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The (re)emergence of aerosol delivery: Treatment of pulmonary diseases and its clinical challenges

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ABSTRACT

Aerosol delivery represents a rapid and non-invasive way to directly reach the lungs while escaping the hepatic first-pass effect. The development of pulmonary drugs for respiratory diseases such as cystic fibrosis, lung infections, pulmonary fibrosis or lung cancer requires an enhanced understanding of the relationships between the natural physiology of the respiratory system and the pathophysiology of these conditions. This knowledge is crucial to better predict and thereby control drug deposition. Moreover, aerosol administration faces several challenges, including the pulmonary tract, immune system, mucociliary clearance, the presence of fluid on the airway surfaces, and, in some cases, bacterial colonisation. Each of them directly influences on the bioavailability of the active molecule. In addition to these challenges, particle size and the device used to administer the treatment are critical factors that can significantly impact the biodistribution of the drugs. Nanoparticles are very promising in the development of new formulations for aerosol drug delivery, as they can be fine-tuned to reach the entire pulmonary tract and overcome the difficulties encountered along the way. However, to properly assess drug delivery, preclinical studies need to be more thorough to efficiently enhance drug delivery.

1. Introduction

Inhalation of medicinal substances is an ancient practice, used since 2000 BCE by various cultures, notably in India [1]. In addition, the Ebers Papyrus, dating around 1554 BCE (ancient Egypt), also mentions the use of black henbane (*Hyoscyamus niger*) by aerosol for its anticholinergic properties. In China, smoking opium for its analgesia, antidiarrhea and antitussive properties, has been documented back to 1100 BCE. In ancient Greece, Hippocrate (460–377 BCE), described a device that enabled the inhalation of vapours of herbs and resins. However, it was only in 1778 that John Mudge coined the term "inhaler" to describe this kind of devices. Before the industrial revolution in 1760, therapeutic aerosols were directly prepared by physicians. With the arrival of mass production and new therapeutic entities, inhalation therapies began to be used for lung diseases, particularly asthma, and for anaesthesia purposes. The advancement of aerosol delivery was marked by the invention of nebulisers and atomizers, with the first atomizer developed in 1849 in France by Dr. Auphon [2]. Around the same time, Ira Warren invented the first known dry powder inhaler in 1852 [3]. Significant new drugs for aerosol applications, including budesonide, salmeterol, and ipratropium, have since been developed for asthma and chronic obstructive pulmonary disease (COPD). The development of Pulmozyme® (Dornase α) by Genentech in the 1990s marked the beginning of the modern era of aerosol therapy, and the use of aerosols in clinical applications has become increasingly widespread [4].

There are several reasons to reconsider aerosol therapy over systemic administration. For instance, aerosol therapy can reduce the degree of invasiveness and facilitate patient compliance, especially in young children [5]. Additionally, some therapeutic agents may not reach the lungs in sufficient quantities to enhance pharmacological, immunological, or metabolic effects when administered in a non-pulmonary way. For example, recombinant human DNAse (Dornase α) is one of these incompatible agents which cannot be administered via the systemic route [4]. Furthermore, aerosol administration of some therapeutic agents, such as aminoglycosides, is more suitable to minimize the toxicity while enhancing the local bioavailability in the airways [6,7].

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Inhalation therapy avoids the hepatic first-pass effect and mitigates possible side effects thanks to a localized effect. Despite local delivery, aerosolization may also be suitable when a rapid effect is required, especially for bronchodilator treatment [8]. Depending on the capability of a drug to cross the air-blood barrier in case a rapid onset of pharmacological action of a drug is desired, pulmonary administration could lead to high plasma concentrations thanks to a rapid absorption [9,10]. In contrast, many hydrophobic drugs are not able to cross the airway-blood barrier, such as the fluticasone, which does not exhibit rapid pulmonary absorption and thereby prevents its circulation in the blood system [11]. This review aims to provide an overview of the current and future use of aerosol therapy, especially in the context of cystic fibrosis (CF), infectious diseases, and lung cancer.

2. Aerosol delivery

2.1. Devices used for aerosol delivery

Aerosol therapy may briefly be defined as a medical treatment delivered directly into the lungs via inhaled aerosolized particles. These particles are administered using devices that can be classified into two main categories: nebulisers and inhalers (Table 1).

2.1.1. Nebulisers

2.1.1.1. Jet nebulisers. The jet nebuliser was the first nebulisation technology developed on the basis of air jet atomisation (Fig. 1a) [12]. Atomisation is the process of converting a volume of liquid into several microdroplets [13]. This physical process is relatively easy to realise for most liquids by passing a gas at high velocity under pressure or a stream of air through a small orifice [14]. A subtype of jet nebuliser is the double Venturi nebuliser, which has saver valves that contribute to reduce the waste of medication by increasing the flow of aerosol during inspiration. This allows a five times higher inhaled dose compared to a constant flow nebuliser [15]. However, these single-use devices are not portable, require a large volume of medication and show a high inter-patient variability [16]. In fact, the amount of aerosolised drug deposited in the lungs is highly variable, having 2 % to 30 % of the original dose finally deposited in the airway epithelium [17]. In general, this type of nebulisers generate microdroplets sized from 4 to 6 μm [16].

2.1.1.2. Ultrasonic nebulisers. The ultrasonic nebuliser transforms the drug preparation into aerosols thanks to acoustic waves (Fig. 1b). These are generated using a piezoelectric crystal that can be electrically polarised when subjected to a mechanical stress or deformed when an electric field is applied [18]. The crystal vibrates at a frequency up to 20 kHz and makes the liquid contained in the nebuliser tank vibrate either directly or via another liquid that conducts the vibrations (i.e., water) [19]. The resulting aerosol is then formed thanks to the phenomenon of cavitation. However, the vibration waves can denature and heat the molecules [20]. This type of aerosol is therefore not suitable for all types of drug solutions and is, in that case, explicitly mentioned in the marketing authorisation of the medicinal product. For example, Pulmicort® (budesonide, an anti-inflammatory corticosteroid) and Pulmozyme® cannot be administered with this type of nebuliser.

2.1.1.3. Mesh nebulisers. The mesh nebuliser uses the high-frequency vibrations of a micro-aperture mesh to transform a liquid solution into an aerosol (Fig. 1c) [21,22]. The size and density of the micro-openings are key parameters that influence the size of the microdroplets formed and the inhaled dose of the drug. With this type of device, particle sizes range from 3 to 5 μm [23]. This system has several advantages over the jet nebuliser, it is portable, silent and requires a lower volume [24]. In addition, this technique can be used for all types of molecules and allows a reduction in time delivery by applying a flow rate two to three times higher to those of conventional nebulisers. As an example, a study reported that in CF patients, the use of eFlow rapid (PARI®), a mesh nebuliser, instead of the LC Plus jet nebuliser (PARI®) drastically reduced the nebulisation time of the tobramycin solution and thus improved patient compliance [25].

Table 1
Examples and comparison of inhalers and nebuliser devices used to treat airway diseases [38–40].

	Inhalation system	Advantages	Limitations	Device example
Inhaler	Soft mist inhaler	Portable Multi-dose device Can be used for children	Not breath-actuated Requires coordination of inhaler activation and inspiration	Respimat®
	Dry powder inhaler	Portable Compact Short treatment time Efficient for various formulations Breath-actuated	Moderate to high inspiratory flow required Can be a single dose	Breezhaler® Ellipta® Aerolizer® Nexthaler® Turbuhaler® Easyhaler® Flexhaler® Jethaler® Genuair® Diskus®
	Metered dose inhaler	Portable Compact Multi-dose device Efficient for various formulations	Not breath-actuated High oropharyngeal deposition Requires coordination of inhaler activation and inspiration	Evohaler®
Nebuliser	Jet nebuliser	High doses can be administrated Dose modification possible	Costs Less portable Pressurized gas source required Long treatment period	Pari LC® Pari LC plus® Pari baby® Marquest Acorn II® Hudson T Up-draft II®
	Ultrasonic nebuliser	High doses can be administrated Small dead volume Faster delivery than jet nebuliser Less drug loss	Costs Requires electrical power Suspensions not well nebulised	OMRON NE-U780®
	Mesh nebuliser	Portable Compact High doses can be administrated Small dead volume Faster delivery than jet nebuliser Less drug loss Higher lung deposition compared to jet nebuliser	Costs Some models may be drug-specific	Durable Sidestream® Pari eFlow®

