Accepted Manuscript

Preclinical evaluation of aerosol administration systems using Positron Emission Tomography

Unai Cossío, Vanessa Gómez-Vallejo, María Flores, Braulio Gañ án-Calvo, Gloria Jurado, Jordi Llop

Please cite this article as: U. Cossío, V. Gómez-Vallejo, M. Flores, B. Gañ án-Calvo, G. Jurado, J. Llop, Preclinical evaluation of aerosol administration systems using Positron Emission Tomography, *European Journal of Pharmaceutics and Biopharmaceutics* (2018), doi: https://doi.org/10.1016/j.ejpb.2018.05.037

TITLE

Preclinical evaluation of aerosol administration systems using Positron Emission Tomography

AUTHORS AND AFFILIATIONS

Unai Cossío[a], Vanessa Gómez-Vallejo[b], María Flores[c], Braulio Gañán-Calvo[c], Gloria Jurado[c], Jordi Llop[a,*]

[a] Radiochemistry and Nuclear Imaging Group, CIC biomaGUNE, Paseo Miramón Nº 182, 20014 San Sebastián (Guipúzcoa), Spain

[b] Radiochemistry Platform, CIC biomaGUNE, Paseo Miramón Nº 182, 20014 San Sebastián (Guipúzcoa), Spain

[c] Ingeniatrics, C/Camino Mozárabe Nº41, 41900 Camas (Sevilla), Spain
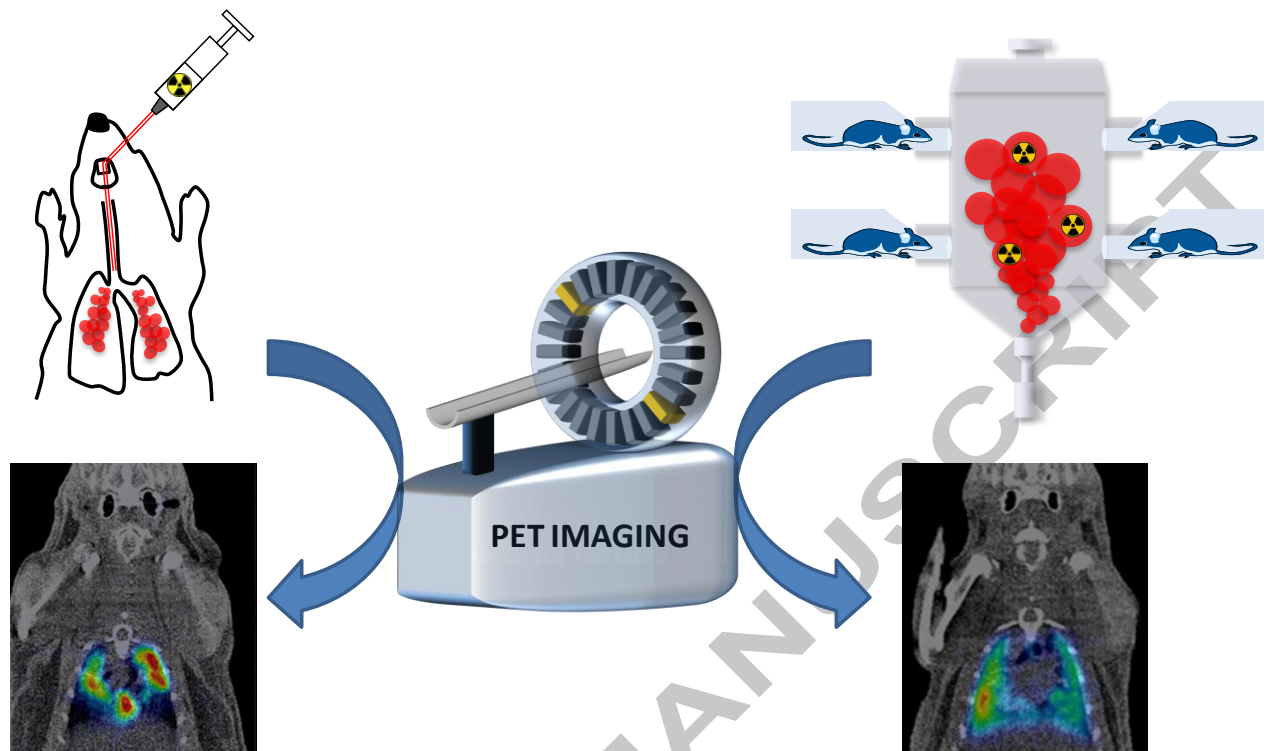
CORRESPONDING AUTHOR

