"Innovative preclinical models for pulmonary drug delivery research"

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Abstract

Introduction: Pulmonary drug delivery is a complex field of research combining physics which drive aerosol transport and deposition and biology which underpins efficacy and toxicity of inhaled drugs. A myriad of preclinical methods, ranging from *in-silico* to *in-vitro*, *ex-vivo* and *in-vivo,* can be implemented.

Areas covered: The present review covers *in-silico* mathematical and computational fluid dynamics modelization of aerosol deposition, cascade impactor technology to estimated drug delivery and deposition, advanced *in-vitro* cell culture methods and associated aerosol exposure, lung-on-chip technology, *ex-vivo* modeling, *in-vivo* inhaled drug delivery, lung imaging and longitudinal pharmacokinetic analysis.

Expert opinion: No single pre-clinical model can be advocated; all methods are fundamentally complementary and should be implemented based on benefits and drawbacks to answer specific scientific questions. The overall best scientific strategy depends, among others, on the product under investigations, inhalation device design, disease of interest, clinical patient population, previous knowledge. Pre-clinical testing is not to be separated from clinical evaluation, as small proof-of-concept clinical studies or conversely large scale clinical big data may inform pre-clinical testing. The extend of expertise required for such translational research is unlikely to be found in one single laboratory calling for the setup of multinational large-scale research consortiums.

Keywords: Nebulization, Aerosolization, Inhalation, Theoretical modelling, Cell culture techniques, Animal models

1. Introduction:

Pulmonary drug delivery is a field of intensive research to deliver drugs topically at their pulmonary site of action to treat the growing worldwide burden of pulmonary disease, but also for systemic targeted drugs. Albeit breathing puts the lung in direct contact with the atmosphere and thus with drugs to be inhaled, the complexity of the multiscale respiratory system makes scientific investigation very challenging. The complexity comprises vertical heterogeneity of a system made of several in series organs (mouth and nose, upper airways and lung) with different anatomic and histologic properties. The complexity also arises from operating at different scales reaching from macroscopic physiology such as breathing flow and rate, neurological and muscular command to microscopic biologic phenomenon such as cellular differentiation and cross-talk. Furthermore, the lung as an asymmetric branched system presents significant horizontal heterogeneity between lung regions particularly in case of lung disease. As patient to patient variability inherent to clinical research further complicates the scientific challenges, pre-clinical modeling has been extensively used to investigate, understand and predict drug transport, deposition, local tissue exposure and biological effects to optimize the translational research path. Pre-clinical modelling has gained tremendous refinement at all levels to better mimic clinical situations at the expense of experimental complexity and cost. The present review covers pre-clinical models of inhaled pulmonary aerosol drug delivery from *in silico*, to *in-vitro*, *ex-vivo* and *in-vivo* models to delineate the benefits and drawbacks of this increased complexity (Table 1).

2. Innovative mathematical and computational fluid dynamics modelization of aerosol deposition

Although several experimental techniques can be used to measure total and/or regional deposition of inhaled aerosols, mathematical models are often required to complement experimental studies under different exposure conditions. These models not only help interpret experimental data but also allow predictions to be made for cases where experimental data are not available. Furthermore, modeling can be used as a tool for interspecies dose extrapolation, an important element in preclinical studies.

Due to the complexities of the respiratory system, most early computational models of aerosol transport and deposition used a simplified representation of airway anatomy [1, 2]. Later models were based on a continuous description of aerosol transport in the lung [3, 4] where a one-dimensional (1D) convective-diffusive equation incorporating a term accounting for deposition was solved. These models have been successful in predicting

overall deposition averages but failed to accurately predict local deposition. This may be because the models use a single "typical" path representing the whole lung (or an individual lobe) in which deposition is computed. Deposition in each airway of the single path is then multiplied by the number of airways in each generation to provide an estimate of total deposition. Such an approach implies that deposition in each airway of a given generation is similar and does not account for any inhomogeneity in the branching pattern and/or subtended volume. As such, this type of models cannot incorporate heterogeneities in airway anatomy and tissue mechanics that are the hallmark of several lung diseases. The development of multiple-path models has partially addressed this limitation. One of the most widely used and best validated multiple path model is the "Multiple Path Particle Deposition" model [5]. The model uses semi-empirical relationships in the extrathoracic airway and solves flow and deposition in the lower respiratory tract made of cylindrical airways. This model provides not only total deposition but also lobar-specific and airway-specific information.

While 1D models have the advantage of being able to predict deposition throughout the entire lung, they lack the ability to describe site-specific deposition within individual airways or in specific locations in the lung. More recent approaches including computational fluid dynamics (CFD) models have taken advantage of the developments in automated reconstruction of lung airways from clinical lung imaging to create highly realistic lung models in which aerosol transport and deposition can be predicted [6-8]. CFD models use three-dimensional (3D) geometries in which the spatial distribution of deposited particles can be predicted using detailed governing flow and particle transport equations. These models, however, are more difficult to implement than 1D models, require extensive computing resources and thus typically only focus on a specific region of the lung [7, 9-13]. Thus, multiscale strategies have been developed to link different models that apply to different regions of the lung to obtain a realistic subject-specific picture of the fate of inhaled aerosols in the lungs. One strategy has been to integrate distal lung mechanics through coupling of the 3D CFD model of the upper airway and large conducting airways with 0D or 1D models at each outlet [8, 14]. 0D models are represented by sets of simple ordinary differential equations representing the compliant mechanics of the airways (Figure 1); 1D models can be represented by single or multiple path models. While these models are still in their infancy, promising preliminary results suggest that hybrid models can accurately predict site- and region-specific deposition of aerosol throughout the respiratory system. Such models can thus be an effective tool to explore and understand the connection between disease, diagnosis and inhaled therapy outcome.