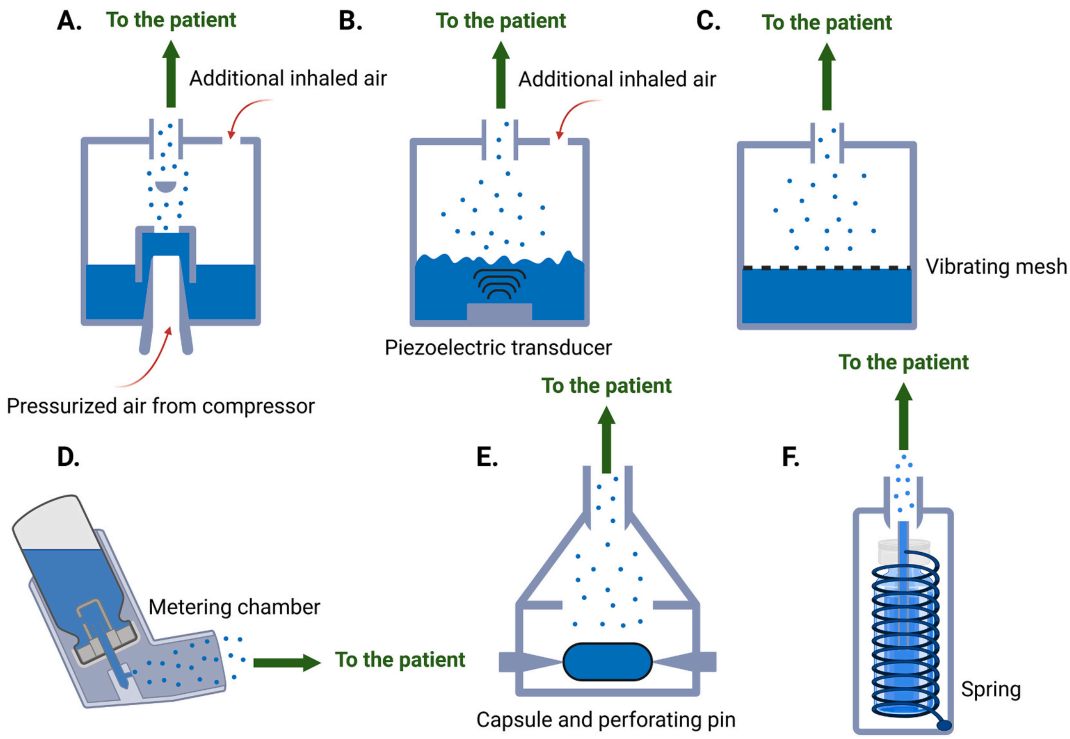

Fig. 1. Schematic representation of nebulisers and inhalers: A) Jet nebuliser: Based on the Venturin effect and Bernoulli's principle, pressurized air from compressor transforms the liquid solution into droplets. B) Ultrasonic nebuliser: Uses a piezoelectric transducer to generate an electric field and acoustic waves, producing droplets. C) Mesh nebuliser: Employs a vibrating mesh with micropores to extrude the liquid solution. D) Metered dose inhaler (MDI): Encapsulates solutions, emulsions, or suspensions in pressure-resistant containers. A propellant is used to form and release the aerosol. E) Dry powder inhaler (DPI): Model designed for single-unit doses containing a capsule with solid micronized or dry powder drug, inhaled using the patient's airflow. F) Soft mist inhaler (SMI): Uses a spring to force the liquid solution through a system of nozzles, producing a fine aerosol. (Figure created with BioRender.com)

2.1.2. Inhalers

2.1.2.1. Metered dose inhalers. Metered dose inhalers (MDIs) or pressurized metered dose inhalers (pMDI) were first used in 1956 after the development of nebulisation (Fig. 1d) [26]. At that time, the MDI gained popularity over the jet nebuliser because it is a small, portable, inexpensive and silent device [27]. MDI requires propellants (e.g., hydrofluoroalkanes) to nebulise the aerosol at a high velocity (> 30 m/s) [28,29]. This drug delivery device is mainly used for COPD and asthma patients, but it is difficult to use properly [30]. Indeed, this inhaler requires good coordination and its incorrect use can be responsible for poor drug delivery efficacy [31]. Only 10 to 20 % of the aerosol produced by the metered dose inhaler is deposited in the lungs [32].

2.1.2.2. Dry powder inhalers. In contrast to MDI, dry powder inhalers (DPIs) do not require propellant gases (Fig. 1e) [28]. Like the other inhalers, they are portable devices and are designed to be easily handled by the patient and therefore cannot be mishandled. However, 20 to 25 % of the medication is retained by the device and only about 12 to 40 % reaches the lungs [33,34].

2.1.2.3. Soft mist inhalers. Soft mist inhalers (SMIs) produce an aqueous aerosol mist without the use of propellant gases (Fig. 1f). Using a spring, the solution is forced through a system of nozzles to produce a fine aerosol demonstrating a good lung deposition [35,36]. For instance, Respimat® (Boehringer Ingelheim) generates 60 % of fine particles equal to or smaller than 5 μm, enhancing drug delivery to the smaller bronchi and bronchioles [37]. In addition, SMIs are independent of the patient's inspiratory effort and therefore reduce errors and misapplications.

2.2. Micro- and nanoparticles for drug delivery

In order to develop new aerosol formulations, inhalable micro- and nanoparticles (NPs) appear to be promising carriers for drug delivery as they improve drug distribution on the lung epithelium [41]. They are able to efficiently protect various hydrophobic and hydrophilic compounds, including small molecules, biological macromolecules (such as nucleic acids and proteins), and vaccine components [42]. Various carriers have already been approved by the United States Food and Drug Administration (FDA), such as lipid-based, polymeric or inorganic NPs [43]. For example, liposomal doxorubicin (Doxil®), initially approved for solid tumours such as Kaposi's sarcoma and breast cancer, was the first liposomal drug used in the treatment of lung cancer [44].

2.2.1. Lipid-based nanocarriers

Among lipid-based nanocarriers, liposomes are small biocompatible vesicles that consist of amphiphilic lipids, such as phospholipids, and can encapsulate hydrophilic drugs in their inner core or hydrophobic drugs in their lipid bilayer [45,46]. Liposomes can be unilamellar or multilamellar and are very similar to natural cell membranes in their physicochemical properties [47]. Other types of lipid-based nanocarriers can also be used for the transport of NAs. Lipid nanoparticles (LNPs) are mainly composed of four lipid components such as phospholipids (DSPC or DOPE), ionisable amine-containing lipidoid, cholesterol and polyethylene glycol (PEG) [48]. LNPs can be used for the transport of mRNA or siRNA. For example, LNP-based mRNA vaccines from Pfizer/BioNTech and Moderna are already used worldwide for the COVID-19 vaccine [49].

2.2.2. Polymeric nanocarriers

Polymeric nanocarriers are widely used for drug delivery purposes. Their charge can be cationic, anionic or neutral. For instance, one of the most frequently investigated cationic polymers nanocarriers for drug delivery is chitosan. It is a polysaccharide derived from the deacetylation of chitin, which forms the exoskeleton of arthropods and cephalopods. Chitosan is a natural biodegradable polymer that can be used for the administration of drugs with low cytotoxicity [50–54]. Poly-L-lysine (PLL) was the first polymer used for gene transfer. It consists of the amino acid lysine, positively charged at physiological pH. Depending on the molecular weight, the size of the polyplexes can change. For example, a 25 kDa PLL forms polyplexes of 50 to 500 nm [55]. However, PLL can induce cytotoxicity [56].

Polyethylenimine (PEI), another cationic polymer, was used for gene therapy for the first time in 1995 by J.-P. Behr and his team [57]. PEI is a polycarbon chain that is interspersed with an amine carrying positive charges every three atoms. This protonation affects only 20 % of amines at physiological pH, but plays a fundamental role in the endosomal escape process thanks to the proton sponge phenomenon [58]. Depending on the degree of polymerisation, PEI can be linear (l-PEI) or in a branched form (b-PEI) with variable molecular weight. Generally, the higher the molecular weight of PEI, the greater the transfection efficiency. However, it may also be responsible for cellular toxicity [59,60]. Although it is oxidizable, the 25 kDa b-PEI is favoured because of its easy accessibility and efficiency in gene transfer [61]. When administered as an aerosol, b-PEI has been shown to be effective in murine as well as in sheep models, especially since it can be re-administered [62,63].

Copolymers represent another type of polymeric nanocarrier. PLGA (poly(lactic-co-glycolic acid) is one such copolymer and has been widely used for drug delivery purposes [64]. PLGA is a biocompatible and biodegradable polymer composed of glycolic acid and lactic acid. This polymer has demonstrated efficiency in gene delivery via aerosol using a mesh nebuliser, as demonstrated by Fernández et al. [65]. Polymers can also be organised into three blocks with a modular conformation. Triblock copolymers, such as Pluronics® (or Ploxamers), are composed of two poly(ethylene oxide) (PEO) blocks intercalated with a poly(propylene oxide) (PPO) block and are capable of forming micelles with sizes ranging from 10 to 100 nm [66]. Tetrafunctional block copolymers can also serve as drug delivery carriers and consist of four PEO/PPO blocks arranged around an ethylenediamine moiety [67]. Copolymer 704 is an example of this and has been successfully tested in vivo via intratracheal administration for gene delivery purposes [68].

2.2.3. Inorganic nanoparticles

To be used as nanocarriers, inorganic particles must meet the following criteria: they have to be (i) biocompatible, (ii) stable, (iii) resistant to microbial degradation, and (iv) exhibit high distribution efficiency [45]. For instance, gold NPs are among the least toxic and most extensively studied inorganic particles. These NPs can take various forms, such as nanospheres, nanorods, nanostars, nanoshells, and nanocages [69]. Additionally, other nanomaterials, like mesoporous silica NPs, are suitable for drug delivery. These NPs can be accumulated inside the lungs and present a larger surface area associated with a strong loading capacity [70]. Silica NPs can also be aerosolised with a nebuliser or inhaler and coated with lipids or polymers such as PEG or PEI [71].

2.2.4. Extracellular vesicles and mesenchymal stem/stromal cells

Extracellular vesicles (EVs) are small vesicular structures secreted by mammalian cells that serve as communication mediators [72]. Research in this fields has increased because extracellular vesicles (EVs) can serve as vehicles for the delivery of therapeutic agents, including small molecules, exogenous RNA (such as siRNA or miRNA), peptides, and DNA [73]. In addition, EVs can be administered by nebulisation into the lungs using a vibrating mesh nebuliser [74]. In a study by Hang et al., the inhaled EVs were well distributed and exclusively present in the lung tract, especially in lung macrophages and airway epithelial cells [75]. In another study, the nebulisation of EVs from mesenchymal stem/stromal cells (MSCs), a type of multipotent adult progenitor cells, were efficiently delivered and tolerated by severe COVID-19 patients [76]. EVs also have shown antimicrobial and anti-inflammatory activities [77–79]. Many (pre)clinical studies have demonstrated that MSC-derived EVs (MSC-EVs) and their parental MSCs had very similar therapeutic benefits [80]. In addition, EVs formulated with excipients and stabilisers (e.g., trehalose, lactose, mannitol) allow a greater stability during freezing [81]. For example, nebulisation of EVs carrying bacterial and viral antigens from microbial lung pathogens has been successfully performed in both preclinical and clinical studies [82,83].

Recently, mesenchymal stem/stromal cells (MSCs) itself, have been of great interest to multidisciplinary medicine, including respiratory diseases [81,84]. While clinical studies highlight the benefits of MSCs injections by the conventional routes of administration, their delivery and elimination in the respiratory tract remain a challenge. In this regard, Averyanov et al. have pointed out that the use of a compressor nebuliser is more suitable than ultrasonic and mesh nebulisers for achieving a high number of viable MSCs after the aerosol process. Furthermore, in an animal model of bleomycin-induced pulmonary fibrosis, comparable results in terms of therapeutic benefit were observed between the groups receiving intravenous versus nebulised treatment [85]. The feasibility of nebulising MSCs has also shown promising results using two microspray devices (i.e., MADgic Airway™ MAD780 (LMA) and MicroSprayer® Aerosolizer (PennCentury)) [86]. In addition to regulating immunological diseases such as fibrosis or asthma [80], the secretome of the MSCs is highly concentrated in antimicrobial mediators such as antimicrobial peptides (AMPs). Indeed, aerosolization of MSCs with a vibrating mesh nebuliser (Aerogen® Solo) has been shown to reduce the number of the Gram-positive bacterium *Staphylococcus aureus* as well as the Gram-negative bacterium *Klebsiella pneumonia*. Nevertheless, further investigations are required, in particular proteomic studies, to attribute the observed antimicrobial effects of MSCs to their secreted AMPs [77].

