table 2 summarises the reported effect of antibiotics on the respiratory microbiome. It has been reported that, in progressive CRDs such as CF, antibiotic use could decrease the bacterial diversity of the respiratory microbiome over the course of a decade, ahead of the effects of age and lung function [30]. In several observations, antibiotic use had a minor impact on microbial communities during clinical exacerbations, suggesting that microbiome composition shifts may instead be a long-term process [32, 41]. Microbiome modifications could also be due to a joint effect of antibiotic treatment and age in CF patients; the influence of age is complex to determine in this population independently from factors such as disease evolution and longer exposure to antibiotic treatments. Indeed, one study reported that, during the first year of life, the nasal microbiome had an increased Shannon diversity index after antibiotic treatment compared to samples collected before antibiotic administration, suggesting bacterial colonisation following antibiotic administration [39]. This increase in the relative abundance of some bacterial families after antibiotics was maintained after more than one antibiotic treatment, indicating some persistent changes in nasal microbiome under antibiotic treatment [39].

Influence of macrolides on the respiratory microbiome

Long-term macrolide treatment is recommended as chronic suppressive therapy in the CRDs investigated in this review. Azithromycin is associated with an antibiotic action, inhibiting bacterial protein synthesis and reducing bacterial virulence [46], as well as anti-inflammatory and immunomodulatory properties [15, 47]. These properties, associated with a prolonged half-life, make azithromycin the preferred alternative for chronic suppressive treatment in several CRDs such as CF, diminishing the occurrence of respiratory exacerbations in chronically infected patients and thus contributing to the improvement of respiratory function [27]. The consequences of macrolide use on the respiratory microbiome have been intensively studied. A reduction in bacterial diversity has been observed after a 48-week treatment with azithromycin in patients with persistent uncontrolled asthma, with a decrease of Gammaproteobacteria including the pathogenic species *Haemophilus influenzae* [35]. Richness reduction and bacterial alteration of the respiratory microbiome were also confirmed in patients with moderate/severe asthma after a 6-month

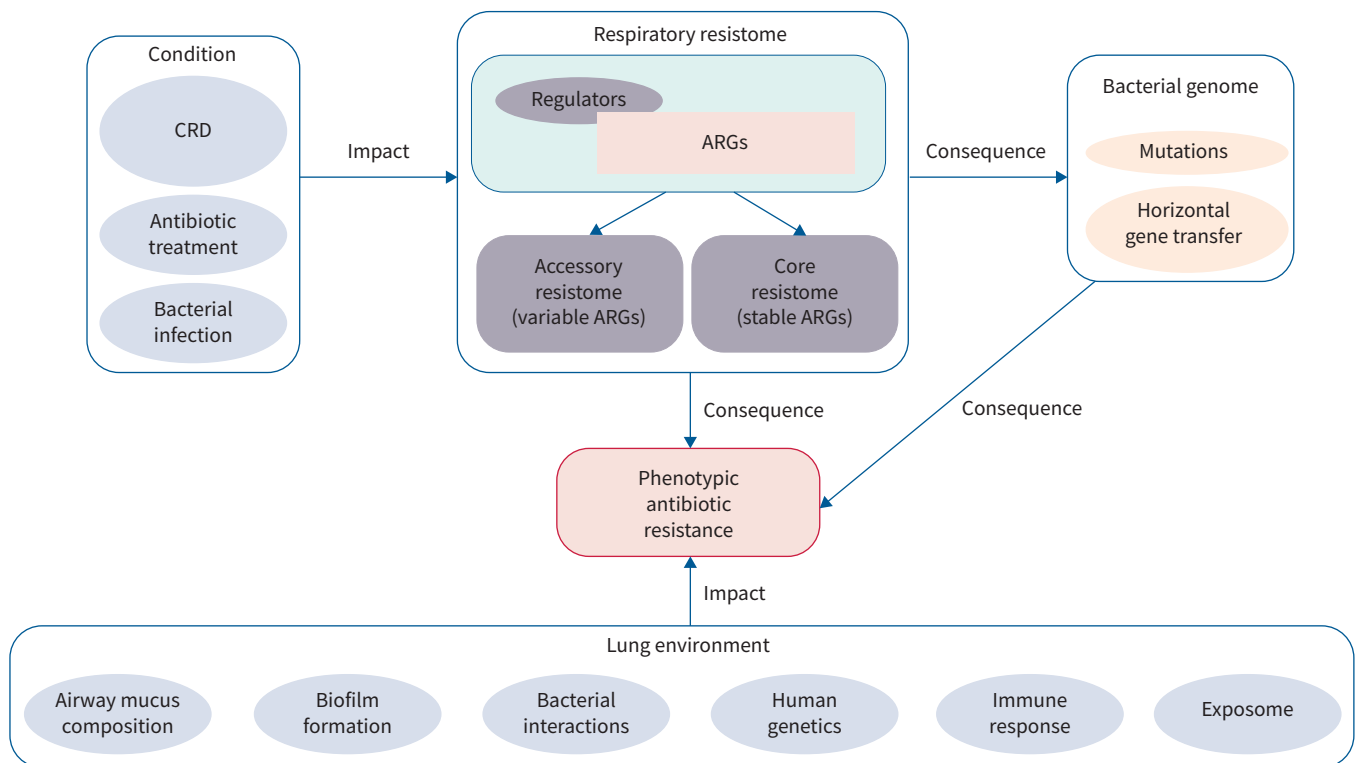


FIGURE 1 The respiratory resistome and its partners. The respiratory resistome is defined as the total of the antibiotic-resistance genes (ARGs) present in pathogenic and non-pathogenic bacteria in the respiratory airways. These ARGs can undergo mutations and spreading by horizontal gene transfer. A “core” resistome has been defined based on the similarity of ARGs found in healthy subjects and people with chronic respiratory disease (CRD). The “accessory” resistome refers to the resistome specific to CRD patients, and is affected by different factors, e.g. CRD pathophysiology, bacterial infection and antibiotic treatment. The resistome has an impact on the phenotypic antibiotic resistance, which is also affected by lung environment factors such as airway mucus composition, biofilm formation, bacterial interactions, human genetics and the immune response.