Indeed, for a locally-acting inhaled medicine, a measure reflecting lung deposited dose or lung deposition pattern will be more predictive of therapeutic performance than delivered (or emitted) dose. Also, the latest computational approaches using subject-specific models can facilitate matching patient (morphometry, disease, …) to aerosol characteristics required for optimal regional drug targeting. Such strategies also hold important promises to address broad inter-subject variability studies to foster the development of clinically efficient strategies across large human patient populations [15]. Conversely, *in-silico* predictions may help interpret the outcome of clinical trials and experimental work by providing detailed information on the theoretical fate of inhaled aerosols. Often, clinical and experimental work indicate if a therapeutic strategy is effective or not but rarely comprehensively investigate why and how it could be improved in case of inefficacy.

3. Innovative *in-vitro* models

Various complementary *in-vitro* models cover the whole spectrum from macroscopic drug inhalation and delivery to microscopic deposition and biological effects. They enable to bridge the knowledge from *in silico* calculations to *in-vivo* experimentation.

3.1 Anatomical models/impactor technology

The existing *in-vitro* test methods for inhaled drug aerosols in the pharmacopeial compendia [16, 17], are based on the multi-stage cascade impactor, because the mass of active drug can be determined by chemical assay on each impactor stage (each stage corresponding to a given particle size), enabling computation of the aerodynamic particle size distribution which in turn, is predictive of likely deposition in the respiratory tract [18]. Although robust and simple [19], this method does not enable the multitude of factors associated with patient use to be investigated [20]. Two simple changes have been proposed to make the measurements more pertinent to support clinical data [21]:

(1) Replace the original induction port with an inlet more representative of the oropharynx;

(2) Operate the impactor at constant flow rate throughout the measurement whilst allowing the inhaler to experience clinically relevant inhalation waveforms.

There are many choices of anatomic inlet to consider:

(1) anatomically correct oro- or naso-pharynx, based on casts made from cadaver airways [22];

(2) bespoke oro- or naso-pharyngeal inlets developed from imaging of individual living patient airways, in polymeric materials, by individual research groups;

(3) anatomically accurate standardized inlets representing small, medium and large airways averaged from imaging of several living adults developed either by the Oropharyngeal (O-P) Consortium (Emmace Consulting AB, Lund, Sweden, www.emace.se) [23] or those modeled by the research group at Virginia Commonwealth University (VCU) (Respiratory Drug Delivery, Richmond, VA, United States, www.rddonline.com) [24-26];

(4) the 'Alberta'-series of idealized inlets, based on CFD modelling of flow characteristics based on several living individuals in a particular age class (infant, small child, adult) (Copley Scientific Ltd., Nottingham, United Kingdom, www.copleyscientific.com) [27-29].

Cadaver-cast prepared inlets tend to collapse *post-mortem*, resulting in unrealistic *in-vivo* flow characteristics [30]. At present, commercially available standardized inlets, in general, formed from rigid polymeric materials, are available only for adults, whereas the idealized 'Alberta' inlets have been produced and validated for infant, small child and adult models. Aerosol particle size distributions measured for both pressurized metered dose inhalers and dry powder inhaler with those idealized inlets are significantly shifted to finer sizes. This suggests that the original compendial inlet underestimates the deposition of larger particles [31, 32].

Anatomic inlets are often used without further attempts to reproduce facial geometry, which is satisfactory when the patient interface of the inhaler is a mouthpiece as in most adult situations. When the inhaler-on-test has a facemask as in most pediatric situations, it is highly desirable to incorporate the inlet into an age-appropriate facial model [19]. Small leakage pathways between the facemask and face can greatly reduce the delivery of medication, particularly where a metered dose inhaler is used in conjunction with a spacer or valved holding chamber [33]. Attention should be paid to the realization of the soft tissues of the face model, as the force applied to the face by application of the facemask can affect both leakage and the internal dead space within the facemask [34]. Infants are generally nose- rather than oral-breathers [35], so that a suitable nasopharyngeal inlet is needed.

The cascade impactor is designed to operate at a constant flow rate throughout the experiment [36], but compendial methods for testing dry powder inhalers in order to mimic an inhalation maneuver, apply vacuum to the impactor to start the measurement, and the transition from zero flow to the target value can take several hundred milliseconds [37]. The Nephele mixing inlet [38] avoids the potential for bias associated with non-

steady state flow through the impactor. The mixing inlet is located between the inhaler and impactor (Figure 2). It has tapered surfaces of the inner tube containing the aerosol stream from the inhaler at the gradual merge with the make-up air for the impactor that avoids particle losses to internal surfaces of the mixing inlet due to turbulence. The inhaler aerosol particle size measurement takes place almost simultaneously with the aerosol generation process as the inhaler is actuated [39]. A further refinement is to operate the inhaler with a patient-generated or standardized inhalation flow profile (Figure 2). Olsson *et al.* have used this inlet to achieve remarkably good *in-vitro in-vivo* correlations [40]. One may further refine such models using disease specific flow profiles.