*Jordi Llop

Tel.: +34 943 00 53 33

Fax: +34 943 00 53 01

E-Mail: jllop@cicbiomagune.es

GRAPHICAL ABSTRACT

ABSTRACT

Pulmonary administration of drugs has recently gained attention because it exhibits numerous advantages compared to oral or intravenous administration. The administration of aerosols for inhalation to animals, however, remains a critical challenge and only a few methods of administration have been developed. Herein, we compare the regional distribution of aerosols in the lungs of wild type rats after pulmonary administration using three different methods: (a) The Penn-Century MicroSprayer® Aerosolizer; (b) an in-house designed aerosol generation system; and (c) the Aeroneb® Lab Micropump Nebulizer. Both the regional distribution and the fraction of aerosol deposited in the lungs were determined by means of radiolabelling of the aerosol followed by *in vivo* and *ex vivo* Positron Emission Tomography (PET) imaging and dissection and gamma counting. Endotracheal nebulisation using the PennCentury MicroSprayer resulted in >85% of the administered dose accumulated in the lungs, with a non-uniform distribution of the radioactivity in different lobes and a low animal-to-animal reproducibility. Administration using the in-house designed and the Aeroneb nebulizers resulted in a uniform distribution over the lungs, but only a small fraction of the nebulized activity (ca. 0.1%) was deposited in the lungs.

KEYWORDS

PET, Positron Emission Tomography, Lung administration, Nebulisation, Aerosol.

1. INTRODUCTION

Lung administration of drugs has recently gained attention (Loira-Pastoriza et al. 2014), especially when the lung is the target organ, e.g. to treat lung cancer (Garrastazu Pereira et al. 2016) or airway diseases such as asthma (Kwok et al. 2014; Hoch et al. 2016), cystic fibrosis (Heijerman et al. 2009) and chronic obstructive pulmonary disease (Ariel et al. 2018). Pulmonary administration offers numerous advantages as a delivery route compared to oral or intravenous administration. The bioavailability of drugs can be enhanced, because contrary to gastrointestinal tract and liver, the lung possesses limited intracellular and extracellular drug metabolizing enzyme activities (Loira-Pastoriza et al. 2014). In contrast, lung administration usually leads to higher absorption rate, reduced drug doses and rapid onset of action (Karathanasis et al. 2005; Loira-Pastoriza et al. 2014).

Preclinical studies conducted with various animal models are critical endpoints in the development of an inhaled drug formulation (Degeorge et al. 1997). In the particular context of aerosols for inhalation, the administration into animals is a critical challenge especially in small rodent species, and only a few methods of administration have been developed so far. Endotracheal and intratracheal insufflations have the advantage that they enable specific deliver of the drug to the lung, with high predictability in terms of delivered dose. Additionally, compared to intratracheal administration (via an incision into the trachea), endotracheal insufflations are non-invasive and can be used in repeated dose studies. Two major disadvantages of this method are the potential induction of lesions due to the introduction of the insufflators and the need to maintain the animals under anaesthesia during the administration, which can cause some problems in certain scenarios (Guillon et al. 2012). Alternatively to endo/intratracheal insufflations, nose-only exposure inhalation towers can be used for pulmonary administration of aerosols to small rodents (Asgharain et al. 2003; Cosnier et al. 2016). In this case, the aerosol reaches the lungs through natural respiration, and hence this method is perceived to result in a

4

homogeneous distribution of the aerosol within the lungs. Additionally, several animals can be exposed simultaneously. One major drawback is the need to keep the animals under anaesthesia during long periods of time. This limitation can be overcome by establishing a training program to ensure that animals are accustomed to the exposure tubes (Fuhst et al. 2013), although this process is time-consuming.

Irrespective of the administration strategy, two critical factors have to be considered in lung administration: (i) the dose actually deposited in the lungs, which is critical when planning dose-dependent pre-clinical studies, such as toxicity, pharmacokinetics and efficacy studies (Duret et al. 2012); and (ii) the regional distribution within the lungs, which is of utmost importance because uniform distribution among the different lung nodes is required both to guarantee an effective treatment and to prevent toxic or side effects due to local, potentially harmful deposition of the drug.