azithromycin course [36]. In COPD exacerbations, antibiotic treatment has also been associated with reduced abundance of many taxa in the respiratory microbiome (mostly Proteobacteria), with two of the four treatments evaluated being azithromycin [38]. These modifications persisted in samples taken 14–94 days after exacerbation onset [38]. These observations highlight the potential negative impact of such long-term antibiotic courses on the respiratory microbiome. This effect has also been shown in bacterial communities close to the respiratory environment. For example, the oropharyngeal microbiome composition of BE patients differed modestly but significantly after a 48-week erythromycin treatment, with a decrease in the relative abundance of operational taxonomic units (OTUs) assigned to *Actinomyces* or *Streptococcus pseudopneumoniae* and an increase in the OTUs assigned to *Haemophilus* in the group receiving erythromycin [33]. Similarly, oropharyngeal samples from patients with severe asthma showed an increase in the relative abundance of *Streptococcus salivarius* and a decrease in *Leptotrichia*, *Actinomyces* and *Fusobacterium nucleatum* after a 6-month azithromycin treatment versus untreated patients [34]. However, some studies also demonstrated similarity in the microbiome composition between pre- and post-treatment samples [34], suggesting a resilience of the respiratory microbiome even after a long-lasting macrolide treatment. The initial composition of the respiratory microbiome could explain these contradictory observations. The BLESS study, which evaluated sputum microbiome composition in BE patients treated for 48 weeks either by macrolides or placebo, detected a difference in the influence of macrolides according to the pulmotype [37]. No difference in the evolution of microbiome composition was recorded between baseline and 48-week samples in patients with airway microbiome dominated by *Pseudomonas aeruginosa*, despite a significant reduction in the frequency of exacerbations [37]. By contrast, more substantial microbiome changes were demonstrated in patients with microbiomes dominated by species other than *P. aeruginosa*, with a significant decline in the relative abundance of *H. influenzae* and a rise in intrinsically macrolide-tolerant organisms such as *P. aeruginosa* [37]. This study warns of potential deleterious consequences of long-term macrolide treatment and raises the question of its relevance

TABLE 1 Summary of antibiotic treatments recommended in CRDs

Indication	Type of CRD			
	Cystic fibrosis	COPD	Severe asthma	Bronchiectasis
Bacterial prophylaxis	<i>Staphylococcus aureus</i> UK: flucloxacillin the first 3 years of life [15, 16] Other countries: not recommended (uncertainty on clinical consequences and on <i>P. aeruginosa</i> colonisation [17])	NA	NA	NA
Bacterial eradication	<i>Pseudomonas aeruginosa</i> First-line treatment: 28 days of TIS or up to 3 months of a combination of nebulised colistin and oral ciprofloxacin [16] Aggressive therapy: <i>i.v.</i> meropenem or <i>i.v.</i> tobramycin [15] MRSA Combined oral and <i>i.v.</i> antibiotic regimen (fusidic acid, rifampicin, teicoplanin and vancomycin) [15]	NA	NA	<i>P. aeruginosa</i> First-line treatment: 14 days of oral ciprofloxacin If persistence of positive culture of sputum samples: <i>i.v.</i> treatment (e.g. β -lactam+aminoglycoside) or inhaled antibiotics (e.g. colistin, tobramycin or gentamicin) with a total duration of 3 months [18, 19]
Chronic suppressive therapy	<i>P. aeruginosa</i> Intermittent therapy: treatment with TIS for 28 days (300 mg twice daily) on alternate months (28-day on/off) Alternatives: TIP, inhaled aztreonam lysine, colistimethate dry powder or LIS [16, 20–22] Continuous daily administration mode also increasingly recommended [23] Maintenance therapy Proposition of 6 months of oral azithromycin with a 3 times a week dose of 500 mg [27]	Key pathogens Antibiotic courses administered 3 times per week [24] Maintenance therapies Infra-dose of azithromycin [25]	Maintenance therapy Oral infra-dose of azithromycin for 48 weeks Azithromycin 3 times per week orally, in addition to inhaled corticosteroids and long-acting bronchodilators [7]	Recurrent (≥ 3 per year) or severe acute PEx [19, 26] Long-term inhaled antibiotics (nebulised gentamicin, TIS, colistimethate dry powder, dry powder or liposomal ciprofloxacin), usually prescribed on alternate months [18] Long-term oral treatment with macrolide or other molecules [18, 26]
Treatment of PEx	<i>P. aeruginosa</i> -induced PEx 4 days with <i>i.v.</i> administration route [16]; oral or inhaled administration route can also be used [15, 23] Other bacterial species-related PEx ≥ 2 antibiotics with different mechanisms of actions [16]	Antibiotic therapy: not systemically recommended [25, 28] Duration of antibiotic treatment: 5–10 days [28]	Antibiotic therapy: not systemically recommended except in cases of documented pneumopathy [29]	Antibiotic therapy: 14 days of antibiotics are recommended First-line: oral antibiotics In case of severity: <i>i.v.</i> \pm inhaled colistimethate dry powder [18, 19, 26]

CRD: chronic respiratory disease; COPD: chronic obstructive pulmonary disease; PEx: pulmonary exacerbations; NA: not applicable; TIS: tobramycin solution for inhalation; *i.v.*: intravenous; MRSA: methicillin-resistant *Staphylococcus aureus*; TIP: tobramycin inhaled powder; LIS: levofloxacin inhaled solution.