2.2.5. Advantages of nanoparticles for aerosol delivery

From a general point of view, NPs offer several advantages for aerosol delivery. For instance, they have a large surface area, enabling the addition of significant quantities of drugs [87]. This surface also allows for specific targeting of cells by incorporating active agents that facilitate NP transport through biological barriers (e.g., mucus, as discussed in section 3.2.3) [88,89]. In addition, nano-formulations have demonstrated potential to increase drug solubility and bioavailability, as well as enhance stability in vivo [90]. This can reduce the required dosage and improve lung retention [91,92]. To prevent pulmonary embolism, particles larger than 5 μm must be excluded from aerosol therapy. The size of NPs also impacts cell internalisation, with pulmonary epithelial cells internalizing particles 10 times smaller (0.5 μm) more efficiently than larger particles (1 μm) [93].

3. The challenges of aerosol delivery

3.1. In vitro and in vivo evaluation

Preclinical studies are required to assess the administration of aerosol into the lungs and several systems have already been developed. Among them, in vitro, ex vivo and in vivo exposure systems are widely used (Table 2) [9]. However, despite the use of these types of models, some challenges still need to be overcome. For example, patient variability cannot be fully reproduced. In addition, physiological ventilation such as respiratory rate differs between humans and animal models. In rodents, for example, there is no lobe division in the left lung. Non-human primates, on the other hand, have lung anatomy more like humans and can therefore be used in preclinical development. However, this type of in vivo experiment has limitations, including high costs, ethical concerns, and challenges such as uncontrolled breathing patterns [43].

Table 2
Evaluation of aerosol drug delivery considering in vitro, ex vivo and in vivo models.

Exposition	Systems	Advantages	Disadvantages
In vitro	Vitrocell® cloud is a diffusion/ sedimentation system using a exposure chamber to deliver liquid aerosols through a mesh nebuliser [94,95]	Possibility to use air/ liquid interface culture that closely mimic *in vivo* physiology	Does not reflect clinical conditions well, as the inspiration/ expiration cycle is not mimicked.
	Vitrocell® automated exposure station or Cultex® radial flow system, allow to adjust flow rate and monitor particles concentration and size distribution [96]		Expensive and restricted to mesh nebuliser
In vivo	Whole-body inhalation	Each animal will receive the same aerosol formulation	Needs high amounts of material; variability of the received dose (about 60 to 80 % of the aerosol is ingested) [97]
	Lung-only exposure	Delivery using MicroSprayer®, limits usage of invasive techniques such as tracheotomy [98]	Mice must be anesthetized; tissue damage might occur
	Nose-only exposure	Uniform exposition and better control of the inhaled dose	Mice are restrained in an immobilization chamber; a few systems are available, (Buxco®, TSE systems®) but remain expensive
Ex vivo	Composed of a model human head connected to a sealed enclosure with ex vivo porcine pulmonary tract [99]	Complete organ, allow to determine lung repartition of the particles	Different species available but tissue viability was restricted to few hours [100,101]

3.2. Barriers to aerosol drug delivery

In aerosol application, the medication must pass through many steps to efficiently reach the epithelium of the airways. The excipients and thus the formulation are of crucial importance to protect the drug from the aerosol process [102]. In addition, formulating solid particles is more challenging compared to formulating liquid solutions [103,104]. Depending on the pathology, especially in lung cancer where high doses are needed, the time required for nebulisation is longer and can be a problem for patients [19]. To efficiently manage the toxicity and efficacy of an inhaled drug, accurate determination of the administered dose must be an important prerequisite [105]. However, this determination is a challenge compared to other routes of administration. This is because the characteristics of the patient, i.e., respiratory parameters, morphology, and physiology, strongly influence the inhalation dose.

3.2.1. Pulmonary tract

From a physical point of view, the morphology, size, and speed of inhaled microdroplets have a direct influence on their fate in the airways. There are several parameters to characterise them: (i) The equivalent aerodynamic diameter (EAD) represents the diameter of a sphere that has the same falling velocity than a particle with a density of 1 g/cm^3 [106]. This value is used to determine the movement of the particle in the inhaled air. (ii) The mass median diameter (MMD) is the particle diameter at which 50 % of the mass of the aerosol is below this threshold [107]. In more schematic terms, this is the diameter at which half of the mass of the aerosol consists of small particles, while the other half corresponds to large particles. (iii) Finally, the mass median aerodynamic diameter (MMAD) corresponds to the median diameter of all particles formed as follows: $\text{MMAD} = \text{MMD x} \sqrt{\textit{liquide density}}$. However, depending on the nebuliser/inhaler used, the microdroplets are not all the same size, and this fact must be considered depending on the application.

In a general application, the optimal aerosol diameter for targeting the lungs is a MMAD between 1 and 5 μm [108]. The deposition of aerosol in the airways is determined by the chemical and physical properties of the formulation, the aerosolization process, the inhalation parameters of the patient and lung anatomy [109]. In general, the smaller the particles, the deeper they penetrate the airways. The MMAD is one of the most important parameters that is decisive for deposition in the lungs [110]. For example, particles with an EAD of 5 μm or larger have an impact at the level of the branches in the upper airways (Fig. 2) [111]. During nasal inhalation, the area behind the nostrils is where the deposition is greatest (75 to 95 % for particles with an EAD of 5 μm or larger in diameter) because the air flows is quickly and turbulently dispersed in this area [112]. Therefore, upper airway physiology is an important parameter to consider when studying the delivery and deposition of inhaled medications [113]. With a particle diameter between 2 and 5 μm, the particles sediment under the influence of gravity in the bronchi and bronchioles. Finally, particles with an EAD smaller than 2 μm diffuse into the deep lung and follow the Brownian motion due to thermal movement [114,115]. The particle size also has an influence on cell targeting. Indeed, particles with a size of about 500 nm are mainly phagocytosed by alveolar macrophages and may be interesting for the treatment of intracellular infections, such as *Mycobacterium tuberculosis* infecting this specific cell type [116].

3.2.2. Immune system and pulmonary epithelium

The lungs have an immune system whose main function is to protect the epithelial cells from inhaled xenobiotics. The latter can be air pollution particulate matter or engineered nanomaterials [117]. Therefore, alveolar macrophages provide a barrier to inhaled elements by direct phagocytosis or indirect mechanisms acting as antigen-presenting cells (APC) [118]. A non-specific inflammatory response mediated by cytokines can also intervene and activate lymphocytes. When gene therapy is used, some cytokines such as interferon-gamma (IFN-ϒ) and tumour necrosis factor alpha (TNF-α) inhibit the gene expression by acting on cytomegalovirus (CMV), respiratory syncytial virus (RSV) or simian vacuolating virus 40 (SV40) promoters, which are required for viral gene therapy [119]. Synthetic vectors can also trigger an immune response and induce in vivo cytotoxic effects. For example, Scheule et al. reported that neutrophil infiltration and proinflammatory cytokines overproduction (IL-6, IFN-ϒ and TNF-α) occurred 1 to 2 days after instillation when the cationic Genzyme lipid (GL)-67, the current gold-standard for in vivo lung gene transfer, was administered by instillation into the lungs of mice. However, this phenomenon disappeared within 14 days after administration [120]. The cationic lipid used has been shown to mediate the inflammatory response, but this response is much less pronounced than with intravenous administration [121]. If the lung epithelium is the main target, the aerosolised particles must also pass through the plasma membrane. To promote this passage, the strategy of using transporters that promote interactions between particles and epithelial cell membranes can be considered [122]. Another element of the respiratory epithelium, the glycocalyx, has also been shown to act as a barrier against inhaled drugs. The latter is a surface carbohydrate-enriched gel-like layer and participate to maintain

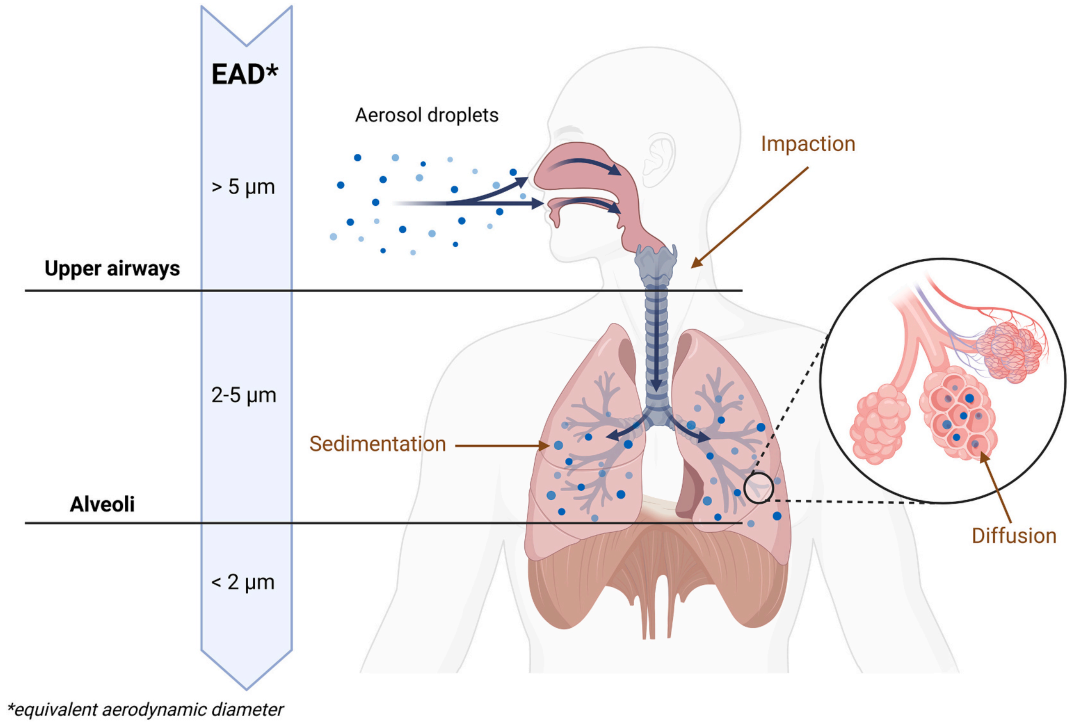

Fig. 2. Progressive lung deposition of inhaled particles based on their size. Particles with an equivalent aerodynamic diameter (EAD) greater than 5 µm are retained in the upper airways by impaction. Particles between 2 and 5 µm are deposited by sedimentation in the bronchi and bronchioles. Finally, particles with an EAD of less than 2 µm can diffuse into the alveoli. (Figure created with BioRender.com)

of cell homeostasis [123]. However, glycocalyx also represents a barrier to drug delivery and the use of molecules capable of removing sialic acid residues, such as neuraminidase, has been demonstrated to increase gene transfer by destroying the glycocalyx of polarised cells [124].