3.2 Advanced lung cell models

Whereas *in-vitro* cascade impactor experiments directly estimate the dose delivered to the patients, aerosol particle size measurement also enable more advanced lung deposition calculations based on *in silico* modelling of particle size and inhalation maneuver driven particle behavior in the lung. However, it completely lacks modelization of biological phenomenon. Cell culture experiments represent a necessary complement in this regard.

3.2.1 Advanced cell cultures

Although respiratory tract epithelia originate from only one anlage, the structure-functional characteristics, architecture and cell-types change significantly from the upper to the lower compartment (Figure 3) [41]. Therefore, defining the lung region relevant for the investigated aerosol as well as the endpoint of interest to implement the optimal cell model is crucial. Many human lung cell culture models have been introduced during the past years, varying from nasal/trachea, bronchial to alveolar barrier cultures, from 2D monolayer cultures to more advanced 3D co-cultures with the aim to provide further insight into cellular communication, cellular responses at a mechanical level or interaction of aerosols, *e.g.* drugs or particles, with cells [42-44].

The pseudostratified epithelium of the conducting airways is usually presented by human primary cultures of nasal, tracheal and bronchial epithelial cells which can be derived by nasal brushings or biopsies [45, 46]. When the cells are grown under optimal conditions, which include transition from standard submerged to air-liquid interface culture conditions, they retain important properties of differentiated airway epithelial cells such as polarized monolayers with extensive tight junction belts and ciliated epithelial cells [46-48]. The advantage of primary cells is not only the typical *in-situ* phenotype but the cultures can be used for long-term

experiments (chronic aerosol exposures) over several weeks to months, and also offer the possibility to use cells from different pathologies such as from patients with asthma [49] or chronic obstructive pulmonary disease [50]. In addition, in many studies bronchial cell lines, albeit not as close to *in-vivo* physiology but easier to culture, such as BEAS-2B, Calu-3 and the 16HBE14o- are used. These cells differentiate into cell monolayers with a cuboidal shape and for Calu-3 and 16HBE14o- cells tight junctions have been reported [47, 51, 52].

The alveolar region is, up to now, more difficult to mimic with cell models. The epithelium in the lung parenchyma is extremely thin and the alveoli are lined by squamous cells, the alveolar type I epithelial cells which cover about 95% of the surface and share a basement membrane with the endothelial cells covering the pulmonary capillaries, and also contain alveolar type II epithelial cells, which secrete lung surfactant to prevent alveolar collapse [41, 53]. Alveolar epithelial type II cells isolated from normal human lung tissue undergo morphological and histochemical changes, differentiating from type II to type I like cells [54] and monolayers with high trans-epithelial electrical resistance (>1000 Ωcm^2) can be generated [54, 55]. However, access to these tissue biopsies is more difficult and reproducibility of the cultures is challenging. Therefore, the cell line A549, which originates from human lung carcinoma [56], belongs to the better characterized and most widely used *in-vitro* alveolar lung models [57]. It has been shown that A549 cells have many important biological properties of alveolar epithelial type II cells (*e.g.* membrane-bound inclusions), which resemble lamellar bodies of type II cells [58] and they can release surfactant [59]. Most recently, two research groups reported the immortalization of human type II cells with type I like phenotype characteristics [60, 61] and this development will hopefully help to design more realistic human alveolar tissue models in the future.

The possibility to culture lung epithelial cells at the air-liquid interface simulates the *in-situ* lung tissue even closer, as the cells can be exposed to air environment from apical side, while fed with nutrients from the basal side [62]. Recently, air-liquid interface cell cultures on elastic membranes have been exposed to cyclic stretch mimicking even more closely the biophysical conditions in a breathing lung than static cell cultures on standard transwell inserts. In addition to the air-liquid interface techniques the possibility to culture different cell types together is important since cells continuously crosstalk *in-vivo* through intercellular signaling to maintain homeostasis and to coordinate immune responses [63]. Multi-cellular systems to simulate the human alveolar-capillary barrier by culturing human pulmonary microvascular endothelial cells, and primary isolated

human type II alveolar epithelial cells on opposite sides of a permeable membrane support, have been established [64, 65]. Other systems have described co-cultures of epithelial and immune cells, *i.e.* macrophages and dendritic cells [66], mast cells [67, 68], fibroblasts [69] or natural killer cells [70]. Lung organoids may represent an interesting model in the future to study such multicellular complex 3D interactions, however aerosol drug delivery is not yet foreseeable for such models [71]. The air-liquid interface culture technique offers the opportunity to be used together with aerosol delivery systems allowing relevant investigation of aerosol delivery on the lung cell surface [72, 73]. Different studies in the literature report about the comparison of lung cell responses under submerged or ALI conditions. For instance, A549 cells cultured at ALI express more inflammatory mediators upon exposure to zinc oxide (ZnO) compared to submerged conditions [74], whereas for silica (SiO2) nanoparticles inflammatory response was less pronounced at ALI [75]. Another study showed faster uptake kinetic for aerosolized Bortezomib, a proteasome inhibitor for inhalation therapy, as for the drug dissolved in cell culture medium [76]. A proteomics investigation of a co-culture composed of epithelial cells (A549 cell line), macrophages (differentiated THP-1 cell line) and lung fibroblasts (MRC-5 cell line) showed that the model exposed at ALI express significantly higher amount of proteins (most enriched pathways were oxidative stress and acute phase response pathways) compared to submerged conditions independently on exposed materials (i.e. negative control, or carbon nanotubes) [77]. Similarly, a co-culture of A549 and THP-1 cells showed higher response to poorly soluble nanomaterials (TiO2 and CeO2) when exposed at the ALI compared to submerged exposure highlighting also the importance of considering the deposition rates when comparing ALI to submerged exposure [78]. To conclude it is important to carefully consider the exposure conditions when comparing results from in vitro studies.