TABLE 2 Impact of antibiotics on the respiratory microbiome in CRDs

Antibiotic	Sample analysed	Type of treatment	Impact of antibiotics on diversity of the microbiome	Impact on relative abundance of bacterial taxa	Disease	Reference
Association of different antibiotic class in treatment of CF exacerbation episodes	Sputum	Diverse antibiotic treatments for a course of 8–9 years	↓ α -diversity (inverse Simpson index)	–	CF	[30]
	Sputum	Diverse antibiotics used for the treatment of acute PEx	↑ Species richness through PEx and return to the baseline state during recovery period	↓ <i>Prevotella melaninogenica</i> and <i>Streptococcus sanguinis</i> ↑ <i>Veillonella parvula</i> during treatment period, but return to baseline state in post-recovery samples	CF	[31]
	Sputum	Diverse antibiotics used for the treatment of acute PEx	Either very little change through exacerbation cycle or return to baseline state on the post-recovery sample	No taxa modification associated with clinical stage, including treatment	CF	[32]
Oral macrolides	Oropharyngeal swab	12 months of twice daily oral doses of 400 mg of erythromycin	No impact on α -diversity measures	Difference between treated and placebo groups: ↓ <i>Actinomyces</i> and <i>Streptococcus</i> ↑ <i>Haemophilus</i> after 48 weeks of treatment	BE	[33]
	Oropharyngeal swab	6 months of 250 mg daily azithromycin for 5 days and then 250 mg 3 times per week	Impact on β -diversity measures	↓ <i>Fusobacteria</i> ↑ <i>Firmicutes</i> during treatment compared with untreated group, but return to pre-treatment state after a 1-month washout period	Severe asthma	[34]
	Sputum	12 months of low-dose azithromycin	↓ Faith's phylogenetic diversity	↓ Gammaproteobacteria (including <i>H. influenzae</i>) after 48 weeks of azithromycin treatment compared with placebo group	Severe asthma	[35]
	Bronchoalveolar lavage of the right upper lung lobe	6 weeks of low-dose azithromycin	↓ Shannon's diversity index	↓ <i>Prevotella</i> , <i>Staphylococcus</i> and <i>Haemophilus</i> ↑ <i>Anaerococcus</i> between pre- and post-treatment state	Moderate and severe asthma	[36]
	Sputum	Low-dose erythromycin	Increase of genus richness between baseline and 48 weeks in treated group but no difference with placebo group	No change in composition of airway microbiome in <i>P. aeruginosa</i> -dominated subgroup ↓ <i>H. influenzae</i> and ↑ <i>P. aeruginosa</i> in non- <i>P. aeruginosa</i> -dominated subgroup	BE	[37]
	Sputum	Treatment of exacerbations exclusively by antibiotics (without addition of corticosteroids) (two treatments azithromycin, one by ofloxacin, one by trimethoprim-sulfamethoxazole)	–	–	↓ of multiple taxa, mainly <i>Proteobacteria</i>	COPD

Continued

TABLE 2 Continued

Antibiotic	Sample analysed	Type of treatment	Impact of antibiotics on diversity of the microbiome	Impact on relative abundance of bacterial taxa	Disease	Reference
β-lactams	Nasal swabs	Various courses of treatments of several weeks, mostly (71%) β-lactams	↑ Shannon's diversity index	↓ <i>Moraxellaceae</i> ↑ other bacterial families (this increase was verified after more than one antibiotic treatment)	CF	[39]
	Bronchoalveolar lavage, sputum or deep throat swabs	Courses of treatment including β-lactams (25 of 31 involving a single β-lactam molecule) for acute PEx	↓ α-diversity between exacerbation and treatment ↓ α-diversity at therapeutic doses between baseline and treatment ↑ α-diversity at sub-therapeutic doses at the same time points	↓ <i>Haemophilus</i> , Clostridiales and <i>Lachnospiraceae</i> ↑ <i>Fusobacterium</i> and <i>Pseudomonas</i> in the group treated at therapeutic doses between baseline and treatment samples No difference was observed in the sub-therapeutic group at the same time points No difference in the bacterial composition was observed in the two groups between post-recovery and baseline samples, or between exacerbation and treatment samples	CF	[40]
	Sputum	Treatment of exacerbation episodes, 19 of 23 treatments including β-lactams	Minimal impact on global community structure	↓ RA of some low abundance taxa with antibiotic treatment: <i>Gemella</i> , two <i>Pasteurella</i> OTUs, two <i>Streptococcus</i> OTUs, <i>Oribacterium</i> and <i>Neisseria</i>	CF	[41]
	Sputum	Treatment of exacerbation episodes by associations including at least one <i>i.v.</i> β-lactam	↑ Shannon's diversity index in the first 72 h of treatment Return to the baseline state after 8–10 days of treatment	↓ <i>P. aeruginosa</i> ↑ anaerobes (<i>Prevotella</i> and <i>Veillonella</i>), in the first 72 h of treatment but return to the baseline state after 8–10 days of treatment	CF	[42]
	Sputum	Cycle of 28 days of AZLI treatment followed by a 28-day period without treatment	No significant change in Shannon's diversity index or Bray-Curtis β-diversity index in one AZLI cycle ↓ Shannon's diversity index ↓ Bray-Curtis β-diversity with ↑ AZLI cycles	–	CF	[43]
Aminoglycosides	Sputum	A 1-month treatment with TIP	↓ average species richness (Shannon and Simpson diversity indices) after 1 week of therapy Return to baseline state after the end of TIP therapy	Most changes noticed between baseline state and first week of treatment occurring among low abundance taxa, mostly facultative and obligate anaerobes (<i>Neisseria</i> , <i>Megasphaera</i> , <i>Granulicatella</i> , <i>Haemophilus</i> , <i>Streptococcus</i> , <i>Gemella</i> , <i>Rothia</i> , <i>Veillonella</i> , <i>Oribacterium</i>)	CF	[44]
	Sputum	TIP or TIS during at least 1 year	No difference in Shannon's diversity index	↓ <i>Parvimonas</i>	CF	[45]

CRD: chronic respiratory disease; CF: cystic fibrosis; PEx: pulmonary exacerbation; BE: bronchiectasis; COPD: chronic obstructive pulmonary disease; RA: relative abundance; OTU: operational taxonomic unit; *i.v.*: intravenous; AZLI: aztreonam lysine for inhalation; TIP: tobramycin inhaled powder; TIS: tobramycin inhaled solution.

in non-*P. aeruginosa*-dominated infections. Furthermore, some prudence should be advised for these long-lasting macrolide treatments, as in the CF population, because there is evidence of an antagonistic action between azithromycin and intravenous tobramycin use for the treatment of pulmonary exacerbations [48].

Influence of tobramycin on the respiratory microbiome

Inhaled tobramycin is used for chronic maintenance therapy for persistent *P. aeruginosa* infections in CF patients. Sputum microbiome composition under a 28-day course of tobramycin inhaled powder (TIP) has been shown to shift between the pre-treatment state and 1 month after the treatment. Surprisingly, this evolution is mainly driven by non-dominant taxa, with a decrease in the abundance of facultative and obligate anaerobes, *i.e.* untargeted bacteria at first sight [44]. The authors suggested that this observation might have been either associated with aminoglycoside activity, although limited, under low-oxygen conditions or *via* an indirect effect through other interacting taxa [44]. However, this conclusion should be balanced with another study, which, in contrast, showed relative stability of the CF sputum microbiome after TIP or tobramycin inhaled solution (TIS) treatment over several months [45]. These disparities could be associated with the differences in tobramycin exposure, with the history of the patients (either naïve or with remote exposures to tobramycin depending on the study) or with the delay between the microbiome analysis and the initiation of the treatment. Further studies are needed to differentiate short- or long-term changes. HEIRALI *et al.* [45] also underlined that, in CF patients, some microbial predictors of the clinical response to TIP and TIS could exist in the respiratory microbiome: people with higher staphylococci relative abundance are better responders than people with higher *P. aeruginosa* relative abundance. This fact highlights the potential importance of respiratory microbiota composition analysis before introducing TIP or TIS chronic maintenance therapy.

