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## Insights on animal models to investigate inhalation therapy: Relevance for biotherapeutics

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1 **Insights on animal models to investigate inhalation therapy: relevance for biotherapeutics**

2

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26

27

28 **Abstract**

29

30 Acute and chronic respiratory diseases account for major causes of illness and deaths worldwide.  
31 Recent developments of biotherapeutics opened a new era in the treatment and management of patients with  
32 respiratory diseases. When considering the delivery of therapeutics, the inhaled route offers great promises with  
33 a direct, non-invasive access to the diseased organ and has already proven efficient for several molecules. To  
34 assist in the future development of inhaled biotherapeutics, experimental models are crucial to assess lung  
35 deposition, pharmacokinetics, pharmacodynamics and safety. This review describes the animal models used in  
36 pulmonary research for aerosol drug delivery, highlighting their advantages and limitations for inhaled  
37 biologics. Overall, non-clinical species must be selected with relevant scientific arguments while taking into  
38 account their complexities and interspecies differences, to help in the development of inhaled medicines and  
39 ensure their successful transposition in the clinics.

40

41

42 **Keywords:** Animal models; Inhalation therapy; Biotherapeutics

43

## 44 1. Introduction

45

46 Over the last decades, the respiratory drug pipeline has broadened to include a large array of  
47 biotherapeutics which have ushered in a new chapter in the management and treatment of patients with  
48 respiratory diseases (Reichert, 2016). As an example, in the past two years two monoclonal antibodies  
49 (mepolizumab and reslizumab) received approval for severe asthma, providing further options for the treatment  
50 of severe asthma. When considering the administration of large drug molecules, like biotherapeutics, matching  
51 the delivery route to the relevant anatomical location is paramount (Desoubieux et al., 2016). The delivery of  
52 drugs for treating respiratory diseases by inhalation has long been considered to allow a rapid onset of action, to  
53 ensure a high concentration of drugs in the vicinity of the relevant anatomical region while limiting systemic  
54 exposure and reducing the likelihood of side-effects. Inhalation is commonly used for small molecule drugs to  
55 improve their therapeutic index, but appears more complicated for biotherapeutics, with only one protein,  
56 Dornase alpha (Pulmozyme™), approved for the treatment of cystic fibrosis by inhalation (Dentice and Elkins,  
57 2016), and few additional inhaled biotherapeutics in clinical evaluation (alpha anti-trypsin, an anti-RSV  
58 nanobody™ and two monoclonal antibody (mAb) fragments). Indeed, inhalation of biotherapeutics raises  
59 technical and formulation challenges (e.g. stability during aerosolization), pharmacological issues related to  
60 their fate and action after pulmonary deposition, and safety issues (Respaud et al., 2015). To improve the  
61 development of inhaled biotherapeutics and avoid failure to translate promising drugs from animal models to  
62 humans, it is crucial to use/develop more predictive preclinical models. For the development of inhaled drugs,  
63 local lung tolerance is one of the most critical aspects and requires the use of several animal species. On the  
64 other hand, assessing *in vivo* lung deposition, pulmonary pharmacokinetics (PK) and pharmacodynamics (PD)  
65 are also essential to understand the fate and specific effects of inhaled liquid or solid (powder) formulations.  
66 Given the variability of the deposition of inhaled particles in the airways, as well as their clearance and  
67 pharmacological effects, a large number of animal species have been used in preclinical studies. Rodents (rats  
68 and mice) are the most commonly used species, but larger mammals such as dogs and primates are often  
69 required for toxicological evaluation. Although animals are necessary, they all display their own limitations as  
70 models of humans. Thus, when considering non-clinical studies of inhaled materials in experimental mammals it  
71 is imperative to take into consideration the anatomy of the airways, the characteristics of particle deposition and  
72 clearance, as well as methods of exposure (inhalation chambers, masks, etc...), and the practical design of the  
73 experimental protocol. Other factors are also important, such as biotherapeutic interaction with the molecular  
74 target and animal host system, ease of handling animal models, and technical constraints linked to animal  
75 housing and/or the availability of specific techniques. This review describes existing approaches to the use of  
76 experimental animals regarding aerosol drug deposition, PK/PD and toxicity, and discusses the limitations of  
77 these models with respect to the anatomical and physiological characteristics of the respiratory system.

78

79

## 80 2. Animal models and aerosol delivery

81

82 Studies dealing with modelling and/or comparison of deposition of inhaled particles by the most  
83 common laboratory species are based on a limited amount of data (Derelanko and Hollinger, 1995; McClellan  
84 and Henderson, 1989; Schlesinger, 1985). It is likely that total deposition of an inhaled aerosol might be similar  
85 in mammals with equal mass, despite distinct anatomical features, whereas there might be greater inter-species  
86 variations in terms of regional deposition (Phalen et al., 2008). Overall, anatomical characteristics of the lungs  
87 and respiratory parameters differ considerably among mammals and clearly matter when evaluating aerosol  
88 deposition and for translating results from animal models to humans.

89

### 90 2.1 Rationale for species selection

91

92 Many species are suitable for studies of aerosol deposition of drugs in the lungs, but a rigorous  
93 comparative analysis of the airway anatomy and physiology is mandatory in the selection of an animal model in  
94 order to extrapolate the results to humans. The structure of the respiratory apparatus changes significantly across  
95 species with regard to the anatomy of the nose, the overall anatomy of the lung, the tracheobronchial tree, and  
96 the lower lung. Interspecies comparisons of breathing parameters have been reviewed in more detail elsewhere  
97 (Cryan et al., 2007) and key differences in individual anatomical, biological and physiological parameters are  
98 discussed below.

99

#### 100 2.1.1 Comparative anatomy of the lung, from the nose to the alveoli

101 Small laboratory rodents are unable to breathe through the mouth, breathing only through the nose,  
102 similar to humans during the neonatal period. In contrast, most other mammals preferentially breathe through  
103 the nose, although they are also able to breathe through the mouth, as do pigs in hyperventilation or non-human  
104 primate (NHP) with nasal obstruction. The nose in humans and other primates consists of several superimposed  
105 shelf-like structures (turbinates) projecting into the nasal cavities with a small olfactory region. The turbinates  
106 cause turbulence of the inhaled air. In contrast, the nasal anatomy of most other mammals is more complex, with  
107 double-scrolled turbinates and large olfactory regions (Schneider and Raabe, 1981). Anatomical comparison can  
108 be misleading because the nasal cavity is not uniformly ventilated. Only 10-15% of the inspired air-stream  
109 follows a dorsal pathway of the rat nose and flows over the complex ethmoturbinate. Thus, whereas the  
110 ethmoturbinate structure in the rat is quite different than in the human, little of the inspire air passes through this  
111 region (Salem and Katz, 2005). Magnetic resonance imaging (MRI) studies have shown that there are many  
112 similarities in the structure of the monkey and human nose suggesting that the rhesus monkey is a good human  
113 surrogate in aerosol deposition studies (Yeh et al., 1997). In contrast to humans, nose-breathing rodents exhibit a  
114 lower pulmonary deposition accompanied by a higher nasal and tracheobronchial deposition, when inhaling  
115 large particles (> several  $\mu\text{m}$ ) (Schlesinger, 1985). When comparing pulmonary aerosol deposition of large  
116 particles, in conditions of nasal breathing between humans and other species, deposition is similar in humans  
117 and NHP, but greater in humans than in rats (Phalen et al., 2008). Interestingly, adapting the aerosol device to  
118 minimize large particles may reduce the nasal particle trapping, as demonstrated in guinea pigs (Wu et al.,  
119 2011).