One of the main challenges in addressing the above mentioned points is that the drugs (or the aerosols containing the drugs) are extremely difficult to detect and quantify once distributed in a biological system. An alternative that overcomes this difficulty consists of labelling the drug or the aerosol with a fluorescent tag, enabling their localization *in vivo* using optical imaging. For example, in a recent study a newly developed aerosol generator was tested for pulmonary administration to the lungs of mice and compared to an endotracheal insufflator (Tonnis et al. 2014), using Fluorescence Molecular Tomography (FMT) and ovalbumin as a model protein labelled with a fluorescent dye. In spite of the suitability of this approach, optical imaging techniques have severe limitations *in vivo*, mainly due to the poor penetration capacity of visible or infrared light in tissues, which limits the application of the technology to small rodents. Additionally, quantification of the images is extremely challenging. An alternative to overcome these drawbacks consists of labelling the drug or the aerosol with a positron emitter (Lizal et al. 2018). The disintegration of the positron emitter leads ultimately to the generation of high energy

gamma rays, which have virtually no penetration limits and can be externally detected and processed to generate three dimensional (3D)-images. Such images provide quantitative information about the spatiotemporal distribution of the labelled specie (Vaquero et al. 2015). This approach has been applied to evaluate changes in the deposition of inhaled aerosols within the lung related to the presence of disease or resulting from inhalation challenge interventions or inhaled therapies in the clinical field (Dolovich 2009).

In the context of the EU funded project PneumoNP (FP7-NMP-604434), our consortium has developed novel nanosystems to treat drug-resistant lung infections, to be assayed in therapeutic experiments in a rat model using lung administration. In order to select the most convenient administration strategy, three different options were considered: (i) endotracheal insufflation using a MicroSprayer® Aerosolizer (PennCentury^{TM}); (ii) inhalation using an in-house manufactured nebulizer developed at Ingeniatrics (Seville, Spain); and (iii) inhalation using a commercially available nebulizer. In all cases, the aerosol was labelled with 2-deoxy-2-(^{18}F)fluoro-D-glucose ([^{18}F]FDG), a radiotracer widely used in the clinical field for the early diagnostic and evaluation of the response to treatment of different cancer types. For the three administration methods, the regional distribution of the aerosol within the lungs and the percentage of administered dose deposited in this organ were evaluated using *in vivo* PET imaging and complementary *ex vivo* techniques such as *ex vivo* PET imaging and dissection/gamma counting.

2. MATERIALS AND METHODS

2.1. Animal experiments: general

All animal experiments were performed in accordance with the Spanish policy for animal protection (RD53/2013), which meets the requirements of the European Union directive 2010/63/UE regarding the protection of animals used in experimental procedures. All

experimental procedures were approved by the Ethical Committee of CIC biomaGUNE and authorized by the local authorities.

2.2. Aerosol administration

Six-to-eight weeks-old female Sprague Dawley rats (Janvier, Le Genest-Saint-Isle, France) weighting ca. 225 g were used in all the experiments.

2.2.1. Endotracheal insufflations:

Endotracheal insufflations were carried out using the Penn-Century MicroSprayer® Aerosolizer (FMJ-250 High Pressure Syringe Model, Penn-Century. Inc. Wyndmoor, USA; henceforth "Penn-Century Aerosolizer"). Deep sedation was induced to the animals (n = 6) by inhalation of 5% isoflurane in pure O_2. The tip of the delivery needle was carefully positioned just above the carina and a pre-defined volume (established by using spacers in the syringe plunger) of injectable [^{18}F]FDG solution (Provided by Iba Molecular Spain and diluted with ultrapure water 1:25; 50 µL; amount of radioactivity around 1.85 MBq) was administered. A small animal Laryngoscope (Penn-Century, Model LS-2) was used for correct visualization of the epiglottis. Immediately after administration, rats were divided in two groups (group 1: n = 3; group 2: n = 3), and submitted to *in vivo* and *ex vivo* imaging experiments, respectively (see below).

2.2.2. Inhalation using an in-house developed aerosol generation system:

The aerosol inhalation system developed at Ingeniatrics (henceforth "Ingeniatrics nebuliser") consisted of the following parts: (a) Aerosol generation unit with Flow Blurring® (FB) technology. The nozzle (FB240) had an equal exit orifice diameter and inner tube diameter of 240 µm; (b) a peristaltic pump to push the labelled solution to be nebulised at a constant flow rate; (c) a compressed air supply, equipped with a pressure regulator and a pressure meter; (d) main nebulisation and distribution chamber; and (e) an exit outlet to prevent overpressure in the main chamber (see Figure 1).