Influence of β -lactams on the respiratory microbiome

β -lactams are recommended for the treatment of CF exacerbation episodes. In CF patients aged 1–21 years old, intravenous (*i.v.*) β -lactam therapeutic exposure during exacerbation episodes has been associated with a decrease in the diversity of the respiratory microbiome, and this modification was prolonged for more than 1 month after the end of the antibiotic treatment [40]. There was also a minimal change in microbial diversity in exacerbation episodes under sub-therapeutic antibiotic exposure, highlighting the role of the β -lactam dosage in these modifications [40]. The abundance of bacterial genera also changed during β -lactam treatment at therapeutic doses, with a decrease in *Haemophilus*, Clostridiales and *Lachnospiraceae*, and an increase in *Fusobacterium* and *Pseudomonas* [40]. The time of exposure to β -lactams above the minimum inhibitory concentrations also reportedly has consequences on the recovery of microbial diversity following antibiotic treatment for pulmonary exacerbations in CF [49]. Another study confirmed a decrease in diversity and a different composition of the core and accessory microbiome during treated CF exacerbation periods, but also resilience to the intervention of antibiotics 30 days after the end of treatment, with a return of the respiratory microbiome to the baseline state, in contrast to the previous observation [31]. Several studies have reported global stability of sputum communities throughout exacerbation episodes and antibiotic treatment in CF patients, with minimal or transient changes in microbial population structures and general stability of the dominant taxa between the onset of exacerbation, after antibiotic treatment and during stable intervals [32, 41]. FODOR *et al.* [41], however, did observe a small decrease in the richness of the respiratory microbiome at the end of antibiotic treatment but this was related to a decrease in the abundance of a small number of taxa: *Gemella*, two *Pasteurella* OTUs, two *Streptococcus* OTUs, *Oribacterium* and *Neisseria*. They also did not report any influence of the type of β -lactam on the respiratory microbiome structure [41]. Conversely, in a study on CF exacerbation episodes associated with *P. aeruginosa* and treated with *i.v.* β -lactams, some significant perturbations of the airway microbiome were noted in the first 72 h of antibiotic treatment, with a decrease in the relative abundance of *P. aeruginosa*, an increase in the overall microbiome diversity and an increase in the abundance of anaerobes such as *Prevotella* and *Veillonella* [42]. However, this perturbation was transient, with a bacterial community profile similar to the pre-treatment state after 8–10 days of antibiotic treatment [42]. Thus, the action of β -lactams through CF pulmonary exacerbations is contradictory between studies, with either a significant or minor impact on the whole structure of the respiratory microbiome during the treatment period, probably depending on the dosage of the treatment and the chronology of the samples. Most studies confirmed microbiome resilience of the bacterial communities after the discontinuation of antibiotic treatment [32, 31] or even during this treatment [42]. The number of β -lactam antibiotic courses should also be considered in these studies because repeated antibiotic exposure is a factor in decreasing microbiome diversity [30]. Variation in the definition of a pulmonary exacerbation could also contribute to the differences in the observations. In some studies the definition of pulmonary exacerbation was based on Fuchs' criteria [40, 41], whereas in others only clinical signs were used [32] or the criterion was the physician's decision to use *i.v.* treatment for an increase in respiratory symptoms [42].

β -lactams are also used for CF long-term maintenance therapies, with cycles of inhaled aztreonam lysine. A study by HEIRALI *et al.* [43] revealed that even if the CF airway microbiome seems quite stable through one cycle of inhaled aztreonam lysine, the α -diversity assessed by the Shannon diversity index tends to decline with repeated exposure to this antibiotic, suggesting that increased exposure to β -lactams through repeated cycles is related to a decrease in microbiome diversity. Finally, the age of the patients, especially in CF in which paediatric and adult populations are studied, could also have an influence on the impact of β -lactams on the microbiome and on its resilience, with the hypothesis of persistent changes in the respiratory microbiome following antibiotic treatments in the paediatric CF population [39, 40].

The spread of ARGs through the respiratory microbiome can play a role in its behaviour under antibiotic treatment and its resilience following discontinuation. Therefore, analysis of all ARGs within the respiratory microbiome, *i.e.* analysis of the respiratory resistome, seems crucial for a global understanding of the consequences of antibiotic treatment on the respiratory microbial community.

Deciphering the respiratory resistome

Different approaches have been used to explore the respiratory resistome: targeted methods (using quantitative real-time PCR (qPCR)), sequence-based analysis (using mNGS), functional metagenomics and three-dimensional modelling [50].

Targeted analysis techniques

Targeted approaches such as qPCR have been used to explore respiratory ARGs. These methods track the presence of specific ARGs within the microbiome and determine the relative abundance of these genes using a semi-quantitative technique [50]. The qPCR approach has been shown to be more sensitive than mNGS for detecting and estimating ARG abundance [51]. RAMSHEH *et al.* [52] used a custom set of 296 primer pairs that targeted, among others, 283 ARGs to explore the airway resistome in COPD and healthy subjects. Some strategies have also associated 16S rRNA gene amplicon sequencing, with multiplex PCR targeting ARGs, for a more comprehensive approach [33].

Combining mNGS with validity qPCR analyses provides new perspectives for resistome analysis [53]. The qPCR strategy is limited because it targets identified resistance genes, missing potential unknown or untargeted ARGs, and point mutations involved in the resistance phenotype. Besides, with these techniques it is impossible to identify the host carrying the ARGs, thus providing an incomplete view of microbial resistance. However, qPCR can be helpful as a more precise and efficient tool for specific ARG detection or quantification [53]. Therefore, qPCR should be helpful not as a particular technique given the limitation of this method, but to complement metagenomic analyses.