3.2.2 Aerosol delivery to cell cultures

Many of the currently available aerosol-cell delivery systems suffer from spatially non-uniform aerosol deposition or insufficient levels of delivery (or dose) efficiency (ratio of cell-delivered to minimal invested dose) or dose rate for preclinical drug testing [73]. The following commercially available devices have a track record in preclinical drug testing. The "*Vitrocell-Cloud*" system (VITROCELL Systems, Waldkirch, Germany) is an easy-to-use, one-button system, which employs a clinically relevant vibrating mesh nebulizer to deposit a dense cloud of liquid (~100 g/m^3) onto standard transwell inserts for air-liquid cell cultures. With

an exposure time below 5 minutes and a dose efficiency of up to 20%, the system provides delivery rates of about 0.2 µl/cm^2/min [79, 80]. For dry powder formulations, the "*PreciseInhale*" system equipped with the so called "*DustGun"* utilizes a focused high-pressure air pulse to disperse a small amount of powder (~200-5000 µg) into a 300 mL holding chamber from which powder aerosol is delivered via a defined air flow to exposure systems for either *in-vitro* cell cultures (or cell-free dissolution in lung lining fluid), *ex-vivo* or *in-vivo* models (see below) [81-83]. High aerosol concentrations (~g/m^3) and slow-settling particles (less than 5 µm diameter) favor high dose efficiency and delivery rate. However, delivery is typically 1-20% depending on operational parameters and characteristics of the specific exposure system (P Gerde, personal communication, Inhalation Sciences 2019). For real-time dose control these cell exposure systems can be equipped with a quartz crystal microbalance [79, 84].

Assessing the solubility/dissolution and interactions with cells of inhaled drugs after they deposit into the respiratory tract may be important during pre-clinical evaluation as these processes can influence pharmacokinetics, pharmacodynamics. Several issues have to be considered: the clinical and biological relevance of the substitute used to mimic the respiratory tract lining fluid and the methods to collect the aerosol and measure drug release and interaction with cells. Aside of using chemical surfactant, phospholipid-containing fluids and lung surfactant preparations, recent advances led to the development of a synthetic simulated lung fluid which displays similar physico-chemical properties (e.g. pH, conductivity, viscosity and surface tension), as the respiratory tract lining fluids and demonstrated biocompatibility with A549 lung epithelial cells. The relevance of such developments was investigated measuring as similar dissolution rate of inhaled fluticasone propionate as compared to the use of lung surfactant preparations [85]. Whereas 2D cell culture models and aerosol exposure systems enable extensive biological evaluation of drug efficacy and toxicity they are limited in complexity with respect to precisely mimic the distal lung where 3D anatomical factors combine with complex multicellular biological interactions, cyclic fluid flow and tissue strain in a complex environment. The challenge to target and investigate drug delivery to this specific micro-environment requires bioengineering input to create relevant comprehensive 3D models.

3.2.3 Lung-on-chip and microfluidic models

Following the seminal model of Huh *et al.* nearly a decade ago [86], the field of *lung-on-chips* has witnessed a dramatic surge in the number of designs of microfluidic *in-vitro* platforms that strive to mimic more closely

the human pulmonary environment [87]. Such efforts have been motivated by the need to move beyond the limitations of traditional cell culture and concurrently tackle the limitations of *in-vivo* animal models for clinical relevance [88]; a point that has been most recently highlighted in a seminal review emanating from a consortium of leading pharmaceutical players in the R&D sector [89]. In parallel, a number of comprehensive reviews [87, 90-92] have extensively discussed the bioengineering efforts at hand to realize such *in-vitro* lung models, spanning the microfabrication processes involved (*e.g.* photolithography, etching techniques, etc.) to the challenges of integrating lung cell cultures with porous membranes (*e.g.* primary cells, co-/triple- cultures, etc.).

The appeal of *lung-on-chip* platforms revolves around state-of-the-art bioengineering strategies to integrate broad features spanning anatomical mimicry at true scale (*e.g.* branching tree structures, alveolated airways, etc.), respiratory breathing motion and ensuing tissue strains [93] (*e.g.* elastic membranes), in conjunction with physiological respiratory airflows along with continuous nutrient perfusion that translates into mechanosensory shear stress-driven cues. As such, these *in-vitro* systems are offering a tangible path towards the most realistic *in situ*-like inhalation assays to date mimicking spatially non-uniform local aerosol deposition and associated biological outcomes with particular emphasis on hot spot regions of aerosol deposition [94]. These advanced *in-vitro* inhalation assays are for example suited to explore the role of carrier design (*e.g.* particle size, shape, etc.), inhalation maneuvers and therapeutic compound (*e.g.* concentration, composition, formulation) on biological endpoints including cytokine secretion, viability, gene expression, etc. as well as lung tissue barrier properties (*e.g.* permeability, electrical resistance, etc.). Moreover, they offer unprecedented biological read-outs as exemplified amongst other in monitoring the stiffening of an elastic membrane during airway epithelial formation [95]. Due to their complex characteristics and functions, the design, handling and robustness of such *lung-on-chips* represent concrete challenges that must be overcome such that end users are encouraged to opt for such complex tools [96]. The upcoming years will demonstrate whether microfluidic lung models will constitute a new gold standard for *in-vitro* models in pulmonary pharmaceutical research.