The nebuliser was placed at the bottom of the nebulisation chamber, which was directly connected to four rat holders coupled with rubber adaptors to enable appropriate positioning of the animals and only-nose exposure to the aerosol. An exit outlet was also installed at the top of the nebulisation chamber to avoid overpressure in the system and to trap the radioactive aerosol exiting the main chamber. For this purpose, two washing bottles were connected in series and a vacuum pump was installed to keep the system at slight under-pressure (-50 Pa), thus ensuring that all the radioactive material was properly trapped.
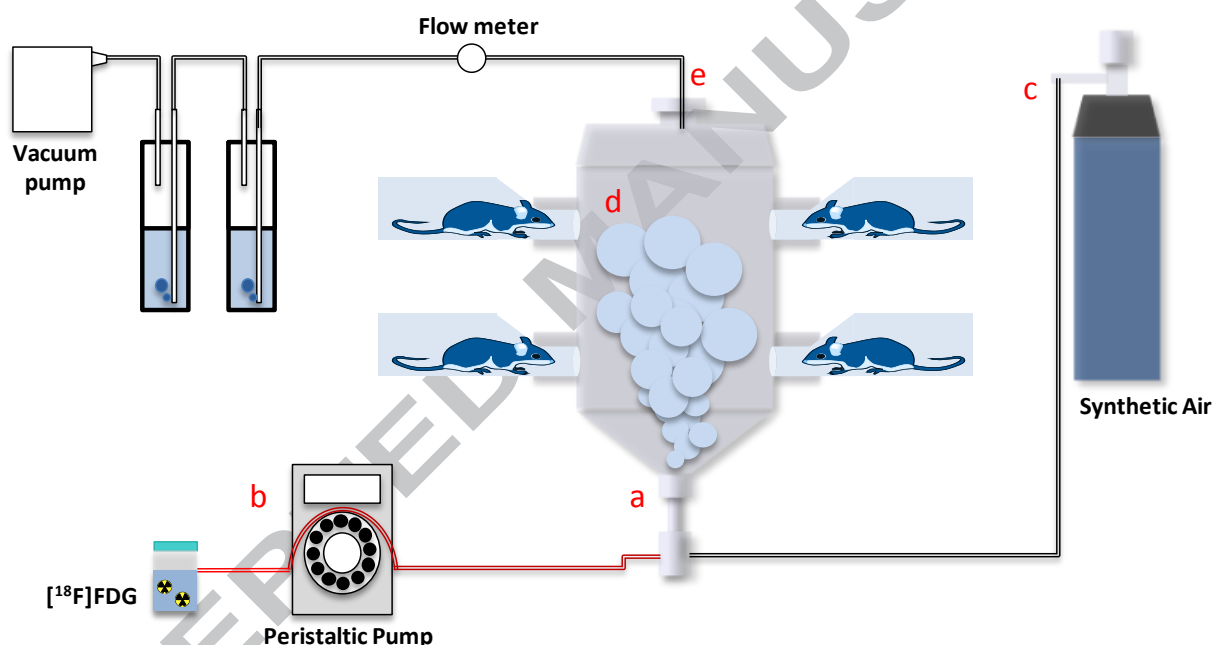

Figure 1. Aerosol generation system designed to simultaneously expose 4 rats: a) FB240 Nozzle; b) peristaltic pump; c) compressed air bottle; d) nebulisation chamber with four rat holders; and e) exit outlet.

To conduct inhalation studies, rats (n = 6) were anesthetized by an intraperitoneal (IP) injection of a mixture of medetomidine, midazolam and fentanyl (0.6, 6 and 0.02 mg/Kg, respectively). Once animals were under sedation, they were introduced into identical cylindrical oro-nasal

exposure holders. Then, a solution (1.5 mL) containing [^{18}F]FDG (Provided by Iba Molecular Spain and diluted with ultrapure water 1:12; amount of radioactivity: 370 ± 10 MBq) was aerosolized using the system described above. Animals were exposed in groups of 2, using the holders located at the lower part of the nebulisation chamber. Experimental conditions were: Liquid flow = 0.15 mL/min; air pressure = 4500 mbar; air flow = 1.6 L/min. After finalizing the exposure, animals were randomly divided in two groups (group 3: n = 3; group 4: n = 3), and submitted to *in vivo* and *ex vivo* imaging experiments, respectively (see below).

2.2.3. Inhalation using the Aeroneb® Lab Micropump Nebulizer:

For inhalation studies using the commercially available Aeroneb® Lab Micropump Nebulizer (henceforth "Aeroneb nebulizer"), animals (n = 6) were submitted to the same procedure as described above (section 2.2.2), using an inhalation configuration as depicted in Figure 2. Actually, the system used in our experiments was an adaptation of a device currently used in humans, the Aerogen® Pro nebulizer (Aerogen, Dangan, Ireland) and allowed exposure of one animal at a time, by using a rat nebulizer Delivery System (AG-ALSMHLD-RAT, Kent Scientific Corporation, Connecticut, USA). In this case, the generation of the aerosol relies in the presence of a vibrating membrane just below the nebulizer's reservoir, enabling fast administration of medicines to patients with very low residual losses (Knoch et al. 2005; Lass et al. 2006). The droplet size can be tuned by appropriate selection of the membrane's pore size, which in our case was chosen to generate droplets within 4-6 µm in diameter. The air flow, used to push the aerosol towards the animal, was set to 100 mL/min. The total nebulised volume of FDG (Provided by Iba Molecular Spain and diluted with ultrapure water 1:4) was 0.5 mL containing 370 ± 10 MBq (total exposure time = 5 min). After exposure, animals were randomly divided in two groups (group 5: n = 3; group 6: n = 3), and submitted to *in vivo* and *ex vivo* imaging experiments, respectively (see below).