Shotgun metagenomics (mNGS)

Within genomic techniques, mNGS offers a potential alternative to decipher the lung resistome, with high-resolution profiling of ARGs. This method can generate millions of DNA reads from one sample, without prior amplification of a targeted gene [54]. mNGS should be considered a valuable clinical adjunct in introducing antibiotic therapy in CRDs by helping to predict the evolution of antibiotic resistance. In addition, the presence and abundance of ARGs can be monitored in the long term and analysed based on the clinical evolution. Exploration of the genetic environment of ARGs, achievable with this technique, also allows for investigation of the potential for horizontal gene transfer and spreading of these genes. Metagenomics and exploration of the resistome in different habitats can help to identify highly mobile resistance genes and hotspots for lateral gene transfer [55]. However, if mNGS is associated with the prospection of the lung resistome, some issues still need to be settled. mNGS data analyses rely on databases that do not necessarily reflect the exhaustive panel of ARGs, only the reference genes, limiting the interpretation. Comparisons based on similarities with reference ARGs allow the detection of known ARGs or variants but can rarely predict totally new ARGs. Also, some resistance predictions arise from specific gene mutations, which are more challenging to analyse, even if known chromosomal mutations conferring resistance can be detected [56]. Thus, although the prediction of phenotypic resistance based on mNGS results seems a tempting prospect, some false-negative results may still occur when compared with phenotypic methods [57]. In addition, the interrelationship between ARGs predicted by mNGS and the resistance phenotype may be unclear. It has been shown that some putative ARGs fail to confer resistance when expressed, such as the putative aminoglycoside kinases Rv3817 and Rv325c from *Mycobacterium tuberculosis* when expressed in *Escherichia coli* [12]. Some technical concerns also have to be accounted for when using mNGS, *e.g.* the high proportion of human DNA in the respiratory tract complicating the extraction of microbial sequences, and thus ARGs [1]. The relatively low abundance of ARGs also requires an adequate sequencing depth for an exhaustive and meaningful assessment of the respiratory resistome [58]. Another restriction is the high price of mNGS if applied to large patient cohorts, which may impede

routine application for antibiotic stewardship. To increase the sensitivity of resistome analysis, some studies propose ARG enrichment using a targeted capture method with a set of probes designed from reference sequences of the Comprehensive Antibiotic Resistance Database [59]. This method, applied for the analysis of gut ARGs, might be helpful to analyse the respiratory resistome, resulting in a better performance than mNGS with reduced depth of sequencing.

Functional metagenomic techniques

Some authors suggest applying a combination of metagenomic sequencing and culture methods as a functional analysis to explore the lung resistome. Functional metagenomics is an effective technique for analysing gene content and gene function in a polymicrobial sample. It allows a more complete analysis of the resistome, identifying the sequence of genes responsible for antibiotic resistance [56]. ALLEMANN *et al.* [60] used this technique based on amplification and fragmentation of whole genomic DNA, with ligation of DNA fragments from metagenomic libraries into a cloning vector, to explore the respiratory resistome. This method was based on previous studies that developed functional metagenomics to characterise antibiotic resistance [13, 50, 61]. The cloning step was then performed in an *E. coli* cloning host. After clone amplification by culture, the ones corresponding to ARGs were selected on agar plates containing antibiotics, using the same antibiotics as for the treatment of infants with CF. Up to 50 clones per sample were then set, for which plasmids were extracted and the corresponding DNA sequences amplified and analysed using nanopore MinION sequencing (Oxford Nanopore Technologies) [60]. “Classic” mNGS recovered some resistance genes linked to antibiotics that were not tested with the functional metagenomic approach (lincosamide, fosfomycin, macrolides and aminoglycosides). However, the read coverage obtained with mNGS was relatively low compared with the alternative [60]. Thus, this functional metagenomic approach seems quite powerful and allows phenotypic features to be correlated with the identification of ARGs, with the capacity to discover new resistance genes. This method is, however, restricted to the analysis of resistance genes associated with the antibiotics used to select the clones, limiting the exploration of the resistome. The *E. coli* cloning system used also presents some limitations for exploring gram-positive ARGs [62]. Besides, the possible presence of regulators of ARGs that are not recognised by the cloning host’s gene machinery can introduce bias in this functional analysis, by altering the expression of genes and generating either false-positive or false-negative results [63]. The choice of an appropriate vector seems therefore crucial in the design of the study. Additionally, culture conditions such as the medium, the antibiotic concentration and the conditions of incubation can affect the selection of resistant clones [63]. Overall, despite some constraints, this seems a novel complementary approach that allows a functional perspective attractive for respiratory resistome analysis.

Three-dimensional modelling

Other functional predictive approaches could be adapted from the exploration of the intestinal resistome to predict ARGs from metagenomic datasets. RUPPE *et al.* [64] developed a method based on protein homology modelling named “pairwise comparative modelling”. This method increases the functional prediction of proteins by building structural models and enables the classification of predicted antibiotic-resistance determinants. These can then be assigned to bacterial phyla and located and investigated for their mobility potential, distribution in the human microbiome, and dynamics within the antibiotic exposure being investigated [64]. This approach complements metagenomic analysis with the functional prediction of ARGs. Furthermore, three-dimensional modelling helps predict the harmful consequences of specific mutations in the reference protein [56]. However, while this method may be useful for predicting genes homologous to known antibiotic-resistance determinants, it could also exclude ARGs without homology to reference genes.

Currently, there is no gold-standard method for the exploration and prediction of the resistome. However, using several complementary approaches might allow a deeper insight into the respiratory resistome.

Respiratory resistome

Bacteria constituting the lung microbiome of patients with CRDs are associated with a diverse set of resistance mechanisms. Significant promotion of antibiotic resistance is observed in patients with CF and advanced pulmonary disease, which is related to long-term antibiotic treatments [65]. Lists of ARGs documented in respiratory samples in healthy and CRD populations are presented in the supplementary material. Some studies have also reported the presence of a complex nasal resistome in the first year of life in CF patients not yet exposed to antibiotics, suggesting that exposure to antibiotic treatments might not be the only factor in developing the bacterial resistome [60]. A wide respiratory resistome has also been identified in patients with COPD, BE or severe asthma, with a higher repertoire of resistance determined in patients with COPD and BE [66]. Another study identified that ARGs were 37% more prevalent in the sputum from COPD patients than in expectorations from healthy subjects [52], and this was mostly

attributable to the increase in the bacterial burden in COPD patients compared with healthy individuals [52]. This attests to the issue of assessing the ARG reservoir in these CRDs. Furthermore, the instability of the respiratory resistome has been highlighted, with “losses” of resistance to antibiotic classes reported simultaneously by metagenomic and in-culture analyses [67]. Therefore, mechanisms underlying the evolution of the respiratory resistome would be interesting to dissect. This review focused on whole respiratory resistome analyses, excluding specific PCR on bacterial isolates obtained by standard culture.