3.2.3. Mucociliary clearance and airway surface liquid

Despite the advantages of aerosol administration, the inhaled drug has to overcome many biological barriers [125]. The most important of these is the airway surface liquid (ASL), which is composed of both

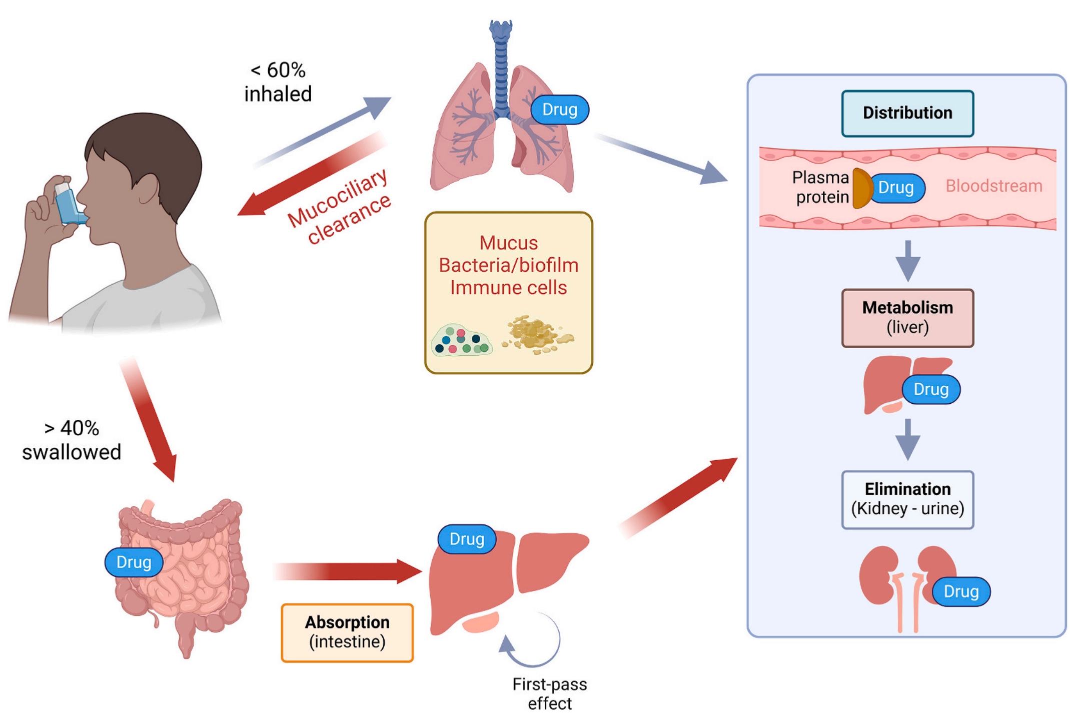

Fig. 3. Pharmacokinetic of inhaled drugs. In the upper airways, mucociliary clearance moved the inhaled drug via cilia and mucus displacement into the pharynx. As a result, NPs are swallowed down and end up in the digestive tract where they are absorbed in the intestine, distributed through the bloodstream, metabolised in the liver and excreted. This phenomenon is particularly marked in pulmonary diseases such as CF, COPD, or lung infections, where mucus, bacteria/biofilm and immune cells form a barrier to inhaled drugs by reducing the amount retained in the lungs. (Figure created with BioRender.com).

periciliary fluid and mucus layer. Mucus is a dynamic structure mainly composed of water and the glycoprotein polymeric mucins secreted by goblet cells [126]. Two major mucins, MUC5AC and MUC5B, are present in airway mucus. Both form a network through the formation of disulfide bonds, with pores approximately 500 nm in size [127]. This mesh directly influences the viscoelastic properties of mucus [128]. In the context of aerosol therapy, mucus, along with the movement of cilia on the apical surface of ciliated cells (i.e. mucociliary clearance, MCC), plays a key role in pulmonary excretion by acting as a barrier to inhaled drugs [129]. In fact, the physiochemical properties of aerosols (particle size distribution, density, shape, surface area, hydrophobic/hydrophilic properties) significantly impact particle deposition in the respiratory tract as well as mucus diffusion. Since mucin is negatively charged, it can interact with the charges of NPs, preventing them from penetrating the lung epithelium [130,131]. To summarize, large, hydrophobic, and positively charged NPs tend to strongly interact with mucus, leading to entrapment, whereas small, neutrally charged NPs are more likely to reach the underlying cells.

In terms of deposition sites, the upper airways have many ciliated cells coupled with a thick mucus layer that facilitates the elimination of NPs. Inhaled drugs are poorly absorbed in the pulmonary tract and instead end up in the digestive tract. In fact, the movement of cilia transports mucus containing trapped particles to the pharynx, where the NPs are subsequently swallowed and enter the oesophagus (Fig. 3) [132,133]. It takes about 10 min for half of the inhaled particles deposited in the oropharynx to be excreted through this process [134]. In the lower airways, MCC remains active, although ciliary density decreases. Therefore, NPs in this region persist longer before being cleared. In the alveolar region, where ciliated cells are absent, NPs bypass MCC. However, alternative mechanisms, such as phagocytosis, may contribute to their clearance [135].

In diseases such as COPD or CF, mucus leads to bronchial obstruction and negatively influences the distribution of aerosolised particles. For example, it has been shown that mucus in the airways during NAs administration significantly reduces the transfection capacity of liposomes, but also of adenoviruses and adeno-associated viruses (AAVs) [118,136,137]. The investigation of the physical properties of mucus is therefore an important step for the development of new strategies or formulations that can overcome this major barrier [138]. Various strategies can be explored to facilitate mucus diffusion. The use of PEG, for example, facilitates the passage of lipoplexes into the mucus by stabilizing the positive charges and providing steric stabilization through a hydrophilic layer which preventing aggregation with the negatively charged mucins. This phenomenon is favoured by NPs densely coated with low molecular weight PEGs [139]. The decrease in surface charges also reduces cytotoxicity. Conversely, PEGylation reduces the transfection efficiency of lipoplexes. Pre-treatment of mucus with mucoactive drugs and subsequent administration of drugs with NPs is also a possible approach. Suk et al. tested this strategy by treating CF sputum with *N*-acetyl cysteine to break the disulphide bridges that maintain the mucin network. The results showed a better diffusion of these NPs through pre-treated than untreated sputum [140]. Similar outcomes were observed by pre-treating vaginal mucus with Pluronic® F127 [141].

3.2.4. Specific cases of chronic infections and antimicrobial formulations

Chronic lung infections are in most cases exacerbated by host factors that make the lung environment suitable for long-term microbial colonisation by opportunistic pathogens that are normally eliminated in healthy individuals. These factors are diverse in nature and may be related to a defective or weakened immune system or an abnormal physiological microenvironment of the lung [142]. In this context, patients suffering from bronchiectasis, non-CF bronchitis, non-CF bronchiectasis (NCFBE), CF and COPD are particularly affected by chronic infections that are generally challenged by biofilm-forming bacteria [143]. By producing an extracellular matrix (ECM) mainly composed of water, exopolysaccharides (EPS) and extracellular DNA, biofilms allow a long-term persistence in the lung environment and promote the establishment of polymicrobial communities [144–146]. Overall, biofilms confer a high degree of tolerance to antibiotics, sometimes requiring the use of 100-fold higher doses compared to the treatment of their planktonic counterparts [147]. In addition to this mechanism, the entrapped bacteria themselves can generate many resistance phenotypes to antibiotics by promoting their genetic transfer and accelerating their mutational emergence [148,149].

Regarding the nebulisation of therapeutic drugs, the microbial layer simultaneously represents a challenge for antimicrobial drugs, which need to reach the bacteria and exert their antimicrobial effect, and for curative drugs or intracellular antibiotics, that require an intracellular diffusion through the epithelial membranes to be effective. Indeed, in CF patients, aerosolised protein- or gene-based therapeutics would need to cross this biofilm barrier before reaching the epithelial cells [150]. To overcome this barrier, mainly composed of anionic polymeric substances as in the mucus layer, the NPs must fulfil certain criteria [128,151]. Some studies have reported the importance of using small-sized NPs (100–500 nm) with neutrally charged surfaces to avoid biofilm/mucus stagnation and improve their penetration through this viscous matrix [152,153]. Free antimicrobial drugs show usually limited efficacy under conditions that mimic these barriers [154]. Anti-infective and healing agents could be incorporated into NPs to overcome these limitations. To get through the mucus and biofilm barrier, antimicrobial agents can be administered together with an enzyme degrading the matrix (i.e., EPS, mucin, DNA) or by using "all-in-one" NPs [153]. Pulmozyme® (Dornase α), a DNase that significantly reduces the viscosity of sputum, is able to efficiently disrupt biofilm formation when the DNase-coated NPs contain fluoroquinolones (levofloxacin and ciprofloxacin) [155,156]. Such a strategy has been successfully reproduced with EPS-degrading enzymes, such as alginate lyase and serratiopeptidase [157,158].