4. Innovative *ex-vivo* models:

In order to capitalize on advantages of controlled *in-vitro* experimental settings yet incorporating *in-vivo* like anatomical relevance of the bronchial tree, an innovative *ex-vivo* chimeric model has been developed. Such a

model may achieve very high multiscale anatomic relevance without the complexity of lung on chip bioengineering requirements for being set up, but it still relies on animal and not human tissue. The model comprises a realistic upper airway human cadaver based plastinated and/or 3D printed inlet attached to a porcine *ex-vivo* lung [97]. Porcine lungs are placed in a hermetic box simulating the thorax and ventilated though negative pleural pressure simulation using a pump. Ventilation scintigraphy studies showed a relevant ventilation pattern adequately mimicking human ventilation which makes this model very interesting to investigated regional lung deposition of inhaled aerosols [98]. A similar pediatric model has also been developed using *ex-vivo* rabbit lungs [99]. Beyond their novelty precluding extensive validation studies which will need to be carried out, main limitations of those models are represented by the lack of perfusion of the lung which therefore has a very limited life span with major cellular and histological processes going on over the experimentation period which effects need to be investigated more throughout fully.

5. Innovative *in-vivo* models:

To date, preclinical evaluation in animal models is mandatory for regulatory approval of novel drugs, repurposed drugs for inhalation and excipients, which were not previously delivered through this route. For instance, animal models are crucial to evaluate the pharmacokinetics and toxicity of inhaled drugs. Non-human primate, sheep and pig models are most similar to human lungs, but the most widely used models for drug testing are rats for preclinical toxicity assessment and mice for pathway-specific understanding of pathomechanisms and identification of therapeutic targets due to the wide selection of genetically modified mouse strains (*e.g.* knock-out and knock-in models) [100]. In regulatory pharmacological studies, several species have been used as surrogate models to mimic features of human respiratory diseases for pharmacodynamics: guinea-pigs for airways inflammation and bronchial hyperresponsiveness, preterm lambs/rabbit for surfactant deficiency and ferrets for viral lung infections. Regulatory toxicity or toxico-kinetics usually requires both a rodent and non-rodent animal model. Interestingly, it is often the rat and the dog that are used for inhaled drugs. As reviewed elsewhere [101], animal models display distinct inter-species anatomical characteristics and respiratory parameters that clearly matter for pulmonary drug deposition and the delivery methods in animals often poorly replicate the drug distribution encountered in humans. Furthermore, airway geometry, lung mechanics and thus gas flow rates and velocities are greatly influenced by respiratory disease. In addition to aerosol aerodynamical properties, inhaled drug deposition

depends on respiratory parameters and airways anatomy, which are subjected to inter-individual differences and can be modified due to respiratory diseases [102]. However, those changes are very difficult to implement in animal models relevant for human respiratory disease. In vitro models are easier to modify in order to mimic respiratory disease and potentially more predictive [103].

The present review focuses on techniques used to deliver inhaled drugs to animal models and evaluate drug deposition and pharmacokinetics.

5.1 Methods of pulmonary drug delivery to animal models

Various methods for pulmonary drug delivery in animal models are available for both liquid and dry powder formulations. Liquids can be given as bulk liquid or aerosols, while dry powder can only be applied in aerosolized form.

5.1.1 Liquid formulations

Bulk liquid application without aerosolization is the most widely used experimental method mainly due to ease-of-use, delivery efficiency, and dose control. Liquid may be delivered through intranasal or oropharyngeal aspiration as well as through intratracheal instillation. For intranasal aspiration a drop of liquid is pipetted onto the nostril of an animal [99]; with the next breath the liquid is sucked into the nasal cavity where it turns into a spray which is transported via the air flow into the lungs [99]. Similarly, for oropharyngeal aspiration a drop of liquid is pipetted into the back of the pharynx or the glottis from where it is sucked into the trachea. For intratracheal instillation, animals are orotracheally intubated, a liquid-containing syringe is connected to the intubation cannula and the bulk liquid is squirted directly into the trachea.

Alternatively, the “Microsprayer” technology (Penn-Century, United States) allows for orotracheal release of drugs as a spray directly into the trachea. Intra-tracheal spray can be considered an intermediate method between bulk application and aerosol inhalation, since the liquid is aerosolized, but not inhaled (only squirted into the lung) since most of the droplets are too large to be inhalable (~20-100 µm). Whereas in rodents, a somewhat more uniform drug distribution than standard bulk liquid application methods [104] has been observed, in larger animals drug distribution appears very heterogeneous compared to aerosolization [105]. Albeit still widely used, this aerosol delivery technology is not commercially available anymore, apparently resembling devices available on the market may in fact not implement the same high-level technology and will require validation. All non-aerosol inhalation methods (including sprayer technology) suffer from non-

clinically relevant pulmonary drug distribution and potential, transient and localized disruption of homeostasis due to the relatively large amount of liquid delivered mainly to central regions of the lung (see below; Figure 4).