The “core” respiratory resistome

The concept of a core respiratory resistome has emerged in the last few years. Indeed, a core respiratory resistome common to healthy individuals and patients with COPD, BE or severe asthma has been identified [66]. It is composed of ARGs from β -lactams (*oxa-255*, *cfxA2*), diaminopyrimidine (*dfrA1*), fluoroquinolones (*pmrA*), macrolides (*msrD*, *ermB*, *ermX* and *ermF*), lincosamides (*lnuC*), chloramphenicol (*catS*), aminoglycosides (*aac(3)-VIIa* and *aph(3)-IIIa*) and tetracycline classes (*tetW*, *tetA*, *tetB*, *tetD* and *tetO*) [66]. In another study comparing the resistome of COPD patients and healthy subjects, carbapenemases, extended-spectrum β -lactamases (ESBLs) and *ampC* genes were identified in the respiratory resistome of the healthy control group [52]. The presence of a core resistome with abundant macrolide-resistance genes is a recent and crucial finding for the future management of CRD. The notion of a core resistome questions the diagnostic implications and the potential benefit of screening ARGs in the respiratory microbiota of CRD patients prior to introducing long-term antibiotic therapies such as macrolides.

The “accessory” resistome in CRDs

There are distinctive features in the composition of the respiratory resistome in the context of CRD. It has been shown that patients with COPD and BE have a higher load of ARGs than patients with severe asthma and healthy individuals [66]. One explanation could be the different physiopathology of these diseases, and the fact that patients with severe asthma are often less subjected to multiple courses of antibiotic treatment [68]. However, surprisingly, recent antibiotic exposure does not seem to result in a higher abundance of ARGs in COPD or BE [52, 66]. In these previous studies, antibiotic treatment was either stopped 6 weeks before resistome analysis [52] or used 6 months before recruitment [66]. It is possible that the impact of antibiotic therapy on the resistome is a short-term effect but longitudinal analyses of the resistome are needed to corroborate this hypothesis. However, these metagenomic data concerning the impact of antibiotics on the ARG reservoir must be balanced with phenotypic analyses. Long-term antibiotic therapy for the prevention of COPD exacerbation episodes is associated with a rise in antibiotic resistance, with a significant increase of moxifloxacin, doxycycline and azithromycin minimal inhibitory concentrations (with a three to six times increase) compared to pre-treatment values after a 13-week treatment [69]. In COPD records, no correlation was established between exacerbation episodes (based on forced expiratory volume in 1 s and the modified Chronic Respiratory Disease Questionnaire (mCRQ) score) and ARG prevalence [52]. These contradictory data identify an area for further study that might help elucidate the chronology and evolution of the respiratory resistome with antibiotic use and development of the pathology.

Multidrug efflux-mediated systems

Multidrug efflux-mediated systems are the most represented gene function within the resistance mechanisms in the CF lung resistome [60, 65], representing >50% of identified respiratory ARGs in this population [67], and thus seem to play an essential role in bacterial resistance (supplementary material). Some are associated with bacteriophage sequences in the CF population [70], leading to speculation of horizontal transmission of these genes in the bacterial population. In functional metagenomic analyses performed on the nasal resistome of CF patients in the first year of life, multidrug-resistance efflux pumps of the ATP-binding cassette (ABC) and major facilitator superfamily transporters were the most common resistance mechanisms identified (in 4.6% and 7.7% of the nasal swabs, respectively) [60]. But while these genes seem to be present in the early life of CF patients, the abundance of these resistance genes can vary through disease progression. A metagenomic functional analysis performed on the sputum samples of 12 CF patients with either mild or severe lung disease indicated enrichment of multidrug-resistance efflux pumps of the ABC and resistance-nodulation-cell division superfamilies in the group with severe disease [65]. In this same study, resistance genes were associated with only three bacterial species: *Achromobacter xylosoxidans*, *F. periodonticum* and an unclassified member of the *Bordetella* genus [65]. In BE patients, some multidrug efflux pump genes, such as *hmrM*, appear to increase significantly after treatment with erythromycin [53]. However, qPCR analyses revealed that this gene was significantly correlated with *H. influenzae* because it is chromosomally encoded on this species. Thus, as in this example, the influence of antibiotic treatment on the abundance of resistance genes can instead be associated with variation in the abundance of the bacterial load following antibiotic treatment [53].

The resistome and β -lactam-resistance genes

β -lactam resistance is frequently identified within the CF respiratory resistome. In their study of the nasal resistome in the first year of life in CF patients, ALLEMANN *et al.* [60] found that 64 predicted sequences were associated with β -lactamases among the 171 resistance genes identified. Within these sequences, eight had <85% identity over 90% of sequences to any known β -lactamase, bringing to light the existence of an undiscovered resistome. Surprisingly, eight proteins with a β -lactamase profile were associated with the ESBL phenotype [60]. The β -lactamases, highly abundant in the CF respiratory resistome, have also been identified in the respiratory virome of CF patients, suggesting a potential phage-mediated spread in the bacterial community [67, 70]. This interesting result underlines the interest in resistome analysis for a greater understanding of ARG dissemination mechanisms. ALLEMANN *et al.* [60] also reported that the β -lactam-resistant phenotypes were more frequent in infants who received one or more antibiotic therapies, although with a nonsignificant trend. The presence of β -lactamases, including ESBLs, at an early stage in the life course of CF patients might impact further antibiotic treatment outcomes. The β -lactamases from classes A and B were mainly associated with the phyla Proteobacteria, Firmicutes and Bacteroidetes. β -lactamases from classes C and D were exclusively associated with Proteobacteria [60]. With regard to the COPD resistome, the *pbp2x* gene related to the *Streptococcus* genus has been detected at least 20% more frequently in COPD patients than in healthy subjects [52]. In this population, ESBLs and AmpC β -lactamase genes showed a modest excess compared with healthy subjects [52]. A high copy number of *bla_{VIM}* genes has been associated with bacteriophages in sputum samples of CF patients [71]. Carbapenemases have also been detected in the COPD respiratory resistome, including the New Delhi metallo- β -lactamase-1 (NDM-1) gene [52].