120           Some of the main anatomical features of the respiratory tract of laboratory species are compared to  
121 those of humans in Figure 1. The size of the thoracic cavity varies according to the species, which consequently  
122 affects the size and volume of the lungs. The overall shape of the airway is of particular significance, as seen in  
123 the relatively dichotomous and symmetrical branching scheme of the tracheobronchial system of primates as  
124 compared to the long tapering monopodial airways of small rodents. These anatomical variations may introduce  
125 changes in direction of the airflow favoring aerosol impaction and thus alteration in deposition pattern (Warheit,  
126 1989). Such particular impaction “hotspots” at bifurcations in the human tracheobronchial tree have been  
127 described in detail elsewhere (Balashazy et al., 2003). In species with monopodial branching patterns, and more  
128 particularly in the rat, the particles carried in the airflow continue into the major daughter airways with little  
129 change in velocity or direction, with deposition occurring largely by sedimentation (Warheit, 1989). Rodents  
130 also differ from humans and NHPs in that they have no respiratory bronchioles. In the rat, the terminal  
131 bronchioles, the smallest conductive part of the respiratory tract, are connected directly into the alveolar ducts  
132 (Phalen and Oldham, 1983; Tyler, 1983). Mammals are also distinguished by the number and size of the  
133 pulmonary acini. The size and number of alveoli and the lung area increase with the size of the animal  
134 (Derelanko and Hollinger, 1995). Lobulation of the lung is more or less pronounced among species. The left  
135 lung is generally divided into two lobes (e.g. humans, pigs, rabbits, guinea-pigs) or three (e.g. rhesus or  
136 cynomolgus monkeys) and the right lung into three. The left lung remains single-lobed in certain rodents such as  
137 the rat and the mouse, while the right lung is divided into four lobes.

138

#### 139 *2.1.2 Impact of specific physiological and biological characteristics on aerosol administration*

140           In addition to inter-species anatomical differences, there are considerable inter-species physiological  
141 and biological differences in lungs that may impact relevance of animal studies to predict aerosol deposition in  
142 humans. First, there are significant differences in the lung volumes and breathing pattern across different  
143 species. As summarized in Figure 2, the tidal volume increases with body weight, but in contrast, the respiratory  
144 rate drastically decreases with an increase in body weight. Thus, extrapolation of results from lung deposition  
145 studies in mice to humans is challenging. Second, the rate of inhaled particle absorption is not homogenous  
146 across species. Absorption of lipid-insoluble drugs varies importantly according to species, whereas lipid-  
147 soluble drugs are equally absorbed into the bloodstream of various animal species and are poorly affected by  
148 clearance mechanisms (Phalen et al., 2008; Schanker et al., 1986). Moreover, non-clinical studies are often  
149 carried out in animals at an early stage of life while the rates of lung absorption vary according to age (Schanker  
150 and Hemberger, 1983).

151           Complex mechanisms of non-absorptive clearance following lung deposition can also modify the  
152 bioavailability of drugs in the lung, such as mucociliary clearance, protease digestion and/or phagocytosis.  
153 Indeed, morphometric investigations in rats, dogs, baboons, and humans revealed variations in the distribution  
154 of alveolar macrophages (Crapo et al., 1983). Human lungs contained significantly greater number of alveolar  
155 macrophages which may have implications for non-absorptive clearance (Crapo et al., 1983). Inhaled particles  
156 are cleared from the tracheobronchial region by the ciliary action of the epithelial cells lining the airways, the so  
157 called “mucociliary escalator”. The speed of tracheobronchial clearance is difficult to compare between species  
158 since the size and length of the tracheobronchial tree are highly heterogeneous. Furthermore, small rodents  
159 (mice, rabbit) lack bronchial submucosal glands (March et al., 2000). Finally, distribution of the

160 pharmacological effectors/modulators may be distributed differently within the lungs in animal species and limit  
161 result translation to human. As an example, the neonatal Fc receptor (FcRn), known for its role in IgG  
162 homeostasis, is a critical factor in the biodistribution and PK of mAbs and is differently expressed across species  
163 (Roopenian and Akilesh, 2007). FcRn is expressed by numerous cell types such as epithelial cells, endothelial  
164 cells, and macrophage/monocytes, in which it prevents IgG degradation in the lysosomes and contributes to IgG  
165 recycling and transcytosis. While FcRn is expressed by the alveolar macrophages of all species, there are  
166 differences in the sites of FcRn expression in the respiratory epithelium. FcRn expression is restricted to the  
167 epithelial cells of the upper airways in primates, whereas it is also detected in the epithelium of the alveolar  
168 region in rodents.

169

## 170 2.2 Methods of aerosol administration

171

### 172 2.2.1 Non-invasive aerosol delivery in spontaneously breathing animals

173 *Whole-body inhalation.* Whole-body inhalation systems are particularly convenient for inhalation  
174 studies using large numbers of animals. Another advantage of whole-body inhalation systems is the ability to  
175 expose conscious animals without restraint, thereby limiting experimental artefacts. However, a large proportion  
176 of the aerosol is deposited on the animal's fur, with low lung deposition (<10% of the administered dose) and  
177 confounding PK results due to intestinal absorption. For biotherapeutics such as mAbs, protein degradation in  
178 the stomach is probably associated with a low transfer of the intact drug in the bloodstream. Thus, one would  
179 assume that the gastrointestinal-tract absorption may poorly interfere with PK of inhaled biotherapeutic drugs.  
180 In addition, the size of inhaled particles cannot be controlled, and the exact quantity of drug administered to the  
181 lung is difficult to determine (Kawajiri et al., 2004; March et al., 2004; Pauluhn, 2006). Furthermore, this  
182 system is not recommended for expensive drugs, like biotherapeutics, because of important drug loss. Another  
183 confounding factor is coming from the fact that animals are in a horizontal position (standing on four legs)  
184 during aerosol delivery, which favors ventral lung deposition. For these reasons, whole-body inhalation systems  
185 are rather used to simulate airborne virus infection (Belser et al., 2015) or cigarette smoke exposure (Guillon et  
186 al., 2015b).

187 *Nose-only inhalation.* Nose-only inhalation systems are generally preferred to reduce absorption by  
188 non-respiratory routes. This system also affords measurement of respiratory parameters by plethysmography  
189 during exposure, thereby allowing variability in dosing due to differences in individual animal breathing  
190 patterns to be addressed (Pauluhn and Mohr, 2000). Nonetheless, a large amount of aerosol is still deposited in  
191 the nasal cavity, due to nasal breathing of experimental animals. This fraction may be transferred to the  
192 gastrointestinal compartment following mucociliary clearance where it can be absorbed into the bloodstream.  
193 This may impact the interpretation of the results of PK or toxicity studies. As previously mentioned  
194 gastrointestinal absorption may poorly interfere with PK of protein therapeutics. In contrast, biotherapeutic  
195 deposition in the nose and further direct adsorption may biased PK analysis. Indeed, recent data supports  
196 expression of FcRn in the nasal epithelium and its role in antibody/IgG transcytosis through the nasal epithelium  
197 (Heidl et al., 2016).