9

Figure 2. Illustration of the Aeroneb Lab Micropump Nebulizer connected to the rat nebulizer Delivery System and the rat holder.

2.3. Droplet size measurements

Aerosols generated by Penn-Century Aerosolizer and the Ingeniatrics nebuliser were characterized using a Sympatec Helos/BF Magic laser diffraction system (HELOS, Sympatec, Clausthal-Zellerfeld, Germany). In brief, the nebulizer was clamped into a stand and the mouthpiece exit of the nebulizers positioned 50 mm from the Fourier lens face and 100 mm from the laser beam. The formulation (appropriate dilution of completely decayed [^{18}F]FDG solution with ultrapure water) was then nebulised through the laser beam. The measurements were performed with six runs of 100 ms duration each. The droplet diameter distributions were parameterised by their Mean Droplet Size Distribution (MDSD) and Geometric Standard Deviation (GSD).

2.4. In vivo imaging experiments

Without recovering from sedation, animals from experimental groups 1, 3 and 5 were positioned in an eXploreVista-CT small animal PET-CT system (GE Healthcare, USA) to perform *in vivo* studies. For animals in groups 3 and 5, still under the effects of IP anaesthesia, no additional

sedation was required during image acquisition. For animals in group 1, anaesthesia was maintained by inhalation of 1.5-2% isofluorane in pure O_2.

During imaging, rats were kept normothermic using a heating blanket (Homeothermic Blanket Control Unit; Bruker). Dynamic PET images (energy window: 400-700 KeV) were acquired with the following frames: 4 x 15 s, 4 x 30 s, 3 x 60 s and 3 x 90 seconds. In all cases, four beds were defined to acquire whole body images (total acquisition time = 42 min). After each PET acquisition, a CT scan (X-Ray energy: 40 kV, intensity: 140 µA) was performed for a later attenuation correction in the image reconstruction and for unequivocal localization of the radioactivity. Random and scatter corrections were also applied to the reconstructed image (filtered back projection reconstruction algorithm), generating a 175x175x220 dimension image, with a 2 mm axial FWHM spatial resolution in the centre of the Field Of View (FOV).

PET-CT images of the same animal were co-registered and analyzed using PMOD image processing tool. First, Volumes of interest (VOIs) were manually delineated on the whole lungs to assess the amount of radioactivity deposited in this organ. Time–activity curves (decay corrected) were obtained as cps/cm^3 in each VOI and values were then normalised to the starting amount of radioactivity. For each experimental dataset, an exponential equation was adjusted in order to determine the residence time of [^{18}F]FDG in the lungs. Additionally, small spherical VOIs (diameter = 1 mm) were drawn in the different lung lobes, and the average concentration of radioactivity per VOI within each lung region was determined, in order to assess the regional distribution of the radioactivity over the lungs. Values were expressed as the amount of radioactivity per VOI normalised to the total amount of radioactivity in all VOIs, in percentage.

2.5. Ex vivo imaging experiments

Without recovering from sedation, animals from experimental groups 2, 4 and 6 were sacrificed by cervical dislocation immediately after administration of the labelled aerosol. In all cases, the time gap between the end of the exposure to the aerosol and the sacrifice time was less than 10

seconds. The respiratory system of the rats (trachea and both lungs) was fixed with formalin (10% formalin solution, neutral buffered) and subsequently harvested. PET-CT images of the extracted organs were acquired for 60 minutes (n = 3 per group). Image reconstruction and analysis was performed as above, although in this case exponential curves were not adjusted because static images were acquired. After finishing the imaging session, the lungs were dissected into different lobes, and the amount of radioactivity in each lobe was accurately determined by gamma counting (WIZARD2 Gamma Counter, PerkinElmer, Waltham, USA).