The resistome and macrolide-resistance genes

The influence of macrolide chronic maintenance therapy on antibiotic resistance has been discussed earlier; bacterial isolates from the respiratory microbiome are significantly more resistant to macrolides in CRDs treated with this therapy [72, 73]. However, macrolide resistance cannot solely be associated with macrolide chronic maintenance therapy because macrolide-resistance markers have been described not only in the core respiratory resistome of patients with COPD, bronchiectasis or severe asthma, but also in that of healthy individuals [66]. In the same study, the rRNA methylase gene *ermX* was associated with several taxa, mainly consisting of upper airway commensals. Some taxa, such as *Actinomyces* and streptococci, were directly associated with *ermX* and *ermB*, and a strong association was also found between *ermF* and *Bacteroides thetaiotaomicron* [66]. Some associations between *msrD* and *Clostridioides difficile* or *Morococcus cerebrosus* were also found in respiratory samples of patients with respiratory diseases [66]. The association between macrolide-resistance genes and some gut species whose presence is unusual in the respiratory microbiome, such as *B. thetaiotaomicron* and *C. difficile*, suggests seeding of the airway resistome. Silent aspiration or gastro-oesophageal reflux has indeed been proposed to occur in CRDs [74–77]. These mechanisms could be an interesting explanation for the exchange of species and resistance genes between the gut and the respiratory microbiome. The *mefA* efflux pump system gene has also frequently been identified in respiratory samples of patients with COPD and is one of the most commonly detected ARGs [77, 78]. Thus, resistance genes from the macrolide–lincosamide–streptogramin axis seem highly abundant, dominating the core airway resistome [66]. This result is important, given the high use of macrolides in the context of CRDs: long-term macrolide therapy is recommended to reduce rates of pulmonary exacerbations. The selective pressure exerted on the respiratory microbiome by this class of antibiotics seems critical. Exploring the core macrolide resistome with mNGS before introducing long-term macrolide therapy might help monitor the treatment. Long-term macrolide therapy also has an impact on the respiratory resistome, resulting in a significant increase in macrolide resistance, especially in viridans streptococci, with an increase in the relative abundance of *ermB* or *ermF* methylase genes as well as *mel*, *msrE* and *mefA* efflux pump system genes [35, 69, 77, 79]. The increase in abundance of transmissible plasmid-encoded genes following macrolide therapy, such as *ermB*, is worrying and requires further study of the macrolide resistome in long-term therapy [53].

The resistome and fluoroquinolone-resistance genes

Fluoroquinolone-resistance genes have been identified as being associated with bacteriophage sequences in the CF virome [70], underlining a role for prophages in disseminating resistance genes in the respiratory resistome. In BE patients, a decrease in the relative abundance of *pataA*, a chromosomally encoded fluoroquinolone-resistance gene, was observed following macrolide treatment, but this decrease correlated with a reduction in the abundance of *S. pneumoniae* [53].

The resistome and tetracycline-resistance genes

Tetracycline non-susceptibilities seem rare in the nasal respiratory microbiome of CF infants; the ARGs most frequently identified are *tetK* and *tetM* within Firmicutes [60]. *tetM* has also been commonly detected in the respiratory samples of patients with COPD diseases [78]. In severe asthma, long-term azithromycin treatment (500 mg three times a week for 48 weeks) was related to an increase in *tetW* and

tetM resistance genes, significantly associated with resistant viridans streptococci [35]. In this previous study, *tetM* was found predominantly combined with *ermB* on a Tn916 mobile genetic element in streptococci, clarifying the increase of this gene after macrolide therapy [35]. Similarly, *tetW* is also reportedly associated with *ermB* in transferable genetic elements in streptococci [80]. These observations underline the vast potential consequences of macrolide usage in CRDs on antibiotic resistance.

The airborne-associated respiratory resistome

Some studies have reported the co-occurrence of ARGs in the context of CRDs in the environment, *e.g.* in inhalers [66], illustrating the potential of such therapeutic devices to act both as reservoirs for antibiotic-resistance determinants and a place for the exchange of these determinants between the environment and the airway. Another study using face-mask sampling highlighted the presence of ARGs in the aerosols of 69% of COPD patients, and these were also present in sputum [78]. These studies attest to the airborne dissemination of ARGs from the respiratory resistome in COPD patients and the complex interactions between the respiratory tract of patients with CRDs and their environment, which require further study.

Advantages, challenges and limits of resistome analysis

The genotypic prediction of phenotypic bacterial resistance is an expected benefit from resistome analysis (figure 2). Recently, a scoring system based on mutation-driven and horizontally acquired resistance analysis by whole-genome sequencing has been established for several antibiotics in *P. aeruginosa* [81]. Other profiling strategies have used machine learning to generate a predictive model of antibiotic resistance in this same species, obtaining high sensitivity and high predictive values by combining whole-genome sequencing and transcriptome analyses [82]. These methods would consolidate resistome analyses by developing tools for predicting antibiotic resistance from metagenomic data, thus laying the foundations for targeted antibiotic therapy based on NGS. The long-term monitoring of resistome data of patients with CRDs would also help to rapidly adapt antibiotic therapy to the evolution of ARG composition.

Therefore, respiratory resistome analysis provides access to a broad range of information regarding bacterial antibiotic resistance. However, it is a complex process to compare the results of respiratory resistome analysis between different studies. Heterogeneous results are a result of the different clinical features of the patients, the types of sample collected, the processing of the samples and the kind of techniques used for the analysis [83]. The control of oral contamination is a key element in interpreting respiratory microbiome analyses, with a variable extent of oral contamination depending on the sampling method [84]. No gold-standard sample has been defined for lung microbiome and resistome studies [83]. Differences in bacterial communities have been demonstrated between the types of respiratory specimens in CF patients, with distinct communities in the upper and lower airways [81]. Oral contamination of respiratory samples could, therefore, also have an impact on lung resistome results. Similar to studies of pulmonary microbiota, it is therefore difficult to envisage a single sample that could reflect the entire pulmonary resistome, particularly given the compartmentalisation of certain chronic pulmonary infections and pulmonary biogeography.