198 *Face-mask inhalation.* Large mammals can breathe spontaneously through a face mask connected to an  
199 aerosol generator (Supplementary figure 1) (Marchand et al., 2015). However, because the facial features of the

200 animal differ from those of a human, a mask interface is necessary to connect human devices, such as pediatric  
201 aerosol generators, nebulizers or pMDIs with inhalation chambers. Although, a quantity of aerosol is lost in the  
202 device and the mask interface, a significant quantity of the aerosol is deposited in the respiratory tract.  
203 Interestingly, face-mask inhalation in macaque has been recently used for the preclinical development of a drug  
204 and device system envisioned for the delivery of a mAb into the deep lungs of adults. As expected, mAb  
205 deposition in the lungs of macaque was lower than the dose predicted by cascade impaction, because macaque  
206 have ventilation parameter comparable to infants (Respaud et al., 2016).  
207 Other limitations include the requirement of animal training prior to aerosol administration and the use of  
208 anesthetics that can affect breathing parameters (Iizuka et al., 2010). Despite the limited relevance of these  
209 methods to evaluate the relationship between the effectiveness/toxicity and the dose administered, and the  
210 difficulties to accurately predict lung deposition, these methods are used and validated for lung tolerance and  
211 regulatory toxicity studies. Like for other methods described above, gastrointestinal deposition is important by  
212 face-mask inhalation - probably consecutive to direct swallowing or licking and subsequent swallowing of the  
213 drugs deposited in the mouth and/or on the face – but may have a limited impact on protein therapeutic  
214 pharmacology (Respaud et al., 2016). For example, face-mask inhalation has been used during the preclinical  
215 development of ALX-0171, an anti-RSV nanobody™, to assess PK and anti-viral effects in a neonatal lamb  
216 RSV infection model (Larios Mora et al., 2015). It is also worth noting that these methods could be particularly  
217 useful to study lung tolerance following repeated drug exposure (Pauluhn, 2014).

218

#### 219 2.2.2 *Intra-tracheal aerosol delivery*

220 *Intra-tracheal delivery in small rodents.* To bypass the oro-pharynx during administration and ensure a  
221 full dose delivery in the lung, intra-tracheal instillation has been widely described in anesthetized animals using  
222 either the Saffioti's method (Saffioti et al., 1968) or alternatively following tracheotomy or orotracheal  
223 intubation. To overcome issues associated with intra-tracheal instillation, a large number of devices allowing the  
224 delivery of a spray/aerosol directly into the trachea of rodents have been developed (Chen et al., 2002;  
225 Hamacher et al., 2008; Rivera et al., 2005; Spoelstra et al., 2007; Todo et al., 2003). Intra-tracheal  
226 administration in rodents using spray/aerosol devices, such as the MicroSprayer® Aerosolizer and Dry Powder  
227 Insufflator™ (Penn-Century Inc., US), allows the rapid and reliable delivery of a controlled quantity of drug  
228 directly into the lungs (Supplementary figure 2) (F. Gagnadoux et al., 2005; Mercier et al., 2014). These devices  
229 can be used with liquid or solid drugs (e.g. anti-cancer agents and anti-fungals) or excipients at single or  
230 repeated doses, to assess efficacy, tolerance and PK (Chandenier et al., 2009; Frédéric Gagnadoux et al., 2005;  
231 Gagnadoux et al., 2008; Maillet et al., 2011; Montharu et al., 2010). The Microsprayer™ device has been used  
232 with success to assess the efficacy of several mAbs in murine models (Guilleminault et al., 2014; Hervé et al.,  
233 2014; Maillet et al., 2011). It should be noted however that tolerability issues have been raised concerning intra-  
234 tracheal administration with these devices in small rodents (slight and transitory lung inflammation (Dubois et  
235 al., 2013; Montharu et al., 2010) and lung lesions (Guillon et al., 2012)). A nebulization catheter device  
236 (AeroProbe™, Trudell Medical International, Canada) was also commercialized to administer aerosols directly  
237 into the airways and to provide a complementary approach to existing methodologies for pulmonary drug  
238 delivery in small animals (Tronde et al., 2002). However, to the best of our knowledge, these devices are no  
239 longer commercially available. Aerosol delivery systems for tracheal intubated and mechanically ventilated

240 mice have also been described (Robichaud et al., 2015). These nebulizers are integrated into the inspiratory  
241 branch of the ventilator; however, laboratory-related protocol variations greatly affect the efficiency of the  
242 aerosol generation process and avoid study comparisons (Robichaud et al., 2015).

243 In certain experimental scenarios (e.g. when a very mild anesthesia is necessary), alternative  
244 administration techniques, such as oropharyngeal aspiration or intranasal administration may be used.  
245 Oropharyngeal aspiration is limited to the administration of liquid solutions or suspensions to the lungs, as it  
246 involves applying a small droplet (typically 25 to 50  $\mu$ L per mouse) at the top of the pharynx and allowing the  
247 droplet to be aspirated into the lungs during normal inhalation (Foster et al., 2001; Lakatos et al., 2006; Rao et  
248 al., 2003). Although, this dosing technique is primarily used in the generation of murine disease models (such as  
249 chemically induced asthma (De Vooght et al., 2009; Morgan et al., 2008) or bleomycin-induced lung fibrosis  
250 (Babin et al., 2012)), it was used with success to deliver biotherapeutics (Respaud et al., 2016). It was also  
251 demonstrated that oropharyngeal aspiration generated similar lung deposition profiles as MicroSprayer®  
252 Aerosolizer instillation, notwithstanding a quicker overall process and the requirement for milder anaesthetic  
253 regimes (Patel et al., 2016; Woods et al., 2015). However, this method may induce a bias in PK studies due to  
254 intestinal absorption. Overall, commercial intra-tracheal administration devices, unlike traditional aerosol  
255 administration devices (inhalation chambers), allow the dose of the product to be controlled, ensuring a high  
256 deposition rate (70 to 80% of delivered dose) and a relatively uniform distribution of the product in the lungs of  
257 larger rodents, such as rats. They also allow rapid and repeated administrations of the drug into the lungs, which  
258 is suited to evaluate the efficacy of inhaled biotherapeutics. Finally, one has to consider that intra-tracheal  
259 aerosol delivery in obligate nasal breathers is not exempted of drug deposition in the nasal cavity. Indeed, a part  
260 of the aerosol still floats and remains in upper airway after the aerosol delivery. When the animal exhaled  
261 through the nose, the aerosol could also be transported on the walls of nasopharynx (Yamamoto et al., 2017).