3. RESULTS AND DISCUSSION

3.1. Aerosol droplet size measurements

For inhalation, the most important characteristic of an aerosol is the respirable dose, which is the fraction of droplets in the respirable range. With the aim of having a homogeneous distribution along the whole respiratory track, main requirement in terms of droplet size is having a MSDS below 4 microns with a high percentage of droplets with a diameter in the range 0.9-5.25 μm (Patton et al. 2007).

The droplet size obtained after nebulization depends on the electrolyte concentration of the solution, as recently reported (Beck-Broichsitter et al. 2017). For this reason, in the current work the aerosol droplet size was determined with a proper dilution of the [^{18}F]FDG solution with purified water, in order to obtain representative results for *in* vivo experiments. The [^{18}F]FDG solution provided by IBA Molecular Spain contained 10 mg/mL of NaCl, 1.5 mg/mL of NaH$_2$PO$_4$, and 0.4 μg/mL of ethanol, and dilutions with purified water 1:25, 1:12 and 1:4 were carried out to assess droplet size using the Penn-Century Aerosolizer, the Ingeniatrics nebuliser and the Aeroneb nebuliser, respectively.

For the system developed at Ingeniatrics, the aerosol is produced using a nebulizer based on Flow Blurring® technology, which is a purely mechanical atomization technique that produces highly turbulent pre-mixing between gas and liquid streams. As a consequence of this special regime,

very good quality aerosols with ultrafine droplets are obtained. In this scenario, and given a specific geometry, droplet size distribution can be selected by choosing the proper set of operational parameters. The influence of such parameters in droplet size distribution was investigated using the Ingeniatrics nebuliser (Table 1). Several liquid flow rates and air pressures were evaluated using water as a model liquid. As it can be seen, a wide range of experimental conditions accomplished MSDS<4 µm and a high percentage of droplets in the range of 0.9-5.25 µm. The conditions corresponding to entry 3, with an air flow of 1.6 mL/min, were used for inhalation experiments using the radiolabelled aerosol. Under these conditions, MSDS values of 2.1 µm with 75.7% of the droplets in the desired range could be achieved.

Table 1. Summary of the properties of the aerosols produced with water using the Ingeniatrics nebuliser at different liquid flow rates and air pressures (ΔP); MDSD = Mean Droplets Size Distribution.

Entry	Liquid Flow rate (mL/min)	ΔP (bar)	MDSD (µm)	% droplets 0.9< MDSD <5.25 µm
1	0.25	4.5	2.1	75.0%
2	0.20	4.5	2.1	75.3%
3	0.15	4.5	2.1	75.7%
4	0.25	4.0	2.2	73.9%
5	0.20	4.0	2.2	74.1%
6	0.15	4.0	2.2	74.4%
7	0.25	3.0	2.4	70.3%
8	0.20	3.0	2.4	70.7%
9	0.15	3.0	2.4	70.9%
10	0.25	2.0	2.9	63.8%
11	0.20	2.0	2.8	64.3%

The aerosol produced by the Penn-Century Aerosolizer resulted in significantly higher droplet size, in the range of 20 µm, which was in great accordance with the value specified by the manufacturer. In this case, only a small fraction (2.6%) of the droplets fell within the desired range (0.9-5.25 µm). Evaluation of the droplet size in the aerosol generated with the Aeroneb nebulizer resulted in MSDS of 4.52±1.59 µm, confirming that most of the droplets were within

the respirable range. These results are well aligned with previously reported values using the same system (Ghazanfari et al. 2007; Zhang et al. 2007; Beck-Broichsitter et al. 2012; Beck-Broichsitter et al. 2017).

3.2. In vivo imaging experiments

Evaluation of the dynamic images obtained after administration by endotracheal insufflation using the Penn-Century Aerosolizer showed progressive elimination of the radioactivity from the lungs. However, more than 85% of the starting radioactivity was still in the lungs after 10 minutes, suggesting slow clearance from this organ.