Lastly, particular attention should be given to the microbiome analysis. In several studies, the variation in the composition of the respiratory resistome following antibiotic treatment was associated with the influence of these treatments on single bacterial taxa. Variation in ARG abundance was thus related to the intrinsic resistance of specific bacterial species that emerged after antibiotic treatment [53]. This highlights the corresponding requirement of combining microbiome and resistome analyses for a comprehensive understanding of the mechanisms underlying ARG evolution.

Points for future research and clinical practice

Question for future research

- How to correlate resistome data with phenotypic antimicrobial resistance?

Points for clinical practice

- In the future, resistome analysis will be key for the introduction of a personalised antibiotic strategy in CRDs and support for antimicrobial stewardship.

Conclusion

CRDs are associated with antibiotic regimens administered for the prophylaxis and treatment of exacerbation episodes, which might shed light on the emergence of antibiotic-resistance mechanisms spreading throughout the lung bacterial community. This lung bacterial community is now considered to be a complex interactive network of bacteria. In terms of resistance, these microbial community interactions seem decisive with the horizontal transfer of genes or delivery of resistance enzymes that can

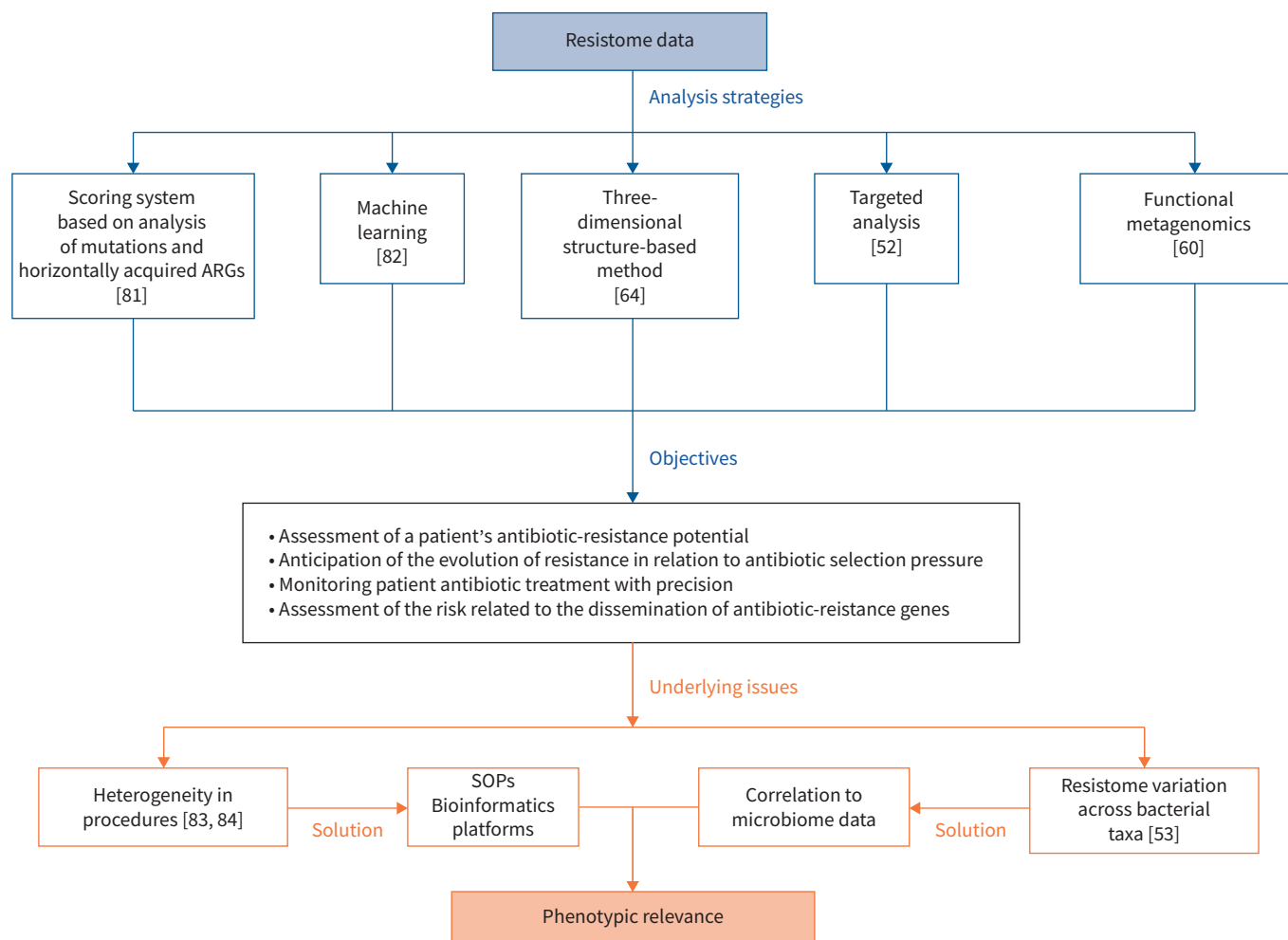


FIGURE 2 Advantages, challenges and issues of resistome analysis in chronic respiratory diseases. Resistome data interpretation for the implementation of antibiotic resistance prediction relies on different strategies. A scoring system, based on analysis of mutations and horizontally acquired antibiotic-resistance genes (ARGs), has been developed [81]. Another method uses machine-learning strategies to predict phenotypic resistance by combining sequencing and transcriptome analysis [82]. Three-dimensional modelling, by building structural models, can also help in protein function prediction [64]. Functional metagenomics, which correlates phenotypic resistance and metagenomics data, can also provide insight into the interpretation of bacterial resistance [60]. However, some issues need to be settled. The variation in respiratory resistome data depending on the methodology used complicates inter-study comparison [83, 84]. Also, some variation in ARG abundance and resistome data can be associated with the intrinsic resistance of a single bacterial taxa, and needs to be correlated with microbiome analysis [53]. Finally, some difficulties can emerge in the phenotypic prediction of resistance to antibiotic molecules. SOP: standard operating procedure.

shield other species from the action of antibiotic molecules [85, 86]. Thus, classical techniques for determining bacterial susceptibility to antibiotic agents in the context of infections in CRD appear to have some limits. In the last few years, resistome analysis has made it possible to decipher the genes involved in resistance mechanisms within the complex respiratory microbiome of patients with CRD. This provides interesting perspectives for the future and the possibility of implementing new strategies for predicting and managing bacterial resistance in these diseases, potentially allowing the implementation of personalised antibiotic therapy depending on the composition of the respiratory resistome.

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