262 *Intra-tracheal delivery in large mammals.* Intra-tracheal administration in intubated large animals is  
263 particularly relevant to mimic aerosol delivery during invasive mechanical ventilation in humans. Primates can  
264 be intubated and mechanically ventilated using respiratory parameters mimicking “pediatric patients”. Pigs are  
265 commonly used as a model for aerosol administration to ventilated patients in intensive care units (Rouby et al.,  
266 2012). In these models, human devices can be used without any modifications and synchronized to inspiratory  
267 time if needed. Despite their tracheal bronchus and the difficulty of oro-tracheal intubation due to their long  
268 mouth cavity and curved larynx, the lung anatomy and physiology of pigs are close to that of humans, which  
269 enable extrapolation of experimental results of aerosol deposition to humans (Supplementary figure 3).  
270 Presently, this model has mainly been used to evaluate nebulization of topically-acting small drugs (e.g.  
271 antibiotics) during artificial ventilation (Rouby et al., 2012). Indeed, pigs are becoming an increasingly  
272 important species for translational research and may be a relevant model in which to test the efficacy of anti-  
273 infectious biotherapeutics. A major advance was made in the field of respiratory medicine with the generation of  
274 the porcine cystic fibrosis model (Guillon et al., 2015a; Rogers et al., 2008). Sheep have also been used, but less  
275 frequently, as a model for aerosol delivery during mechanical ventilation. One of the best examples has been  
276 provided during the preclinical development of a 45kDa recombinant alpha anti-trypsin (rAAT) that  
277 demonstrated the feasibility of using aerosolization to the pulmonary surface to administer rAAT in sheep  
278 (Hubbard et al., 1989). Direct intra-tracheal administration of liquid or powder aerosol is also possible in  
279 intubated large animals thanks to dedicated sprayers (Guillemineault et al., 2014). Dry powder aerosols can be

280 administered using a Dry Powder Insufflator™ without any concerns for tolerance considerations associated  
281 with the device (Guillon et al., 2016); the volume of insufflated air generated by the device is considerably  
282 lower than the animal's tidal volume and therefore does not cause lung lesions (Guillon et al., 2012). Dry  
283 powder inhaler devices have also been developed for use in beagle dogs via oro-tracheal intubation (Sellers et  
284 al., 2015).

285

### 286 *2.3 Aerosol delivery methods in animals and transposition to human*

287

288 It is important to keep in mind that inhalation therapy is atypical in its difficulty to find relevant animal  
289 models, compared to other routes of delivery. Pulmonary delivery is probably the most challenging route in  
290 animal models because drug delivery is highly dependent of the anatomy and respiratory physiology of the  
291 animal species. Drug deposition in animal is also affected by the aerosol-generating systems, which poorly  
292 mimic the aerosol distribution encountered in human, even when using devices intended for human.  
293 Consequently, (i) the gap between human and animal models anatomy and respiratory pattern, and (ii) the  
294 differences between devices engineered for experimental purpose and actual human devices, are two important  
295 limiting factors for successful translation to the clinic. It is clear that further refinement of current experimental  
296 models is necessary. The development of animal devices that would reproduce the distribution of the drugs in  
297 human, taking into account the respiratory parameters, the physiological properties and anatomy of the lungs of  
298 the animal, may be helpful for the translation of results into the clinics.

299 Despite those limitations, two animal models of aerosol delivery have a high translational potential for  
300 specific clinical applications and should be highlighted. First, aerosol delivery during invasive mechanical  
301 ventilation in large mammals, such as pigs, is particularly relevant to mimic aerosol delivery during invasive  
302 mechanical ventilation in humans. As the oropharynx is bypassed by the tracheal probe, the anatomic  
303 differences are not anymore issues. Moreover, ventilators settings could be identical between humans and pigs.  
304 In a recent systematic review assessing the aerosol delivery during invasive mechanical ventilation according to  
305 the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines, 36 studies  
306 were finally included among 234 articles assessed for eligibility, and comprised 26 clinical studies and 10  
307 experimental studies (Dugernier et al., 2017). Nine of these experimental studies were performed on pig models  
308 and one a dog model. So far, these models have never been used for the development of a biotherapeutic. The  
309 second relevant model is the macaque (*Macaca Fascicularis*), in the weight range of a full-term newborn  
310 human, which enable to make accurate prediction of aerosol lung deposition in infant (Dubus et al., 2005;  
311 Réminiac et al., 2017). No modification of human facemask is necessary; infant are also nose breather not able  
312 to synchronize inspiratory time with drug delivery.

313

### 314 **3. Methods to assess lung deposition after aerosol delivery**

315

316 Distribution and deposition of the aerosol in the different regions of the lung depend on particle  
317 properties, but are also affected by the subject's breathing pattern pathophysiological context. Hence, the dose  
318 of the delivered aerosol is likely to vary substantially in the lung and imaging provides a non-invasive way to  
319 measure *in vivo* the regional distribution of inhaled drugs.

320 Assessment of aerosol deposition in airways is mainly performed using imaging techniques which are  
321 summarized in Table 1. Among them, planar (2D) scintigraphy is the most relevant and standardized method,  
322 providing robust results (Newman et al., 2012). It relies on the use of either direct or indirect radiolabeling of  
323 the material to be measured using a gamma camera, and provides a 2D image of the distribution of radioactivity  
324 in the airways. Technetium-99m ( $^{99m}\text{Tc}$ ) is the most common radioactive element used for labeling due to its  
325 suitable half-life regarding the duration of image acquisition and radio-protection. It can be also used in  
326 combination with diethylene triamine penta-acetic Acid (DTPA) to trace the unlabeled drug and avoids quick  
327  $^{99m}\text{Tc}$  diffusion into the systemic circulation (Guillon et al., 2015c).

328 Nebulizer delivery methods are well adapted to  $^{99m}\text{Tc}$ -DTPA 2D scintigraphy because the active drug is  
329 directly accessible for labeling (Behr et al., 2009). Biotherapeutics can also be used as theranostic agents  
330 through the attachment of a radiolabel chelator to the biotherapeutic. For antibodies, the use of the short-lived  
331  $^{99m}\text{Tc}$  improves physical imaging because of high proton flux, high resolution and low dosimetry (Andersson et  
332 al., 2016). It is also particularly relevant for Fab fragments which exhibit accelerated clearance resulting in  
333 better target-background ratio (Wahl et al., 1983). Quality specifications of a labeled biopharmaceutical must be  
334 assessed and in particular the conservation of its biological properties (Eisenhut and Haberkorn, 2006). In  
335 addition, the characterization of peptide impurities following labeling or instability processes is mandatory as  
336 such by-products can generate toxic or unexpected pharmacological effects. Labeling is more challenging when  
337 complex devices or formulations are involved, such as pMDIs or DPIs, as this requires manufacture and filling  
338 of the radioactive formulation into the delivery device (Aug et al., 1991). The validation of aerosol drug  
339 radiolabeling and radiotracing must be done prior to the 2D gamma camera imaging. It must be demonstrated  
340 that the radiolabel or radiotracer does not affect the aerosol characteristics compared to the non-labeled system  
341 (Guillon et al., 2015c). Recommendations have been established for the validation of a  
342 radiolabeling/radiotracing method, utilizing similar criteria to those recommended by the EMA guidelines and  
343 the ISAM/IPAC-RS Workshop Report (European Medicines Agency Committee for Medicinal Products for  
344 Human Use, n.d.).