PET images acquired immediately after administration of [^{18}F]FDG using the three nebulisation methods showed different distribution patterns within the lungs (Figure 3).
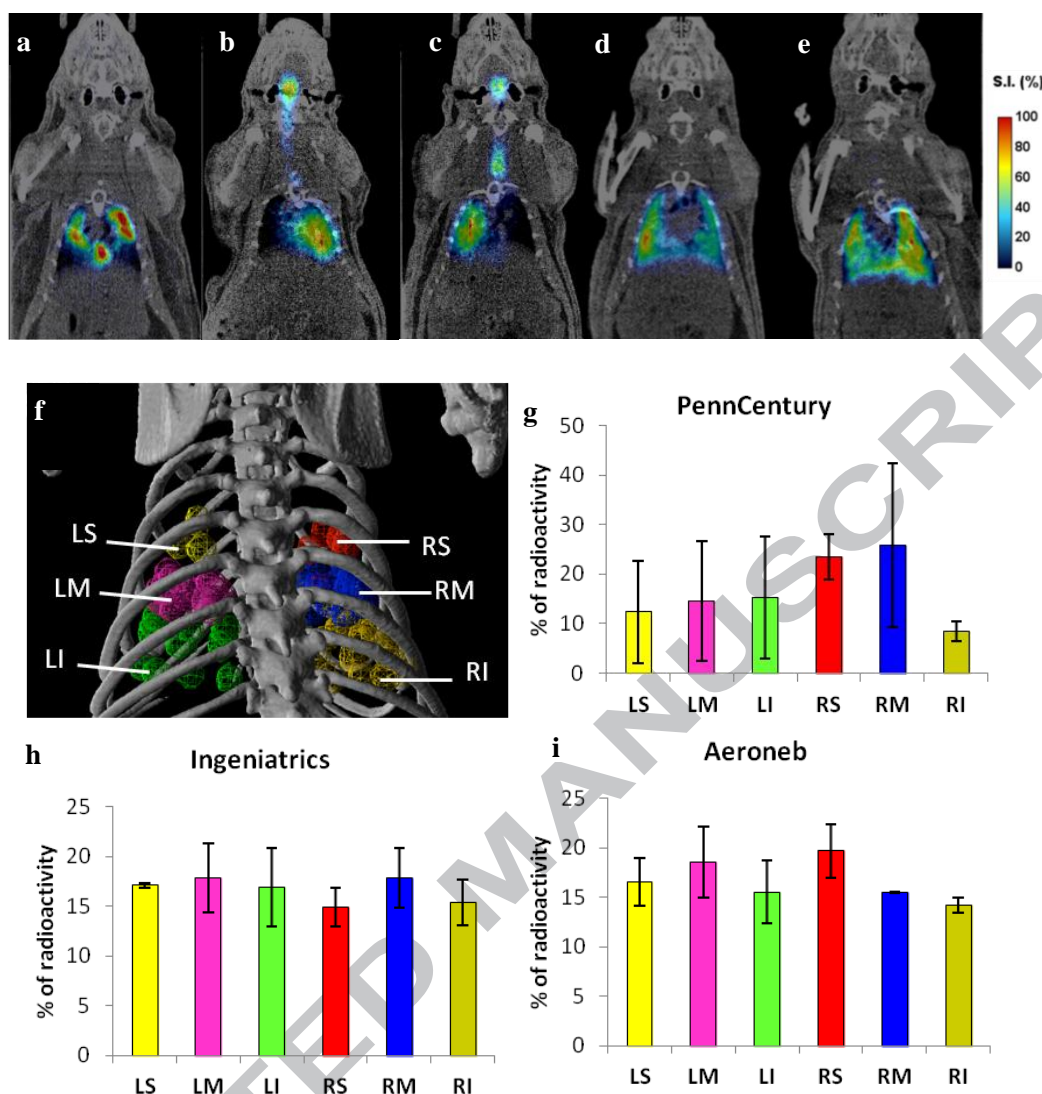
Figure 3. (a-e) Representative coronal PET-CT rat images obtained immediately after administration of [^{18}F]FDG by (a-c) endotraqueal insufflation using the Penn-Century Aerosolizer (3 different animals); (d) inhalation using the Ingeniatrics nebuliser; and (e) inhalation using the Aeroneb nebulizer; (f) VOIs drawn in the different regions of the lungs; (g-i) percentage of radioactivity in each VOI after administration using the Penn-Century Aerosolizer (g), Ingeniatrics nebuliser (h) and Aeroneb nebuliser (i). LS: left superior; LM: left medium; LI: left inferior; RS: right superior; RM: right medium; RI: right medium; values in (g-i) are expressed as mean±standard deviation, n=3.

When [^{18}F]FDG was administered by endotraqueal insufflation using the Penn-Century Aerosolizer, the radioactive aerosol was not uniformly deposited in the whole lungs. Indeed, minute involuntary tilts of the microsprayer when administering the dose resulted in the

deposition of the aerosol in only one lung, a phenomenon which could be observed even after mastering the technique (Figures 3a-3c). Of note, even when the aerosol clearly reached both lungs (Figure 3a), the regional distribution was not uniform within the lungs, and the presence of hot spots and under-exposed regions could be observed by visual inspection of the images. Generally, the lower regions of the lungs received systematically less amount of aerosol that the upper regions.