4. Pathologies and applications of aerosol delivery

4.1. Monogenic disorder: cystic fibrosis as an emblematic example

CF is an autosomal recessive disease caused by mutations coding for the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) protein. Treatment options for this genetic disease include pharmacological treatment, lung transplantation and gene therapy (Fig. 4) [159].

4.1.1. Antibiotics

The benefit of aerosolised antibiotics has been observed mainly in CF, but also in NCFBE and ventilator-associated pneumonia (VAP) patients. For example, Cayston®, a formulation of aztreonam with lysine for inhalation, is available for the treatment of CF infections caused by *Pseudomonas aeruginosa*. Indeed, improvements in respiratory symptoms, lung function and microbiological analyses have been reported in patients who have or have not previously received inhaled tobramycin [160,161]. However, these effects were not observed in patients infected with *Burkholderia cepacia* complex [162]. In addition, a subsequent randomised phase II study including 151 CF patients infected with *P. aeruginosa* showed that 28 days of treatment with levofloxacin led to an increase in respiratory capacity (forced expiratory volume in the first second (FEV)1 of 8.7 %) [163]. Tobramycin, an aminoglycoside antibiotic, can be used by inhalation either from a solution (TIS or TOBI) or from a powder (TIP). The latter has the advantage that it can be administered very quickly and effectively using a Podhaler™ inhalation device. In the phase III EVOLVE study, TIP significantly improved FEV1, reduced the sputum density of *P. aeruginosa* and reduced respiratory-related hospitalisations [164]. The efficacy of TIP was compared to TIS in a clinical trial and does not appear to differ in terms of FEV1 improvement and hospitalisation rate [165].

Colistin or polymyxin E can also be administered as a dry powder.

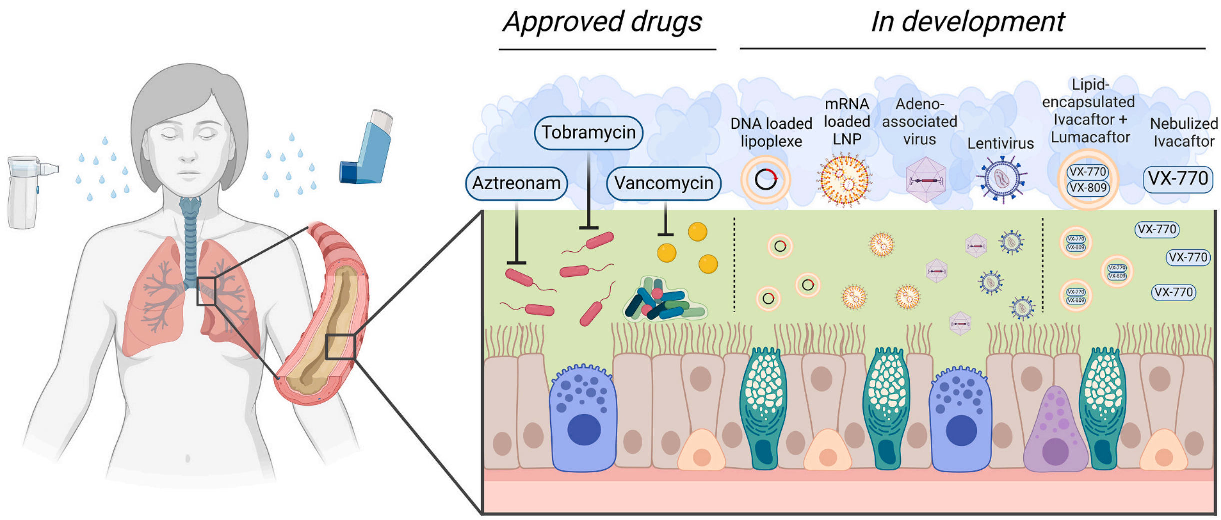

Fig. 4. Overview of the main aerosol applications for the treatment of CF, including approved drugs and approaches in development. Approved drugs, including aztreonam, tobramycin and vancomycin, target either *P. aeruginosa* or *S. aureus* to reduce bacterial exacerbations. Other types of drugs are under development such as nucleic acid delivery using viral vectors or non-viral vectors (lipoplexes or LNs). The latter are also being investigated for aerosol delivery of CFTR modulators. The thick mucus present in the CF airways also needs to be crossed by the inhaled drug. (Figure created with BioRender.com)

Colobreathe® is an encapsulated dry powder formulation (1 inhaled capsule = 1 nebulised dose) administered with a portable inhaler (Turbospin®). Its use has been compared with TIS and does not appear to provide more benefits [166]. Inhaled liposomal amikacin, also known as Arikace®, an aqueous amikacin encapsulated in a liposome, allows better biodistribution and ability to overcome biological barriers such as mucus and biofilm [167]. In this direction, Phase II studies were conducted with various doses of Arikace® in chronic CF patients. Overall, the treatment was well tolerated, an increase in FEV1 was reported (even over extended periods) and the mean frequency of *P. aeruginosa* in sputum also decreased significantly [168]. Inhalable antibiotics against *S. aureus*, such as vancomycin (glycopeptide) (AeroVanc®), are currently being investigated (NCT01594827).

4.1.2. *Pharmacological treatments*

Pharmacological therapies or CFTR modulators are novel therapies that act on the CFTR protein to restore its function. There are different types of modulators, depending on their mechanism of action. Ivacaftor, for example, is a potentiator that increases CFTR activity [169]. Guan et al. have developed inhalable particles for pulmonary delivery of ivacaftor (VX-770) and is commonly used for CF patients with G551D mutations [170]. They used a leucine microparticle-based formulation to release ivacaftor via aerosol to human lung cancer cell line Calu-3. Furthermore, Garbuzenko et al. reported a reduction of CF lung manifestation after repeated inhalation of nanostructured lipid carriers containing lumacaftor (VX-809) and ivacaftor [171]. In addition, a novel combinatorial approach containing a mixture of ivacaftor, elexacaftor (VX-445) and tezacaftor (VX-661) (Trikafta®) has shown promise in increasing CFTR activity and reducing mortality and morbidity rates in CF patients [172]. However, this treatment is mutation-dependent, 10 % of patients are not eligible [173]. Additionally some patients report serious side effects [174].

4.1.3. *Gene therapy*

Gene therapy by aerosol delivery is an attractive strategy for restoring CFTR function as it is mutation independent. In this approach, the conducting airways are the main target for CF gene therapy. Thus, Labiris et al. have demonstrated that the MMAD has to be between 2 and 3 µm to efficiently reach the airway epithelial cells of CF patients and achieve the greatest therapeutic effect [10]. Several solutions have been investigated to efficiently deliver NAs into the airways and clinical studies have been conducted with recombinant viral vectors, such as adenoviral vectors or with cationic lipoplexes [175].

Different clinical trials with non-viral vectors in CF have been performed, but mostly related to strategies for nasal application, due to the complexity of formulating substances that can resist during the aerosolization process [176]. However, two clinical trials based on a spermine-derived cationic lipid, GL67, have been successfully conducted by aerosolization using jet nebuliser in CF patients [177,178]. The GL67A formulation (Sanofi-Genzyme) consists of (i) a cholesterol-derived lipid, GL67, (ii) a zwitterion-type lipid, DOPE (1,2–dioleoyl-sn–glycerol–3-phosphoethanol-amine) and (iii) a PEGylated lipid, DMPE-PEG5000. A CpG-free plasmid, pGM169, encoding optimised *hCFTR* was used and results showed that after one administration per month during one year, lung function is stabilised compared to placebo, regardless of age, gender or mutation [179]. Non-viral gene therapy for CF by aerosol delivery is therefore promising, but still needs to be optimised to increase the efficiency of vector transfection. The goal is to reach at least 20 % CFTR expression (the estimated baseline needed to observe beneficial clinical effects) in epithelial cells, while overcoming the various intra- and extracellular barriers [180]. For example, analogues of GL67A have shown great potential for CF gene therapy by aerosol delivery when used in vivo [181].

The properties of liposomes are directly related to their lipid part, which influences the fluidity and permeability of the bilayer membrane and therefore the efficiency of the transfection [182–185]. Heye et al. have also shown that the presence of asymmetry in the two hydrophobic chains is more efficient for the transfer of NAs [186]. In addition, branched cationic amphiphilic compounds, when administered by jet nebuliser, showed higher gene transfection efficiency on air/liquid interphase (ALI) cells compared to the branched PEI used as a reference [187].

Non-viral gene therapy also offers the advantage that optimised nanocarriers can be used by grafting or coupling chemical components/agents directly onto their surface, which could reduce the microbial burden while ensuring gene transfer efficiency [150,188]. So far, no study has demonstrated that such nanocarriers can prevent the formation of biofilms. However, arsonium-containing lipophosphoramides have shown that gene transfer can be achieved in a coculture model with lipid-based nanocarriers active against *S. aureus* [189]. The antibacterial properties of these vectors were improved by grafting an N-heterocyclic carbene-silver complex onto their structure to extend the antibacterial spectrum to *P. aeruginosa* while maintaining their efficacy after nebulisation [190].

Delivery of viral recombinant vectors into the lung, such as lentiviruses, has also several advantages. A lentiviral vector pseudotyped with Sendai virus envelope proteins F and HN has been developed and tested in mice [191]. Conventional lentiviral vectors pseudotyped with the vesicular stomatitis virus G-glycoprotein (VSV-G) cannot efficiently transduce airway epithelial cells. However, the F/HN pseudotyped SIV (F/HN-SIV) is able to transduce rodent airway epithelial cells in vitro [192]. Moreover, repeated administration to the lower respiratory tract of mice can be performed with a long-term safety profile of this viral vector [191,193].

Gene therapy for CF remains highly relevant, particularly with the initiation of two new clinical studies. The first study, launched in 2022 by 4D Molecular Therapeutics (NCT05248230), include patients who are ineligible for or intolerant to CFTR modulator therapy. This trial employs a single inhalation administration of 4D-710, an AAV vector with a transgene cassette encoding *hCFTR* with a deletion in the regulatory domain. Another clinical trial initiated by Vertex Pharmaceuticals in 2023 is a phase 1/2 clinical trial (NCT05668741) using VX-522 mRNA therapy via oral inhalation by nebuliser for patients who are not responsive to CFTR modulator therapy. VX-522 is an inhaled messenger RNA therapy aims to deliver full length copy of CFTR mRNA using lipid nanoparticles.