345 Multiple planar images at different positions around the body can be processed to generate a 3D-image  
346 of the location of a radiolabeled drug. This principle is at the basis of both positron emission tomography (PET)  
347 and single-photon-emission computed tomography (SPECT) imaging. Before PET became available, SPECT  
348 was the primary imaging modality, especially to assess mAb distribution. SPECT imaging relies on similar  
349 radioelements as planar scintigraphy, including  $^{99m}\text{Tc}$ ,  $^{111}\text{In}$  or  $^{131}\text{I}$ . Both fragment and full-length mAb have  
350 been labeled and the rationale supporting the choice of the radioelement is based on the matching between its  
351 half-life and the plasma residence time of the mAb. For example, long-lived  $^{111}\text{In}$  or  $^{131}\text{I}$  have been used to label  
352 whole antibodies, while the short-lived  $^{99m}\text{Tc}$  has been used to label antibody fragments and all have been  
353 successfully imaged in humans (Goldenberg et al., 1978; Machac et al., 2002; Nagengast et al., 2011; Yao et al.,  
354 2007). PET uses radioisotopes that emit a positively charged electron, which when once annihilated produces  
355 two gamma rays in exactly opposite directions allowing for a better resolution than SPECT. Among the PET  
356 radioisotopes,  $^{89}\text{Zr}$  has been widely used in the clinic for labeling anti-tumor mAbs. Short-lived positrons like  
357  $^{18}\text{F}$  or  $^{76}\text{Br}$  are mainly used for the imaging of antibody fragments and small antibody-like molecules (Lamberts  
358 et al., 2015). Of note, scintigraphy acquisition required careful adjustments and corrections that are not detailed  
359 here. These correcting factors may vary depending on the anatomical features of the animal species (Frédéric

360 Gagnadoux et al., 2005; Gagnadoux et al., 2006; Guillon et al., 2014). In many cases, anatomical images can be  
361 added to deposition images using X-ray computed tomography (CT) or magnetic resonance imaging (MRI) to  
362 provide a better spatial distribution of the aerosol and evaluation of aerosol treatments (Conway et al., 2013).  
363 These methods provide considerable amounts of information in terms of aerosol distribution in the lung and they  
364 are mainly used for basic scientific research. Nevertheless, they are more complex methods than 2D  
365 scintigraphy using a gamma camera and the algorithms for calculating aerosol deposition are less well  
366 established. MRI, on its own, has also been considered in assessing lung aerosol deposition and has the  
367 advantage of measurement without radioactive agent exposure (Thompson and Finlay, 2012). However, the  
368 oxygen in the air that filled lungs complicates MRI, thus making necessary helium inhalation to enhance  
369 contrast in the lungs. For a long time, the signal intensity of the contrast agent in the lung and the lack of a  
370 standardized method of calculation limited MRI imaging to accurately measure aerosol deposition in the lung  
371 (H. Wang et al., 2016). A recent method using ultra-short echo (UTE) sequences and gadolinium-labeled aerosol  
372 resulted in an enhanced signal in the lung parenchyma, compatible with a precise quantitation of regional  
373 distribution of the aerosol deposition.

374

#### 375 **4. Methods to assess PK and toxicity after aerosol delivery**

376

377 Although inhaled drug deposition and lung anatomy/physiology may be different between animals and  
378 humans, making extrapolation across species difficult, animal models are crucial to analyze the PD, PK and  
379 toxicity of aerosolized drugs. The methods used for pulmonary delivery, reviewed above, are critical because  
380 they involve variable drug losses in the delivery system and into the environment, specific sites of deposition  
381 (within or outside the respiratory tract), all of them altering therapeutic efficacy, PK and toxicity of the inhaled  
382 drug.

383

#### 384 *4.1 Determination of drug concentration in blood and lung environment*

385

##### 386 *4.1.1 Determination of aerosolized-drug in the bloodstream*

387 As drug concentration cannot be easily assessed in the lung, PK studies are done by measuring the  
388 levels of drug in the systemic circulation, both for local and systemically acting drugs. However, for drugs  
389 acting locally, high serum concentrations are usually related to unwanted systemic exposure and potential safety  
390 concerns, and thus provide little information on efficacy. Anatomical lung differences, mucociliary clearance  
391 rates and the number of alveolar macrophages, which may all contribute to drug clearance, are known to vary  
392 significantly between species and may influence PK determination. PK studies assessing drug concentrations in  
393 blood are performed by collecting blood from a vein or alternatively via implantation of a catheter to avoid  
394 repeated venipuncture. Although NHPs are the closest to humans, with similar anatomy, physiology and  
395 genetics, rodent models are often used for preclinical PK studies. Nevertheless, mice are technically challenging  
396 as a species to undertake multiple blood sampling from lung dosimetry studies. In larger animals, multiple blood  
397 sampling is no longer an issue and allows a more accurate non-destructive profiling of the systemic  
398 serum/plasma levels in each animal. PK parameters describing the absorption, distribution and elimination of  
399 the drug and its bioavailability can be generated from appropriate mathematical methods (compartmental and/or

400 non-compartmental analysis) and using the concentration measurements in plasma at relevant time points.  
401 However, indirect modelling of lung concentrations from drug concentration-time profiles in plasma using  
402 mathematical compartmental models, taking into account the delivery route may not be enough to predict the  
403 clinical outcome (Eichler and Müller, 1998; Paintaud, 2009; Presant et al., 1994). Indeed, many drugs, such as  
404 anti-infective agents, biotherapeutics and controlled release systems designed to prevent systemic exposure, do  
405 not distribute uniformly in different tissues and the dose-effect relationship is dependent on the distribution of  
406 the drug at the target site. Analyses of the drug concentration in the bloodstream may also be affected by the  
407 swallowed portion of the drug and subsequent absorption across the gastrointestinal tract. Clearance from the  
408 lung to the blood has been shown to be a linear function of the molecular weight of the drug regardless of the  
409 species (Matsukawa et al., 1997). Once in the systemic compartment, biopharmaceuticals like full-length  
410 immunoglobulin, with a molecular weight of 150kDa remain in the circulation for 3-4 weeks and are slowly  
411 metabolized. Monovalent fragments (e.g, single-chain antibody or scFv) have a blood clearance of ~10h with  
412 primarily renal excretion of 2-4h. Molecules with intermediate weight, ~70kDa (the threshold for glomerular  
413 filtration) have longer residence times. These discrepancies are therefore another strong reason for developing  
414 better methods to measure drug concentrations in the lung tissue to understand the distribution of drugs in the  
415 relevant anatomical compartment.

416

#### 417 *4.1.2 Determination of aerosolized-drug concentration in the lungs*

418 Drug concentration can be measured in the airways and epithelial lining fluid (ELF) usually by  
419 bronchoalveolar lavage (BAL), a procedure which consists of injecting saline solution into the lungs, followed  
420 by re-aspiration. BAL can be performed on the whole lungs for small animals or portions of the lungs for larger  
421 species after euthanasia, or on live animals, usually non-rodents, via bronchoscopy (Mankikian et al., 2014).  
422 Because injection of solutions into the lungs interferes with local drug concentrations and thus PK parameters,  
423 BAL usually only gives useful information for one time point in one animal. However, it is possible in larger  
424 animals, to collect, throughout the experiment, several BAL samples from different respiratory tract regions,  
425 using bronchoscopy or tracheal swabbing through the nose (Jacobson et al., 2017). In this case, it is important to  
426 use an endogenous marker diffusing passively throughout the different tissue compartments as an internal  
427 reference (Kipnis, 2005). Alternatively drug concentration can be measured in the lung tissue, at autopsy, which  
428 requires resection, homogenization of lung tissue and extraction of the drug with the appropriate solvent. For  
429 peptide-based therapies, the main drawback is that their lung residency may be hindered by their instability due  
430 to the presence of proteinases.