Various aerosolization techniques may be used in animals. Typically, in small animals (rodents), aerosolized delivery relies on nose-only aerosol inhalation where each animal is placed in a restrainer chamber designed to expose only the nose of the animal to a continuous flow of aerosol-laden air. Although this method is more dose efficient than whole body aerosol exposure, its dose efficiency of <<1% (often <0.1%) is still too low for expensive experimental drugs [106]. Low pulmonary dose efficiency is mainly due to substantial, inadvertent exposure of the nasal mucosa by whole-body and nose-only aerosol inhalation (*e.g.* for rodents often >90% of the inhaled dose is deposited in the nose) and subsequent drug transport into the gastrointestinal tract as a secondary exposure route may limit data interpretation [106]. Hence, these methods are prohibitive for most preclinical drug efficacy studies. Consequently, two other methods are typically used for pulmonary drug delivery via aerosol inhalation. For larger animals (rabbits or larger), aerosol inhalation is feasible using facemasks covering nose and mouth [101, 107]. Alternatively, small animals (rat, rabbit) and large animals (non-human primates, piglets) can be intubated and connected to a mechanical ventilator for pulmonary delivery of aerosolized drug via a clinically relevant nebulizer. These methods provide dose efficiencies up to 30%.

In light of the prominent role of inhalation therapy in clinical settings it is intuitively evident that preclinical inhalation studies are likely to be more predictive for clinical outcome than bulk liquid applications especially for drugs targeted to the peripheral alveoli. While this has been demonstrated for plasmid DNA-mediated gene delivery and for prevention of ricin-induced pulmonary legions in mice [108], there is also conflicting evidence for e.g. virus activity [109], which is likely due to the dependence of aerosolized drug efficacy on numerous factors including (partial) degradation of drugs during the nebulization process, additional therapeutic or toxic effects due to delivery of large dose fractions to non-pulmonary sites (nasal and gastrointestinal deposition for nose-only, whole-body inhalation) and the lack of exact determination of the lung deposited dose as biologically relevant dose metric [80, 106].

5.1.2 Dry powder formulations

Aerosolization of dry powders is often technically more challenging than nebulization of liquids, since the dispersion energy of dry powders depends on numerous parameters including particle size, type of drug, electrostatic charge and humidity conditions. Thus, dry powder application typically requires conduction of pre-experiments for optimized drug delivery. Amongst the most widely used dry powder delivery devices are the “Insufflator” (Penn-Century, United States) [110], which is the powder analogon of the “Microsprayer” for liquids (caveat: it is also not commercially available anymore) and the “*DustGun*” implemented in the “*PreciseInhale*” system (Inhalation Sciences, Huddinge, Sweden). Both methods utilize a single high-pressure air pulse for powder dispersion, but while the “*Insufflator*” delivers the aerosol directly into the trachea the “*PreciseInhale*” fills a holding chamber with aerosol from which aerosol-laden air is drawn to an aerosol inhalation system for animals (nose-only or intubated ventilated inhalation setup) [81]. The “Insufflator” requires pre-experimental determination of a minimum threshold dose (about 2 mg depending on the powder) to enable efficient implementation. Pre-experimentation for dose optimization is less of an issue for the “*PreciseInhale-DustGun*” system, since it relies on an optimized, multi orifice dispersion system, which focuses a short high-pressure air pulse onto a small amount of powder followed by rapid aerosol decompression in a small orifice. When performing efficacy studies with dry powder formulation only the dissolved dose fraction is biologically active. While this issue is well recognized, there are currently no regulatory accepted methods to determine the dissolution fraction and rate [83].

5.1.3 Dose efficiency and guidance on selection of delivery methods

The choice is based on the type of drug, disease of interest, animal model and performance characteristics of the drug delivery method. The latter includes drug delivery efficiency (material consumption/cost), delivery rate (duration of exposure, personnel cost), reproducibility/accuracy of delivered dose (determines number of animals required), uniformity of drug distribution in the lung (clinical relevance) as well as ease-of-use and degree of invasiveness (animal welfare).

From a clinical perspective, aerosol inhalation is the most relevant delivery method, since it most closely resembles the drug delivery characteristics associated with clinical inhalation therapy. Aerosol inhalation has been shown to potentially affect the bioactivity of drugs and toxins [111, 112]. For efficacy testing of experimental drugs aerosol inhalation techniques with dose efficiencies < 1% are not cost efficient, which excludes whole-body and nose-only inhalation systems. Aerosol inhalation via face masks among larger

animals (*e.g.* non-human primates) provides drug delivery efficiencies of around 1-5%, which can be improved to about 13% ± 7% by adapting the device and interface [107]. Hence, these methods are prohibitive for most preclinical drug efficacy studies. Animal intubation and mechanical ventilations techniques yield pulmonary delivery efficiencies of 5-10 % for small animals [113, 114] and values up to 30% for larger animals [98] at inter-subject dose variabilities of about 30% (comparable to aspiration) [113]. Nevertheless, the more complex aerosol and animal handling procedures, make aerosol inhalation less attractive than bulk delivery methods.

The disadvantages of bulk delivery methods include potential disruption of lung homeostasis due to delivery of a relatively large volume of liquid and the preferentially central, patchy drug deposition profile (see blow deposition imaging and Figure 4), which may adversely affect the bioactivity of the drug. On the other hand, these negative aspects are often outweighed by the technical simplicity of bulk liquid applications and high degree of dose control, dose efficiency and delivery rate. For instance, intranasal and oropharyngeal aspiration allow for 10-40% and 30-70% delivery efficiency, respectively, an extremely high delivery rate (entire dose is delivered within ~1s) and moderate inter-subject dose variability (~30%). Intratracheal instillation allows for even better delivery efficiency (70-90%) and reduced inter-subject dose variability (~15%) at an identical delivery rate (~1s) [115], which explains its wide-spread use in preclinical drug testing especially for more costly drugs. However, the more complicated and more invasive animal handling procedure (intratracheal intubation) makes it less attractive for repeated dosing.