4.2. Respiratory infections and antimicrobial treatment

Lung infections are responsible for a large proportion of deaths worldwide, especially in the case of lower respiratory tract infections (2.6 million deaths in 2019) [194]. Some infectious diseases can be treated with the currently available antibiotics (Table 3), while some other require new antimicrobial treatment strategies due to the development of antimicrobial resistance.

Table 3
Commercially available inhaled antimicrobial drugs [272].

Aerosolized drug - Device	Product - Company	Spectrum - indication	Regimen - Dose
Lanimamivir Octanoate Hydrate- Twin Cap	Inavir ® - Daichii Sankyo	Antiviral - Influenza infection	nd* - 20-40 mg (2–4 capsules); 1 dose
Zanamivir - Diskhaler DPI	Relenza ® - GSK	Antiviral - Influenza infection	5 days - 10 mg (2 blisters) twice a day
Ribavirin- Jet nebuliser	Virazole ® - Valeant	Antiviral - RSV infections	3–7 days - 6000 mg (300 mL) once a day
Pentamidine isethionate- Jet nebuliser	Nebupent ® - APP Pharm	Antifungal - AIDS Patients with PCP	Every 4 weeks - 300 mg (6 mL) twice a day
Tobramycin- Jet Nebuliser	TOBI ® - Mylan	Antibacterial - CF patients	Chronic - 300 mg (5 mL) twice a day
Tobramycin- Podhaler DPI	TOBI Podhaler ® - Mylan	Antibacterial - CF patients	Chronic - 112 mg (4 capsules) twice a day
Aztreonam lysinate- e-Flow	Cayston ® - Gilead	Antibacterial - CF patients	Chronic - 75 mg (1 mL) three-times a day
Levofloxacin- e-Flow	Quinsair ® - Horizon	Antibacterial - CF patients	Chronic - 240 mg (2.4 mL) twice a day
Amikacin sulphate- e-Flow	Arikayce® - Insmed	Antibacterial - NTM infection	Chronic - 590 mg (8.4 mL) once a day
Colistimethate sodium- Jet nebuliser	Coly-mycin M ® - nd	Antibacterial - CF patients	Chronic - 75-150 mg (1–2 mL) twice a day
Colistimethate sodium- Turbospin DPI	Colobreathe ® - TEVA	Antibacterial - CF patients	Chronic - 125 mg (1 capsule) twice a day

* nd: non-define.

4.2.1. Antibodies

Most monoclonal antibodies (mAbs) used in clinical trials for the treatment of respiratory diseases are full-length or engineered immunoglobulin G (IgG). Their indication is approved for the treatment of asthma and non-small cell lung cancer (NSCLC) as well as for the prevention of RSV infection [195,196]. However, during the nebulisation process, mAbs are exposed to physical constraints that lead to mAb aggregation, which can cause cytotoxic effects and/or unexpected immunological reactions [197–199]. To overcome this issue, the use of stabilizing additives in mAbs formulations such as sugars (trehalose, mannose), aminoacids (cysteine), glycols (PEG, PLGA) and surfactants (TWEEN-20, sodium dodecyl-sulfate, pluronics) showed a protective effect against unwanted molecular degradation and crystallization [197,200,201]. Compared to jet nebulisers, mesh nebulisers are better suited for efficient administrations of mAbs [195]. In combination with intravenous administration, inhalation could increase the therapeutic index and overcome the systemic side effects [198]. Recently, nebulisation of IgG has been used as a stimulator of the immune response in the prevention of various infectious respiratory diseases. In fact, two years of nebulisation using PARI® eFlow of IgG in patients with antibody deficiency significantly reduced the incidence of upper respiratory tract infections without side effects [202]. In a mouse model, IgG nebulisation also effectively protected against a fatal pneumococcal infection caused by a low dose ($<10^5$ bacteria) of *Streptococcus pneumoniae* [203]. Many clinical trials with mAbs nebulisations were conducted during the COVID-19 pandemic, although some of them were discontinued due to lack of treatment efficacy [197]. The development of other mAbs with neutralising activity is still necessary [204,205]. Genetic content encoding neutralising antibodies could be a promising way to enable long-term antibody production by nebulisation [206].

4.2.2. Bacteriophages

The pulmonary administration of viruses, in particular bacteriophages ('phages'), is of great interest in modern medicine to prevent and treat bacterial infections. Phage therapy was developed in eastern European countries since the 1920s and was a great success against a wide spectrum of bacterial species, but showed questionable preclinical results [207]. The inhalation of phage therapy developed more recently (1960s) requires numerous optimisations. Firstly, the aerosolization process could have a negative impact on the phage integrity by ejecting the NAs and/or creating a "broken" phage fraction [208,209]. In this respect, mesh nebulisers are better suited than jet nebulisers to maintain phage viability [210–213]. The stability of the phage solutions/powders requires special storage conditions, such as low humidity and stable temperature, as well as excipients [214,215]. The liquid solution of the anti-mycobacterial phage D29 was efficiently introduced into the lungs of mice using a vibrating mesh nebuliser and thereby acted as prophylactic treatment against infection with *M. tuberculosis* for 24 h [212,216]. The internalisation of phages into macrophages was improved by the use of liposomal encapsulated D29 formulations [217,218]. The liposomal formulation consisted of phospholipid (egg lecithin), cholesterol and Twenn-80.

Spray-dried phage powders were also used in most preclinical studies [219,220]. Among them, the *Pseudomonas podoviridae* (PEV) phage PEV20 was prepared with lactose and leucine as excipients, a suitable preparation to reduce *P. aeruginosa* load [221], and able to co-deliver antimicrobial agents such as ciprofloxacin [213,222]. The optimisation of the PEV20 formulation was carried out with trehalose, mannitol and L-leucine used as excipients. Better delivery in the lung was achieved by the spray-dried technique despite significantly lower phage recovery in comparison to the freeze-dried spray preparation [219]. Several case studies of CF patients who received inhaled phage preparations showed varying results. For example, lung function improved in patients with persistent *Achromobacter xylitans* [223,224] but not in

Burkholderia infections after phage therapy [225]. In a pilot cohort of 20 infected patients receiving an intravenous and/or aerosolised phage cocktail, favourable microbial and clinical outcomes were observed, although no significant improvement can be directly attributed to phage inhalation [226].

4.2.3. Other antimicrobials

Light-sensitive molecules, also known as photosensitisers, have been intensively studied for anticancer strategies and have recently been extended to antimicrobial applications [227,228]. In combination with a suitable light source, these molecules are able to generate reactive oxygen species (ROS) via photochemical reactions, which are radicals with bactericidal activity [229]. Some pulmonary pathogens, including viruses, bacteria and fungi, have been reported to be sensitive to photodynamic therapy [230–232]. Although photosensitisers were mostly administered intravenously, some of them (such as indocyanine green, photogem and photodythazine) could be efficiently administered by nebulisation [233]. However, only indocyanine green in combination with an infrared light source was successful in reducing bacterial pneumonia in preclinical studies [234,235].

Pulmonary administration of peptides and proteins not only addresses lung infections, but also a range of diseases such as bone diseases or immunological disorders [236–238]. In general, most (pre)clinical studies on pulmonary administration of AMPs focus on *P. aeruginosa* infections [239]. To overcome the impairment of integrity and efficiency during the aerosol process, formulating peptides with polymeric NPs provides enhanced protection and ensures better drug distribution within the lungs. Polymeric NPs can shield AMPs from degradation during nebulisation and enhances the therapeutic potential of AMP-based treatments by maintaining their integrity and optimizing their bioavailability in the lungs [240–242]. Recently, a machine learning pipeline was developed to generate new AMPs that showed promising therapeutic effects in a mouse model of pneumonia [243]. Currently, only one AMP, ALX-009, a lactoferrin peptide in combination with hypothiocyanite, has been aerosolised in a clinical trial (phase I) in CF patients [244].

4.2.4. Polymers and lipid-based nanoparticles

Antimicrobial embedding in liposomes and polymer structures has been shown in the literature to efficiently deliver and enhance many antimicrobial agents [153]. To date, no antibiotic-containing polymer has been clinically approved, but encouraging results have been obtained with NPs based on poly(lactic-*co*-glycolic) acid (PLGA) [245,246]. Wan et al. synthesised an aerosolizable ultra-small d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS)-PLGA hybrid NP that was able to penetrate the mucus layer, reach the biofilm layer efficiently and thus release the azithromycin in the vicinity of the biofilm-forming *P. aeruginosa* [247]. In another study, inhalable polymer formulations based on porous PLGA microspheres decorated with *N*-acetylcysteine (NAC) were used to successfully release antituberculosis drugs after crossing the mucus layer [248]. Spray-dried powders based on PLGA NPs enriched with chitosan and polyvinyl alcohol (PVA) were prepared and showed eradication of *P. aeruginosa* biofilm by releasing high doses of ciprofloxacin [242].

Liposomal formulations of antimicrobials such as amikacin (Arikayce™), tobramycin (LipoBiEDT-TOB™), ciprofloxacin (Pulmaquin™) and amphotericin B (Ambisome™) have been successfully approved for the treatment of pulmonary diseases [249]. The optimisation of liposomal formulations for a better interaction with the biofilm clearly depends on the composition and the nature of these lipids. In this context, lipids such as phosphatidylinositol (PI), stearylamine (SA), dimethyldioctadecylammonium bromide (DDAB) and 3β-(N(N1N1-dimethylaminoethane) carbamoyl) cholesterol (DC-chol) have been identified as enhancers of liposome-biofilm interactions [143,152]. In addition, biofilm-liposome affinity can be enhanced by the incorporation of ligands targeting biofilm components, such as mannose or lectins [250,251].

4.2.5. Mucosal vaccine strategies

Administration via the lung is a needle-free solution for vaccine delivery and may be a suitable choice for eliciting an immune response due to the large presence of immune cells on the mucosal surface such as antigen-presenting cells, alveolar macrophages and dendritic cells [252,253]. Although vaccines have significantly improved global immunisation against a wide range of pathogens and reduced the risk of spreading childhood infections [254,255], challenges remain in their long-term efficacy and accessibility. These issues are particularly pressing for populations in need, where ensuring consistent and widespread vaccination coverage can be difficult [256]. By relying on traditional routes of administration, such as subcutaneous, intradermal, or intramuscular injections, the needle-based vaccines produce systemic immunisation but can hardly achieve mucosal immunisation. However, this protection limit the diffusion of airborne pathogens from the main part of the respiratory tract into the bloodstream after strong recognition and uptake of antigens in the mucosa [256,257].