431

#### 432 *4.1.3 Imaging methods and microdialysis*

433 Although imaging techniques are widely used to follow drug distribution, they are rarely employed for  
434 PK studies of aerosolized drugs in preclinical studies (Anderson, 2000; Fok et al., 1999; Guilleminault et al.,  
435 2014; Weinstein et al., 2012). Depending on the imaging techniques used, the drug is conjugated to the most  
436 relevant tracer, which involves labeling and purification of the drug conjugate. The major caveats for PK  
437 imaging include short tracer half-life that may limit kinetic follow up, in particular for biotherapeutics, such as  
438 antibodies with long half-life. Another limitation is the possible release of the tracer *in vivo* and passive  
439 diffusion into different tissues, and impairment of drug binding to its target antigen and/or transport receptor due

440 to its conjugation to the tracer. Imaging techniques involve costly equipment and are associated with technical  
441 challenges to conjugate the drug, which limit their wider use for PK studies. We previously described the fate of  
442 a mAb, labeled with a fluorophore and delivered by the airways, using Near Infrared Fluorescent Imaging  
443 (NIRF). Although accurate quantification remains challenging, it was possible combining 3D *in vivo* imaging  
444 and 2D quantification on *ex vivo* lung specimens (Guillemainault et al., 2014).

445 In vivo microdialysis is an interesting and well-established semi-invasive sampling technique that has  
446 been used to study PK of drugs in various tissues (Brunner and Langer, 2006; Dhanani et al., 2010; Elmquist  
447 and Sawchuk, 1997; Zeitlinger et al., 2005). As this technique does not withdraw any body fluids or  
448 compromise homeostasis, it allows the repeated measurement of the unbound and/or soluble drug in the  
449 interstitial space from the same animal, which substantially reduces the number of animals required for  
450 preclinical PK studies. This method is based on the use of probes with a semi-permeable membrane, which are  
451 implanted in the tissue of interest and continuously perfused with a physiological solution (perfusate) at a very  
452 slow flow rate (usually 1 to 10  $\mu\text{L}/\text{min}$ ). The implantation of microdialysis probes is possible under direct vision  
453 during thoracic surgery to avoid pneumothorax and collapse of the lung, and to date no major peripheral tissue  
454 trauma has been reported (de la Peña et al., 2000). The dialysate is collected at timed intervals and  
455 substances/drugs in the interstitial space fluid diffuse passively through the membrane pores, along their  
456 concentration gradient (Supplementary figure 4). Although, it has been used extensively to follow the kinetics of  
457 neurotransmitter levels in neuroscience research, and to monitor small drugs in peripheral tissues, it is now  
458 possible, thanks to higher molecular cut-off membrane and push-pull methods, to also analyze the fate of larger  
459 molecules, such as proteins. However, because the dialysate volume is small, in the microliter range, it requires  
460 highly sensitive methods for sample analysis. An additional limitation of microdialysis is the proper calibration  
461 of the microdialysis probes because the analyte diffusion is performed under non-equilibrium conditions and the  
462 concentration measured in the dialysate will reflect only a fraction of the concentrations in the interstitial fluid  
463 surrounding the catheter (Dhanani et al., 2010). Microdialysis has been shown to be feasible for most tissues,  
464 including the lungs, and in different species (de la Peña et al., 2000; Zeitlinger et al., 2005). Notably, it has been  
465 applied to the measurement of both non-infective and anti-infective agents with success, demonstrating its  
466 relevance for intra-bronchial PK measurement of drugs in the lungs (de la Peña et al., 2000; Zeitlinger et al.,  
467 2005). Microdialysis with monoclonal antibodies has recently been shown feasible (Jadhav et al., 2017).

468

#### 469 4.1.4 Influence of pathological conditions on inhaled drug concentration in the lung

470 Disease states have to be taken into consideration for the accurate determination of drug deposition in  
471 the lung, especially those characterized by bronchoconstriction, inflammation and airway narrowing.  
472 Respiratory diseases, such as cystic fibrosis and chronic obstructive pulmonary disease exhibit changes in the  
473 architecture of the lung with obstruction of the airways, mucus hypersecretion and modification in the  
474 bifurcation angles, which may alter the distribution patterns of therapeutic aerosols, in particular those  
475 containing high-molecular weight protein therapeutics like mAbs. Pro-fibrotic diseases such as idiopathic  
476 pulmonary fibrosis or emphysema, are characterized by the overexpression of proteases in the ELF, which may  
477 cause protein therapeutic degradation and hinder drug penetration to the epithelium. ELF also contains  
478 surfactant that have been shown to interact with exogenous proteins promoting their aggregations (Nag et al.,  
479 2007). Myeloid cells such as alveolar macrophages may also participate in the regulation of inhaled drug.

480 Indeed, they have been described as barrier cells to the transport of large protein (>40Kda) from the airway into  
481 the parenchyma (Lombry et al., 2004). During the acute phase of lung inflammation, alveolar macrophages  
482 undergo rapid apoptosis, thereby reducing their interactions with inhaled drug. This may impact inhaled mAb  
483 clearance because alveolar macrophages express FcRn, which may prevent intracellular degradation of inhaled  
484 mAbs, after they deposited in the airways. Finally, inflammation injures both epithelial and endothelial lung  
485 barriers promoting leukocyte extravasation and infiltration of vascular fluid. This increased pulmonary  
486 microvascular permeability may dilute the drug within the lungs. On the other hand, inhaled drug leakage into  
487 the systemic circulation may occur if the bronchoalveolar barrier is disrupted. This was observed for an inhaled  
488 mAb, after an accidental acute lung injury due to gastric inhalation during an aerosoltherapy session  
489 (Guilleminault et al., 2014).

490

#### 491 *4.2 Scaling-up of animal results to determine first-in-man dose, for drug delivered by inhalation*

492

493 First-in-man dose estimation from pre-clinical pharmacology data for systemically administered drugs is based  
494 from the common use of allometric dose scaling (Nair and Jacob, 2016). Allometric scaling approaches allows  
495 interspecies correlation with an empirical calculated pharmacologic parameter and is the standard way to  
496 approximate equivalent interspecies doses unless extrapolating doses based on other parameters is known to be  
497 more appropriate. For drug delivered by inhalation, scientific data to support the assumption that allometric  
498 scaling is useful to predict the dose is lacking. In a recent review (Phillips, 2017), allometric scaling exponent  
499 has been retrospectively calculated from experimental rodent data and the known inhaled clinical dose that needs  
500 to be deposited in the human lung. The author studied four inhaled compounds currently used clinically  
501 (mometasone, budesonide, salbutamol, ipratropium) and assumed that in all rodent, canine, nonhuman primate  
502 and human, the pulmonary deposition fractions were 10%, 25%, 30%, and 40%. The allometric interspecies  
503 scaling equation obtained was:  $Y_h = Y_a (M_a / M_h)^{-b}$ . Where  $Y_h$  is the human drug dose ( $\mu\text{g}$ ),  $M_h$  is the human  
504 body mass (kg),  $Y_a$  is the animal drug dose ( $\mu\text{g}$ ),  $M_a$  is the animal body mass (kg),  $b$  is the allometric exponent.  
505 The average of the allometric exponents obtained in mouse, rat and human supports the current method of  
506 scaling using a fixed allometric exponent of 0.67 (Phillips, 2017). The utility of fixed exponent allometric  
507 scaling to project an inhaled human dose is pragmatic and could have a place in the drug development decision  
508 making process; however, it should be used cautiously as important simplifications were required (i.e. the  
509 pulmonary deposition fractions) and this calculation being performed on a small dataset ( $n=4$ ). Moreover, it may  
510 not be recommended for inhaled biotherapeutics, such as mAbs. Although the predictive value of allometry for  
511 mAbs is admitted with monkey data when mAb PK profile is linear, scaling up from animals to human is more  
512 complicated for mAbs with nonlinear PK because species differences (antigen density, antigen/FcRn interaction  
513 with mAbs, ...) should be considered (Kamath, 2016; J. Wang et al., 2016) and require more mechanistic  
514 approaches (PKPD models, ...).