Dry powder applications are relatively rare in preclinical studies mainly due to the high variability of drug dispersibility - even for the very same powder, but even more so for different powders - due to dependence of the dispersion energy on numerous in part poorly controlled parameters (see above). For the "*Insufflator*", 50-90% dose efficiency of largely non-respirable aerosol has been reported, if the device is filled with a sufficiently large powder volume. The "*PreciseInhale*" system provides dose efficiencies when utilized for intratracheal inhalation with respirable aerosols of about 1%, but with a substantially reduced tracheal deposition (<0.5% of total deposited dose) as compared to intratracheal insufflation with the "Insufflator" (~20-70%) [81, 110].

5.2 Advanced methods for quantification of drug dose and distribution in the lungs

Among the key criteria for selection of the most suitable drug delivery method are dose efficiency and pulmonary distribution of the drug in the lung. The latter is not only relevant for highly localized diseases (*e.g.* lung cancer), but also for the bioactivity of the drug or a toxin, which is often improved for aerosol inhalation due to its highly uniform distribution profile [111]. Moreover, for pharmacokinetic studies, which are mandatory for regulatory approval of novel drugs in order to characterize the concentration and fate of inhaled drugs and guide clinicians to select the appropriate dose and regimen for clinical trials, even longitudinal (time-resolved) dosimetry is required.

Pulmonary drug dose is often measured in bronchoalveolar lavage samples, biopsies or homogenized lungs using mass spectrometric or radio-/fluorometric analysis [106, 115]. Since these methods are either terminal and/or usually provide information for only one time point per animal, they are time consuming and ethically controversial (animal-consuming). Various lung imaging techniques as well as *in-vivo* microdialysis may overcome some of those limitations.

5.2.1 Lung imaging of pulmonary drug delivery

Imaging technologies are widely used for monitoring of the spatial distribution of drugs applied to the lung. For animal models planar gamma scintigraphy, single-photon-emission computed tomography (SPECT), and positron emission tomography (PET) have been widely used for both 2D and 3D profiles of the lung deposited drug dose. Those methods, which require a radiolabeled drug formulation or a radiation source, typically provide a spatial resolution of 1-10 mm, which makes them not only useful for the clinical setting, but also for animal models (*e.g.* rats, dogs, rabbits, pigs, and non-human primates). For instance, SPECT revealed substantial age-dependent differences in the pulmonary distribution of ^{195}Au (gold) nanoparticles in the lungs of rats after intratracheal inhalation [116]. For spatial resolution down to sub-cellular levels various modes of electron and fluorescence microscopy have been introduced (*e.g.* scanning electron microscopy or confocal fluorescence microscopy). However, these methods are typically limited to small *ex-vivo* sections/slices of the lung. Multi-modal imaging platforms combining macro-, meso- and microscale *in-vivo* and *ex-vivo* imaging techniques have been described to provide synergistic insight into the dynamics of pulmonary drug delivery, drug distribution and bioactivity of the drug [117]. For instance, combination of *in-vivo* propagation-based, phase contrast x-ray imaging with *ex-vivo* light sheet fluorescence microscopy on optically cleared lungs revealed the mechanism of drug distribution for bulk liquid applications [117]. Bulk liquid application delivers

drugs preferentially to the central parts of the lung (conducting airways) and in a patchy, spatially not uniform pattern, while aerosol inhalation provides uniform drug deposition throughout the entire lung even down into the deepest parts of the lung: Figure 4 [113, 117]. Imaging methods are not usually used for pharmacokinetic studies as they do not provide direct quantification of the drug in the lung and in the systemic compartment.

5.2.2 Microdialysis

To overcome limitations of animal-consuming, ethically controversial classical quantification techniques usually giving information for one time point only per animal (broncho-alveolar lavage, biopsies, lung homogenates) *in-vivo* lung microdialysis was set-up in different animal models to quantify dynamically inhaled drugs in the lung interstitium [118-120]. Lung microdialysis is a semi-invasive method that enables the continuous and repetitive sampling of unbound inhaled drugs in a single animal. The principle of microdialysis is based on the passive diffusion of inhaled drugs through a semipermeable membrane included in a probe surgically inserted in the lungs and perfused at a constant flow rate with a physiological buffer (perfusate). The diffusion of inhaled drugs across the membrane relies on their concentration difference between the interstitial lung fluid and the perfusate. Quantitative lung microdialysis depends on the reliable measurement of the drugs throughout the experiment and thus, the ability to follow *in-vivo* the recovery rate of the probe. As a general point of view, the probe recovery should exceed 20% to accurately estimate drug concentration [121]. Retrodialysis is the most popular method to determine the probe recovery rate, assuming that the passive diffusion through the semi-permeable membrane occurs similarly in both directions. *In-vivo*, retrodialysis [120] can be applied simultaneously to microdialysis, using a suitable calibrator added in the perfusate and quantifying its loss over time. Interestingly, lung microdialysis was implemented with success to inhaled small molecule drugs, such as antibiotics to evaluate the pharmacokinetic/pharmacodynamic relationship, as well as for macromolecules [118-120], which may not distribute linearly in the different compartments, thereby limiting extrapolation of indirect estimations based on systemic measurements.