Vaccination by pulmonary administration is of particular interest for enhancing the immunisation of vaccines currently administered by injection and for preventing the transmission of airborne pathogens for which no treatment is yet available [258]. The authorised and approved needle-free vaccines against respiratory infections are mostly administered orally, including those due to the following pathogens *Vibrio cholerae* (Dukoral® and Shanchol™), rotavirus (Rotateq® and Rotarix®) and *Salmonella thyphi* (Vivotif®). Currently, only one intranasal vaccine, a live attenuated influenza vaccine (Flumist®), is licenced [259]. Although no lung vaccine is commercially available yet, a long list of preclinical studies shows that they can cover a broad spectrum of lung infections [260]. Compared to injected vaccines, the administration of therapeutics by aerosol systems in (pre)clinical studies still lacks standardisation and has some limitations and challenges, such as species-specific (lung geometry, depth), and inter-patients variables (frequency, volume, age, microbiota) that make it difficult to estimate the accurate therapeutic exposure dose [261,262].

4.2.6. Viral vaccines

The measles virus (MeV) is still circulating despite the various measures taken by the world health organization (WHO) [263]. Over the last 10 years, preclinical studies have been conducted in non-human primate models that have highlighted the medical benefits of aerosol administration of different types of vaccines using mesh nebulisers. For example, aerosolization of the lipopeptide fusion inhibitor MeV has successfully reached the pulmonary tract, resulting in major efficiency in infection prevention thanks to a strong humoral immune response [264]. Preclinical studies in porcine and ferret models have shown the potential of a nebulised mucosal vaccine (FluMist®) against influenza infections [265–267]. Influenza infection is the first infectious lung disease for which a mucosal vaccine has already been approved (FluMist®).

Gene therapy based on recombinant viruses has also shown promising results, particularly through the simultaneous induction of strong mucosal and systemic immunisation against the influenza virus [268]. The emergence of the COVID-19 pandemic led to the rapid development of mass immunisation programs, despite the complex challenges related to storage stability. In this respect, the dried powder for nebulisation of vaccines presented the best properties [269,270]. Inhalation of an adenovirus-based vaccine encoding the SARS-CoV-2 spike protein administered to rhesus monkeys proved to be a safe strategy to induce mucosal and systemic immunisation [271].

4.2.7. Bacterial vaccines

Pulmonary vaccination by Bacillus Calmette-Guérin (BCG) aerosolization, the live attenuated vaccine form of *Mycobacterium bovis*, has shown promising results in non-primate humans, providing immunisation against tuberculosis comparable to conventional administration [273]. The choice of delivery system remains a particular challenge due to the lack of experimental standardisation in preclinical studies [274]. However, vibrating mesh nebulisers have shown better stability and efficiency in the administration of the BCG inoculum, regardless of the concentration of the inoculum [275]. In addition to the device system, other optimisations have also been performed to the vaccine, such as the formulation. The alginate coating of the BCG inoculum leads to a better bacterial release as well as a better immune response compared to the BCG inoculum without coating [276].

Vaccines based on the use of adenoviral vectors have recently been shown to elicit a stronger immunogenic mucosal response than intramuscular injection in healthy humans (NCT02337270; phase I) [277], encouraging the clinical evaluation of other virus-vectored candidates (NCT04121494 and NCT01497769, phase I) [278]. Preclinical studies demonstrated the successful prophylactic efficacy of live attenuated and attenuated recombinant derivatives of *Francisella tularensis* vaccines administered by nasal spray or nebulisation against pneumonic tularaemia [279,280].

Among chronic lung infections, those caused by *P. aeruginosa* are extremely difficult to combat in CF patients and contribute to the failure of antibiotic therapy due to persistent biofilm formation. Prevention of bloodstream infections by vaccination strategies are also the result of years of research [281]. While oral thermosensitive *P. aeruginosa* vaccines have shown promising results for improving pulmonary clearance in a mouse model [282,283], the overall efficacy of *P. aeruginosa* vaccines remains low [284]. Encouraging results were observed with a recombinant vaccine based on the outer membrane protein (IC43) [285,286], inducing a high immunogenicity in a recent clinical trial (NCT01563263; phase III), although no clinical benefit in term of overall mortality was reported [287]. More recently, Yang et al. have developed an mRNA vaccine against *P. aeruginosa* encoding PcrV-, which is involved in the type III secretion system of the bacterium, and OprF-I, a fusion protein comprising outer membrane proteins OprF and OprI [288]. It has been shown that the combination of these two mRNAs is well tolerated by the pathogen-free BALB/c mice and provides better results than protein vaccines.

4.3. Lung cancers

Lung cancer is one of the most common types of cancer worldwide and can be divided into two categories: NSCLC, and small cell lung cancer (SCLS) with an occurrence of 85 % and 15 %, respectively [289]. Surgical removal or resection is the main treatment option for non-metastatic lung cancer, but remains limited to a few percentage patients with NSCLC [125]. When surgery is not possible, chemotherapy is another option to prolong survival without detriment to the patients quality of life [290]. Other strategies such as radiotherapy, immunotherapy or target therapy can also be used.

4.3.1. Chemotherapy

Chemotherapy administered via the systemic route must penetrate the lung tissue to be effective which is not without consequences on severe side effects including hepatic first-pass effect. In this context, inhaled chemotherapy can promote the local drug accumulation while reducing side effects as demonstrated with the inhaled doxorubicin NPs by simultaneously reducing cardiotoxicity and tumour size in a mouse model [291,292]. In addition, inhalation of topotecan, a topoisomerase I inhibitor, improves its pharmacokinetic properties. This formulation based on dry powder technology with standard excipients (trehalose and L-leucine) increases the half-life of the drug and lung exposure [293]. Another chemotherapeutic agent, erlotinib, an epidermal growth factor receptor (EGFR) inhibitor, has been shown to be resistant to nebulisation with different liposomal drug delivery system in the treatment of NSCLC [294].

The use of nanocarriers may help to target cancer cells and thus improve the internalisation of the chemical agent. For example, Zhu et al. conjugated folic acid motifs to liposomes before encapsulating docetaxel [295]. This targeted strategy is possible because folic acid receptors are highly expressed in lung tumours [296]. Administration of aqueous or dry powder formulation showed better internalisation of docetaxel than its free form. This observation could be explained by the presence of folic acid receptors. They also showed better pharmacodynamics and pharmacokinetics for their drug after spray drying [295]. In addition to liposomes, EVs can also transport chemical agents. In their study, Yuan et al. used this approach to induce apoptosis of cancer cells [297]. First, MSCs were transduced to express tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) enabling the production of EVs carrying high levels of this apoptotic protein. The EVs were then collected and used to encapsulate dinaciclib, a cyclin-dependent kinase inhibitor. The authors showed that it is possible to inhibit anti-apoptotic proteins by the nebulisation of EVs and subsequently induce apoptosis via TRAIL.

Another strategy to induce cell death in cancer cells is to activate a regulated cell death process, such as apoptosis, necroptosis, or the ferroptotic pathway [298]. The latter induces cell death through iron accumulation in cells and lipid peroxidation. Currently, various strategies are being investigated to induce ferroptosis in cancerous cells. For instance, W. Wang et al. directly targeted cancerous cells using a nanocarrier loaded with ferrocene (Fc) to catalyse the Fenton reaction, along with fluvastatin to induce tumour acidosis, thereby boosting Fc catalysis. These NPs were administrated via ultrasonic nebulisation and enhanced antitumour effects in an orthotopic lung adenocarcinoma tumour model [299]. Furthermore, Q. Feng et al. aimed to trigger tumour-associated macrophages (TAMs) with iron-based NPs to regulate iron metabolism within the tumour microenvironment [300]. The authors administrated their NPs using a microsprayer to an orthotopic Balb/c mice lung cancer model. They demonstrated that TAM activation promotes ferroptosis in cancer stem cells, thereby suppressing the initiation and progression of early-stage cancer.

4.3.2. Immunotherapy

The use of immunotherapeutic antibodies against the immune checkpoint molecules cytotoxic T lymphocyte-associated protein 4 (CTLA-4), programmed death protein 1 (PD-1) or its ligand (PD-L1) constitutes different strategies to treat lung cancer. The positive status of PD-L1 is a predictive biomarker for an effective response to immunotherapy in NSCLC but not all patients present an increase in PD-L1 [301]. Liu et al. have developed an aerosolisable liposomal NP mainly composed of cyclohexane IGEPAL® CO-520, DSPC and cholesterol and loaded with cyclic dinucleotides to stimulate the production of interferon genes in macrophages and dendritic cells. Aerosol administration to C57BL/6 mice led to a significant increase in PD-L1 expression in the tumour cells and therefore enabled the use of anti-PD-L1 antibodies in order to prolong the survival of the mice [302]. Another team, Gallotta et al., used an agonist of Toll-like receptor 9 (TLR9) administered by inhalation to activate T-cell responses and complement the effect of PD-1 [303]. SD-101, a TLR9 agonist, induces the formation of tertiary lymphoid structures near the tumours and promotes the infiltration of CD8+ T cells into the tumours. These events improve the presentation of tumour antigens and balances the tumour microenvironment. Another study by Hervé et al. showed that aerosolization of a monoclonal antibody against vascular endothelial growth factor (VEGF) can inhibit tumour proliferation and angiogenesis in K-Ras adenocarcinomas [304]. Aerosol administration was compared with systemic injection and showed differences in pharmacokinetic properties. A low serum level of the monoclonal antibody was reported after the first aerosol administration suggesting low rate of passage into the bloodstream and minimal systemic side effects.