515

#### 516 *4.3 Toxicology of inhaled biopharmaceuticals*

517

518 Aerosolized drugs may potentially have adverse effects ranging from subtle changes in respiratory  
519 function to alterations in lung biochemistry, immunology, permeability and potentially mortality. The accurate  
520 determination of the dose reaching the lung is of particular importance, but as for pulmonary delivery or PK-PD

521 studies, toxicology analysis of inhaled drugs will be susceptible to innate specificity of the animal model used  
522 (Figure 1), as well as the exposure system and the calculation method chosen.

523 As highlighted in Section I.B, it is generally accepted that the drug distribution throughout the lung will  
524 not be comparable to aerosol administration and that the toxicological profile of the aerosolized drug will be  
525 different to that of compounds administered intra-tracheally. Drugs or drug formulations are typically  
526 administered either as a nebulized solution/suspension, or as a dry powder (Owen, 2013). Currently, dry powder  
527 formulations comprise the majority of inhaled formulations under development. Due to the impracticalities of  
528 using clinical dry powder inhaler devices in toxicology studies, alternative aerosol generator devices have been  
529 designed for dosing of nonclinical species. Two systems are currently in use to generate dry powder aerosols for  
530 simultaneous dosing of typically up to 24 rodents in nose-only exposure cones or a variable number of dogs  
531 using facial masks: the Wright dust feeder and the relatively recent capsule-based aerosol generator (Armstrong  
532 et al., 2015; Paul et al., 2012). Regulatory studies based on Wright dust feeder always use aerosol  
533 administration, because this dosing method is considered closest to therapeutic dosing. In comparison, the  
534 capsule-based aerosol generator method is able to deliver similar lung doses over a large dosing range, but  
535 utilizes a lower amount of starting material and is therefore suitable for expensive compounds with limited  
536 available quantities (Paul et al., 2012).

537 The species used for regulatory toxicology studies are informed by regulatory guidelines, which require  
538 all assessments to be made in a rodent and non-rodent species, typically the rat and dog, although NHPs are also  
539 increasingly being used. It has been observed that certain forms of toxicity, both systemic and local, may be  
540 specific to the animal model and have little relevance for humans. Species-specific local toxicity or irritancy can  
541 often be observed in the nasal cavity of many experimental animals, especially obligate nasal breathers. This  
542 form of adverse event is typically not relevant for most inhaled products (i.e. pMDIs and dry powder inhalers)  
543 which are delivered via the oral cavity with no nasal exposure (Owen, 2013). However, nasal effects may be  
544 relevant for certain nebulized drugs for intended use in neonatal populations, as infants are also nasal breathers.  
545 Another adverse event often observed in rats, but not relevant for humans, is squamous metaplasia of the larynx.  
546 This local inflammation results from a high impaction of drug in the rat larynx following inhalation exposure,  
547 due to the nearly linear position of larynx behind the nasal cavity in rats. The immune response to the deposition  
548 may finally dictate the safety of the inhaled biotherapy.

549 Species-specific adverse events in the lung itself are rarer. It is widely recognized that rats are  
550 especially susceptible to lung overload with poorly soluble particulates. Lung overload occurs when the  
551 clearance mechanisms for removal of particulates, typically by macrophages, but also via particle entrapment in  
552 the mucus layer and removal via the mucociliary elevator, are saturated. Related to the phenomenon of lung  
553 overload in rats, is the effect of poorly soluble drug particles on alveolar macrophage populations. It has been  
554 reported that focal macrophage accumulations often with a “foamy” appearance are a frequent observation in  
555 non-clinical toxicology studies of dry powder formulations not only in rats, but non-rodent species as well  
556 (Forbes et al., 2014; Lewis et al., 2014). Above certain particle burdens, a progressive neutrophilic inflammation  
557 will develop with infiltration of inflammatory cells and elevated pro-inflammatory cytokine production. Chronic  
558 inflammation may lead to local tissue damage and fibrosis. In rats this can progress to neoplasia, although this  
559 has not been observed in humans or other nonclinical toxicology species (Owen, 2013; Wolff, 2015). The  
560 immunogenicity of biotherapeutics may also be an important issue. Anti-drug antibodies (ADA) may be

561 generated and have detrimental impact on drug safety, efficacy and pharmacokinetics (Smith et al., 2016).  
562 Although the induction of ADA is in many cases harmless, serious consequences might occur, as shown in  
563 human, by the current epidemic of the antibody-induced severe anemia that is associated with the use of a  
564 specific form of recombinant erythropoietin (Schellekens, 2002). Structural alterations of proteins secondary to  
565 the inhalation process can potentially favor the generation of aggregates that could augment protein-specific  
566 immune response and eventually lead to the formation of ADA (Sauerborn et al., 2010). It is acknowledged that  
567 ADA formation in nonclinical species may not be predictive of clinical immunogenicity. However, it is  
568 accepted practice to assess whether differences in the PK/PD profile of the biopharmaceutical between the first  
569 and last dose occur or whether there is evidence of immune-mediated toxicity in nonclinical studies. In such  
570 cases, ADA investigations may be warranted (McElroy et al., 2013).

571 A further issue of relevance for the design of safety assessment studies for biopharmaceuticals is the  
572 impact of exaggerated pharmacology. Since the toxicity of many biopharmaceutics is often related to their  
573 mechanism of action, for example hypoglycaemia following insulin administration, the dose increases routinely  
574 used in toxicology studies may induce a toxic pharmacologic response, which may be independent of the  
575 intrinsic toxicology. For this reason, ICH S6 guidelines require pharmacodynamic endpoints in nonclinical  
576 toxicology study design (McElroy, 2013). In such cases, the intrinsic and biological toxicity can and should also  
577 be assessed in the pharmacological species of interest, as well as animal models of disease. This can help to  
578 define whether the toxicity profile of the biopharmaceutical is primarily defined by intrinsic toxicity or  
579 exaggerated pharmacology (Cavagnaro, 2013).