6. Conclusion:

Important improvements have occurred in all fields of pre-clinical modelling of pulmonary drug delivery. Implementing most recent hybrid multiscale *in-silico* modeling, combined with improved cascade impactor technology may enable to precisely predict drug delivery and deposition which may then be evaluated through complementary *ex-vivo* experiments. Those exposure data can be used for advanced cell model drug exposure

experiments to investigate precisely biological effects considering the biological complexity of the multi-cellular air-liquid interfaced lung tissue as well as physical cyclic shear stress and strain. Last, various *in-vivo* inhaled drug delivery methods enable to test drug delivery in conditions relatively close to clinical practice in various animal models with an increase in the number of relevant readouts to assess deposition, pharmacokinetics and biological effects. The potential benefits and optimal implementation strategies of those complex tools will progressively emerge through their more and more frequent and complementary use by research teams around the world.

7. Expert opinion

The primary objective of the use of pre-clinical models to evaluate pulmonary drug delivery is to gain sufficient amounts of high-quality information in order to make optimal choices regarding modalities of human testing (clinical studies), which remains unavoidable. As presented in this review, complexity and refinement of models have tremendously increased in the past years with numerous innovations on all aspects of pre-clinical testing. Inhaled pulmonary drug delivery shares with few other research fields in health science the unique combination of physics primarily governing aerosol transport and deposition with biology underpinning drug effects. Furthermore, the phenomena under investigations are multiscale, reaching from macroscopic whole-body to microscopic sub-cellular phenomena. Of note, the numerous different respiratory diseases which all induce different specific changes to the respiratory system and thus to drug delivery adds a supplementary level of complexity if one aims to implement an efficient pre-clinical research path for a specific disease.

The challenge for successful pre-clinical investigation remains to choose the adequate models to answer each specific scientific question. *In-silico, in-vitro, ex-vivo* and *in-vivo* models are fundamentally complementary, however the increased number of models and complexity as well as possibilities in terms of readouts may leave the researcher puzzled as to the best strategy to implement. There is a risk of excessive model refinement and associated cost losing sight of the clinical objectives, but conversely improvement in models were driven by genuine attempts to achieve better predictive power of pre-clinical studies for clinical outcome. Unfortunately, there is no single pre-clinical model to be advocated for translational pulmonary drug delivery research. Depending on the product under investigation, formulation issues, carrier, inhalation device design, disease of interest, targeted clinical population, previous knowledge on similar products through other delivery

routes, etc… all combinations and time scheduling of *in-silico, in-vitro, ex-vivo* and *in-vivo* experimentation may be scientifically sound. Dogmatic classical views such as experimenting from *in-silico* to *in-vivo* as a prerequisite for clinical testing or following the particle path from macroscopy and inhaler performance to microscopy and biological effects are of limited relevance. Small proof-of-concept clinical experiments are also frequently required to validate *in-silico* or experimental work before moving towards larger scale clinical trials. Indeed, scientific knowledge on pulmonary drug delivery rather emerged from trial and error repetition with all types of pre-clinical models and cross-validation of various models. In all cases of translational research toward clinical application of pulmonary drug delivery, the researcher will aim at getting relevant information to estimate drug transport and deposition which will determine drug exposure and to put this exposure in perspective with biological effects to be expected. Thus, a fundamentally multidisciplinary approach is required to adequately tackle the research pathway with expertise among physics, mathematics, engineering, biology, chemistry, veterinary and human medicine. Actually, given the high complexity and variety of pre-clinical evaluation methods as detailed in this review, it becomes challenging to bring together the relevant know how within a single research laboratory which renders collaboration at national and international levels as well as between academic and industrial partners mandatory at the price of more complex large scale project management. Such multidisciplinary experimental research is not to be considered as pre-clinical *per se* as it may give very valuable insights analyzing clinical data to explain failures and/or further build on clinical trials success, thus realizing post-clinical experimental research. The current development of anonymized large-scale clinical databases and the associated information technology based big data analysis may close the loop of translational research realizing post-clinical *in-silico* research.

Acknowledgement:

The authors sincerely thank Mark Nagel and Jason Suggett from Trudell Medical (London, Ontario, Canada) for sharing their expertise from the development of upper airway models. Otmar Schmid was partially funded by the German Federal Ministry of Education and Research (BMBF: FKZ 13XP5069A) and the Proof-of-Concept Initiative of the Helmholtz Association of German Research Centres and the Fraunhofer Gesellschaft (PoC-0033). Chantal Darquenne's contribution was supported by the National Institute of Environmental Health Sciences (NIEHS) of the National Institutes of Health under Award Number U01ES028669. Barbara Rothen-Rutishauser and Hana Barosova acknowledge the support of the Adolphe Merkle Foundation. The

authors thank the ERA-NET EuroNanoMed III program (joint transnational call 2017 for “European innovative research & technological development projects in nanomedicine” program: Agence National pour La Recherche and German Federal Ministry of Education and Research: BMBF) supporting the INAT project that coordinates research efforts to optimize aerosol delivery in mice.

Article Highlight box:

- All aspects of pre-clinical evaluation of pulmonary drug delivery, i.e. in-silico, in-vitro, ex-vivo and in-vivo methods have undergone important improvement and refinements.
- Hybrid multiscale mathematical modeling, improved cascade impactor technology, complex multicellular air-liquid interface cell cultures and associated drug delivery devices, lung-on-chip bioengineering 3D models, reliable and reproducible in-vivo inhaled drug delivery methods are among the most important recent innovations.
- The required multidisciplinary expertise required to cover the whole spectrum of pre-clinical testing calls for setting up multi-national large-scale collaboration consortiums.

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