4.3.3. Gene therapy

Another strategy for treating lung cancer is based on gene therapy, an approach that directly targets the genes implicated into cancerous process. Various strategies are being employed. For example, the cellular receptors and ligands linkage is one approach to achieve successful gene therapy in lung cancers [305]. Luo et al. have developed a folate-decorated, hyperbranched polyspermine complex that suppresses lung tumorigenesis via aerosol administration by downregulating the Akt1 protein involved in tumour cell survival [306]. NPs can also contain hyaluronic acid to act specifically on the CD44 receptor. The latter regulates tumour development, growth, and metastasis. This strategy can be used to deliver siRNA via aerosol [307]. Since the COVID-19 vaccine, there has been a renewed interest in mRNA therapy because of the capability of the transfected NAs to synthesise any type of protein. In cancer treatment, suicide gene therapy is an interesting strategy that can be applied [308,309]. For example, Tang et al. have developed a NP containing p53 mRNA to induce apoptosis in cancer cells. The NPs was also constituted of G0-C14 a cationic lipid, DSPE-PEG-mannose and hyaluronic acid directly that target CD44 and the glucose receptor, two overexpressed receptors in lung cancers [310].

4.4. Benefits in other pathologies

The administration of aerosols could also be beneficial in other diseases, for example in the control of blood glucose. Considering diabetic patients, the glycemia is usually regulated by multiple insulin injections per day to prevent short- or long-term serious health problems induced by hypoglycaemia. Recently, the commercially available ultra-rapid inhalation insulin powder Afrezza® has brought real gains in quality of life and demonstrated the delivery of efficient doses [311,312]. In addition, oligonucleotide-based vaccines could be an effective strategy to deliver genetic content to boost insulin production in the long term. Another heterogeneous disease for which there are also no long-term therapies is asthma. Chronic inflammation of the airways is responsible for respiratory symptoms that can lead to chronic obstructive syndrome [313]. Some studies have shown that the BCG vaccine can prevent bronchial asthma by acting on IgE dysregulation [314,315]. Moody et al. compared the aerosolization of topical bronchodilators used for asthma treatment delivered by jet and mesh vibrating nebulisers but did not report any difference between the two devices [316]. As shown in Table 4, numerous clinical studies focus on aerosol delivery in different diseases (e.g., asthma, CF, alpha-1-antitrypsin deficiency).

5. Discussion, conclusion

Inhalation therapy is gaining more interest with the recent clinical successes of inhaled biological and NP-based therapies, enabled by advanced techniques and formulations as well as a better understanding of therapeutic targets. The diverse applications of aerosol delivery presented in this review highlight its relevance as a route of administration. Pulmonary inhalation ensures localized lung delivery while maintaining biological activity of the drug [10]. Water-soluble drugs are absorbed rapidly, bypassing hepatic first-pass metabolism, thereby reducing the required dose and minimizing systemic side effects [317]. However, this technique remains challenging due to preclinical development complexities and patient variability. To accurately evaluate drug delivery of inhaled therapies, it is essential to dispose of suitable in vitro experimental models that consider physiological parameters [135]. Organoid or chip-based platforms can be relevant models to mimic in vivo conditions before assessing aerosol delivery in animal [318]. The choice of device and formulations are key factors for efficient drug delivery to the pulmonary tract. Tools such as next-generation impactor, Aderson cascade impactor, and glass twin impinger can determine drug deposition in a specific lung region and are useful for preclinical screening of novel inhaled therapies [135].

The need to develop aerosol for new therapies remains crucial. Despite challenges, recent studies demonstrated successful aerosol protein delivery by improving NP formulations. For instance, insulin

Table 4
Inhaled drugs involved in clinical trials listed from clinicaltrials.gouv.*

Aerosolized drug & Device	CT number; Phase (status)	Pathology/ infection	Outcome (or results when specified)
Amphotericin B (liposomal form: Ambisome®)	NCT03656081; Phase III (ongoing)	Chronic pulmonary aspergillosis	Therapeutic efficacy and comparison of the therapeutic (clinical and radiological) of a six-month treatment Itraconazole and Nebulised Ambisome® Versus Treatment by Itraconazole Alone
13 cis retinoic acid and Itraconazole (metabolite)	NCT04577378; Phase II (completed)	COVID-19	Efficacy of combination therapy of Isotretinoin and some antifungal drugs
HH-120 (modified antibody)	NCT06039163; Phase I (completed)	COVID-19	Safety, tolerability, and preliminary antiviral effect of HH-120
Liposomal Cyclosporin A (peptide)	NCT00268515; Phase II (completed)	Lung transplant recipients with chronic rejection and bronchiolitis obliterans syndrome	Aerosolized cyclosporine did not improve the rate of acute rejection, but improve survival and extend periods of chronic rejection-free survival
Aerosol BCG* Challenge (live-attenuated bacteria)	NCT02709278; Phase I (completed)	Healthy individuals	Efficacy of the amount of BCG* recovered from the lungs the skin, following challenge by either the aerosol or the intradermal route
Interferon Gamma (protein)	NCT00021567; Phase II (completed)	*Mycobacterium avium complex* infection	Safety and effectiveness of inhaled interferon gamma-1b and oral antibiotics
MSCs-derived exosomes*	NCT04276987; Phase I (completed)	Severe patients hospitalized with novel coronavirus pneumonia	Safety and efficiency of aerosol inhalation of the exosomes derived from allogenic adipose mesenchymal stem cells
10e5 TCID50 (replication deficient adenovirus vectors)	NCT05094609; Phase I (recruiting)	Healthy individuals	Safety and efficacy by monitoring the immunogenicity of a single dose of Ad5-triCoV/Mac or ChAd-triCoV/Mac
AeroNeb Solo Ad5Ag85A/ ChadOx185A (Adenovirus-based TB Vaccine)	NCT02337270; NCT04121494; Phase I (completed)	Healthy individuals	Safety and Immunogenicity compared to IM* administration
α 1-antitrypsin (protein)	NCT04204252; Phase III (recruiting)	Alpha 1-Antitrypsin Deficiency	Safety and efficacy of the nebulisation by measuring alpha 1-Antitrypsin Deficiency antibody level, FEV*
ALX-009 (protein cocktail)	NCT02598999; Phase I (terminated)	CF and non-CF patients with Bronchiectasis	Safety, tolerability, and pharmacokinetics of a single/multiple ascending doses of Hypothiocyanite, bovine lactoferrin

(*continued on next page*)

Table 4 (*continued*)

Aerosolized drug & Device	CT number; Phase (status)	Pathology/ infection	Outcome (or results when specified)
			and their combination
CSJ117 (antibody fragment)	NCT04410523; Phase II (terminated)	Asthma	Safety and efficacy of multiple CSJ117 doses by evaluating the asthma symptom severity and the immunogenicity level
DAS181 (protein)	NCT03808922; Phase III (recruiting)	Lower Respiratory Tract Infection	Safety and efficacy of aerosolized DAS181 on pulmonary functions
Dornase alfa (protein)	NCT03368092; Phase III (recruiting)	Trauma/ Respiratory Distress Syndrome, Adult	Efficacy to reduce the incidence of moderate to severe ARDS in severe trauma patients during ICU* days
Molgramostim (peptide) eFlow	NCT03597347; Phase II (terminated)	Cystic Fibrosis with Nontuberculous Mycobacterium Infections	Efficacy of inhaled molgramostim
Sargramostim (peptide)	NCT04707664; Phase II/III (completed)	COVID-19	Safety and efficacy of Sargramostim to reduce the severity of the symptoms
Unfractionated heparin (protein)	NCT04545541; Phase II/III (recruiting)	COVID-19	Efficacy the mortality and time to extubation in Patients With COVID-19
PRS-060 (antibody)	NCT03574805; Phase I (completed)	Asthma	Investigate the safety, tolerability, and pharmacokinetics of multiple doses of PRS-060

* "BCG": Bacillus Calmette-Guérin, "MSCs": Mesenchymal stem/stromal cells,"IM": Intramuscular, "FEV": Forced expiratory volume, "ICU": Intensive care units.

inhalation is nowadays possible by using the ultra-rapid inhalation microparticles-based powder (Afrezza®), which have considerably increase the quality of life for diabetic patients [311,312]. In CF, there is a lack of development of inhalable protein modulators to reduce hepatic adverse effect. Recently Garbuzenko et al. demonstrated the potential of inhalable Ivacaftor (+/− lumacaftor) loaded in nanostructured PEGylated lipid carriers by using a jet Collison nebuliser to restore CFTR functions [171]. Co-spray-dried formulations of Ivacaftor-based microparticles incorporating leucine improve epithelial cell assimilation [170]. Antibodies are another type of protein particularly challenging to incorporate into aerosol formulations. Due to the rise of pandemic infectious lung diseases, such as SARS-CoV-2, aerosol administrated antibodies would achieve therapeutic equivalency to much higher doses than those delivered by parenteral routes [197,271].

Aerosol delivery also holds promise for advancing next-generation genetic-based therapies for monogenic diseases, respiratory infections, and lung cancers. Recent clinical trials for CF gene therapy demonstrated the interest of inhaled gene therapies using AAV vectors or mRNA therapies (NCT05248230, NCT05668741). This growing interest can also be used for lung cancer as the delivery of siRNA via aerosol have proved to be effective in lung cancer cells [307].

Finally, the development of NPs therapy allows fine-tuning of physicochemical parameters to improve mucus diffusion, cancer cells targeting, and resistance to shear forces during nebulisation [300,310,319]. These technologies support physical stresses during formulation processes (e.g., freezing, spraying, dehydration) and offer the opportunity to combine therapeutic drugs for multitargeting approaches. For example, arsonium-containing lipophosphoramides or N-heterocyclic carbene-silver complexes have shown encouraging gene delivery efficiency, while lipid-based nanocarriers remain active against *S. aureus* and *P. aeruginosa* in coculture models [189,190]. The development of new advanced NPs like "rocket vectors" could significantly benefit aerosol medications. These multimodal carriers would enable mucus penetration, antimicrobial activity, and efficient cargo delivery to epithelial cells, representing a promising approach for future therapies.

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CRediT authorship contribution statement

Rosy Ghanem: Writing – original draft, Visualization, Validation, Supervision, Conceptualization. **Raphaëlle Youf:** Writing – original draft, Validation, Supervision, Conceptualization. **Tanguy Haute:** Writing – original draft, Visualization, Conceptualization. **Xavier Buin:** Writing – review & editing, Validation. **Martijn Riool:** Writing – review & editing, Validation. **Jérémie Pourchez:** Writing – review & editing, Validation. **Tristan Montier:** Writing – review & editing, Visualization, Validation, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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