580

## 581 **5. Future directions and conclusion**

582

583 Extrapolating data from animal studies to humans is a major challenge which is particularly true for  
584 inhaled medicines delivered through the pulmonary route. Because of the complexities and interspecies  
585 physiological, anatomical and metabolic differences in the respiratory tract, it can be difficult to bridge animal  
586 data to human patients inhaling a drug. This does not mean that animal models for aerosol drug delivery are  
587 irrelevant. They have contributed significantly to the development of all currently used pharmacological classes  
588 of drug that are delivered by topical administration to the lung, and they are helping with the development of  
589 inhaled biotherapeutics. From a regulatory point of view they are mandatory, and both rodent and non-rodent  
590 (usually dog or NHP) species are required for this purpose. Rodents are widely used in preclinical studies and  
591 useful, in particular to decipher mechanisms of action considering the availability of a large number of  
592 tools/reagents and transgenic models, particularly in mice. However, it is now assumed that the expansion in the  
593 use of mice for PD studies, over the past decade, has not been associated with a marked increase in the number  
594 of effective new classes of inhaled drugs. The predictability of murine models is particularly questioned for  
595 biotherapeutics, and accordingly, there would seem to be an urgent need to reassess the use of the most  
596 appropriate non-clinical species, depending on the class of the drug, to predict safety and efficacy in man.  
597 Although there are ethical considerations, larger mammals such as NHPs may be more predictable for inhaled  
598 biologics being closer to humans, on a biochemical, cellular and immunological point of view. Moreover, those  
599 drugs often display a high specificity for their target raising further concerns on the relevant species to select for  
600 preclinical development.

601 The difficulty to translate promising drug candidates for inhalation from animal models to humans  
602 underlines the need for more predictive models. Although the utility of *in vivo* studies is sometime questioned,  
603 animals remain irreplaceable so far, because current *in vitro/ex vivo* approaches still struggle to replicate the  
604 complex environment of the human respiratory tract and encounter similar limits as those of animal models  
605 when it comes to aerosol delivery. However, the progresses of the latest technologies are encouraging, offering  
606 great promises for the development of inhaled biotherapeutics. Among them, the following can be emphasized.  
607 Airways epithelial cells cultured at the air-liquid interface reproduce a physiological respiratory epithelium and  
608 may be combined with a dose-controlled aerosol-cell exposure system (ALICE CLOUD) to evaluate  
609 transepithelial absorption of aerosolized protein therapeutic (Röhm et al., 2017). Additional applications with  
610 this delivery system may include the impact of inhaled biotherapeutics on airways epithelium integrity. Organ-  
611 on-chip technology recapitulates the dynamic tissue-tissue interactions on a microdevice. As an example, lung-  
612 chips integrate several tissue compartments (endothelium, epithelium) where cells are submitted to the  
613 mechanical and fluidic forces they experienced in the body, and can be exposed to exogenous agents (cigarette-  
614 smoke, pathogens,...) to mimic pathological conditions (Benam et al., 2016; Huh et al., 2010). Future  
615 developments to connect this biomimetic microsystem to aerosol delivery systems may be helpful to study the  
616 pharmacodynamics and PK parameters with inhaled biotherapeutics. Today, *in silico* approaches provide  
617 realistic 3D micro-models of the human respiratory system that can be combined with simulation techniques,  
618 like computational fluid dynamics, to explore respiratory airflows and aerosol transport in the lungs, useful to  
619 predict inhaled drug deposition (Hofemeier et al., 2017). Thus, hybrid modeling using these emerging  
620 technologies could be a solution to refine existent animal models of aerosol delivery.

621 Although inhalation is clearly not suited for all medicines and has to be chosen based on solid scientific  
622 arguments, it is time to reconsider the most judicious use of non-clinical species in the development of inhaled  
623 medicines to ensure improved rates of successful translation to the clinic. Rather than opposing *in vivo* models  
624 with other approaches, future challenges will be probably to implement complimentary approaches that more  
625 precisely recapitulate the human respiratory complexity and aerosol delivery in order to help future preclinical  
626 developments of inhaled biotherapeutics.

627  
628

629 **Table 1**  
 630

<b>Comparison of imaging techniques for aerosol-based applications</b>		
<b>Technique</b>	<b>Advantages</b>	<b>Disadvantages</b>
Planar scintigraphy	Less expensive Validated methods for quantification Available standardized operating procedures	Only 2-D representation of lung anatomy
PET	Better resolution 3-D representation of lung anatomy Co-acquisition with MRI/CT	Use of radio-labeled element Limited half-life of positron emitters Expensive facilities
SPECT	Better resolution 3-D representation of lung anatomy Co-acquisition with MRI/CT	Use of radio-labeled element Long imaging time Complex quantification Expensive facilities
MRI	Promising technology, but still in development phase	

631

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1062

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1067

1068 **Figure legends**

1069

1070 **Figure 1.** Integrated scheme of advantages and limitations of animal models used to investigate aerosol drug  
1071 delivery in pulmonary research. Anatomical and physiological features were obtained from (Asgharian et al.,  
1072 2012; Crapo et al., 1983; Phalen and Oldham, 1983; Siegwart et al., 1971); animal pictures were obtained from  
1073 Servier Medical Art.

1074

1075 **Figure 2. Inter-species respiratory physiological differences.** Respiratory rates (A) and tidal volumes (B) are  
1076 expressed according to the body weight in several animal species (Asgharian et al., 2012; Bassett et al., 2014;  
1077 Cryan et al., 2007).

1078

1079 **Supplementary figure 1. Assessment of the lung distribution of a nebulized drug in a conscious non-**  
1080 **human primate.** An aerosol is delivered through a face mask connected to an aerosol generator in a  
1081 spontaneously breathing non-human primate (A). The lung deposition of the drug traced with  $^{99m}\text{Tc}$ -DTPA is  
1082 evaluated with a 2D-scintigraphic imaging sequence. The Regions of Interest (ROI) are: 1.lung, 2. ENT, 3.  
1083 stomach and 4. bladder (B).

1084

1085 **Supplementary figure 2. Intra-tracheal administration of an aerosol of a liquid solution containing a**  
1086 **fluorescent dye, in mice.** Briefly, anesthetized mouse was suspended by the teeth positioned on a platform  
1087 slanted at a  $45^\circ$  angle and a small-animal otoscope used to visualize the vocal cords and facilitate insertion of  
1088 the a MicroSprayer® Aerosolizer (connected to a high-pressure syringe) into the trachea (A). After delivering  
1089  $50\mu\text{L}$  of dye solution, kinetic of dye deposition into the lungs was followed up by near-infrared imaging (B).

1090

1091 **Supplementary figure 3. Important anatomy similarities exist between porcine and human lungs.** The  
1092 comparable size and anatomy of pig and human lungs make this model particularly beneficial for translational  
1093 research. The lungs of pigs are lobulated broadly similar to the human lung: the left lung is composed of a  
1094 cranial lobe and a caudal lobe, the right lung is composed of four lobes (cranial, middle, caudal, and accessory –  
1095 not observed in human). (A) Images of the porcine tracheo-bronchial tree obtained by flexible video-  
1096 bronchoscopy (here, the carena is observed). (B) Ex-vivo ventilated lungs of a 20 kg large-White pig.

1097

1098 **Supplementary figure 4. Methods of lung microdialysis.** A probe with a semi-permeable membrane is  
1099 implanted within the lungs under direct vision during thoracic surgery. The probe is continuously perfused with  
1100 a physiological solution (perfusate) at a very slow flow rate (usually 1 to  $10\mu\text{L}/\text{min}$ ) and the dialysate is  
1101 collected at timed intervals. It contains substances/drugs from the interstitial space fluid that diffused passively  
1102 through the membrane pores, along their concentration gradient.

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