Administration of the labelled aerosol using any of the two nebulisers resulted in a more uniform distribution of the aerosol within the whole lungs, as clearly seen by visual inspection of the images (Figures 3d and 3e). The images were quantified to get precise data regarding the amount of radioactivity present in each of the lung lobes. With that aim, and using PMOD image analysis software, different spherical volumes of interest (VOIs) were drawn in the different regions of the lungs. The lungs were divided in six different regions (Figure 3f): left superior lobe (LS), left middle lobe (LM), left inferior lobe (LI), right superior lobe (RS), right middle lobe (RM) and right inferior lobe (RI), and VOIs were drawn in the different regions. Quantitative data (Figures 3g-3i) clearly show the lack of uniformity in the deposition of aerosol in the lungs when the Penn-Century Aerosolizer was used. Indeed, Figures 3h and 3i show that the relative concentration of radioactivity in the different lobes is similar, while small standard deviation values are obtained, confirming the low intra-subject variability. Contrarily, Figure 3g confirms that endotracheal insufflation results in non uniform distribution of radioactivity, while the high standard deviation values reflect the high inter-subject variability.

The PET images obtained *in vivo* were not used to determine the fraction of the dose deposited in the lungs, as these values could be reliably obtained from *ex vivo* studies. In our case, image acquisition was not synchronised with the respiratory and cardiac cycles, and hence absolute quantification of the whole lung could be subjected to motion artefacts. Such artefacts can be neglected during quantification of the spherical VOIs, as these were strategically located within

the region of the organ not affected by lung and heart motion. Hence the determination of regional distribution is reliable. Indeed, our results are well aligned with previous works (Tonnis et al. 2014), in which the authors reported a lack of uniformity within the lungs when the Penn-Century insufflators was used in mice. Such lack of uniformity need to be considered when e.g. therapeutic studies are conducted in the preclinical setting, as unexposed areas may remain undertreated while over-exposed regions may result in localised toxicological effects.

3.3. Ex vivo imaging experiments

Ex vivo imaging experiments were carried out to confirm *in vivo* findings and to obtain accurate information regarding the fraction of the dose deposited in the lungs. With that aim, immediately after finishing PET acquisition, animals were sacrificed, lungs were fixed with formalin and ex vivo PET-CT images were acquired. The advantage of *ex vivo* imaging with respect to *in vivo* imaging relies in two facts: (i) there are no motion artefacts due to animal respiration, and (ii) images can be acquired for long times; this is not the case of *in vivo* imaging, where the acquisition time is limited by the total time that the animals can remain under anaesthesia. Hence, higher resolution images can be expected from *ex vivo* imaging. Taking into account that >85% of the administered [^{18}F]FDG remains in the lungs at t=10 min after administration and that the time gap between end of exposure and animal sacrifice was less than 10 seconds, we assumed that lung clearance during this time gap could be neglected.

Visual inspection of the images showed similar results to those obtained *in vivo*, this is, uniform deposition within the lungs when the Ingeniatrics and the Aeroneb nebulisers were used (see Figures 4a-4c for example of images) and low uniformity when the Penncentury Aerosolizer was used.

The lungs were finally divided into different lobes and the amount of radioactivity was determined in a gamma counter (Figures 4d and 4e). Again, gamma counting results confirmed the results obtained *in vivo*. When [^{18}F]FDG was administered using the Penn-Century

Aerosolizer, the amount of radioactivity in the different lobes of the lungs varied significantly. Additionally, high standard deviation values confirm the high inter-subject variability. When the radiotracer was administered by inhalation, the amount of radioactivity was homogenously distributed within the lungs.

The results also confirmed that endotraqueal insufflation was the most efficient administration method, leading to almost quantitative deposition of the aerosol in the lungs ($85\pm3\%$). Indeed, the rest of the radioactivity could be either absorbed on the walls of the syringe or in the trachea (see Figures 3b and 3c), the latter being contaminated during introduction or withdrawal of the delivery needle.

On the contrary, both aerosol inhalation systems resulted in poor deposition efficiency. Indeed, only 0.07 ± 0.01 and 0.08 ± 0.02 of the aerosolised dose reached the lungs when Ingeniatrics or Aeroneb nebulisers were used. The low dose deposited in the lungs using the Ingeniatrics nebulizer is due to a combination of factors, mainly: 1) The tidal volume of Sprague Dawley rats is around 0.29 mL/100g in resting conditions (Strohl et al. 1997). Considering that under our anaesthetic conditions rats maintained an average breathing frequency of 60 breaths/minute, the inhaled air volume for each rat was estimated to be around 39 mL/min for a rat weight of 225g. Taking into account that the air flow rate in the nebuliser was set to 1.6 L/min (required for a proper generation of the aerosol) a simple mass balance provides a maximum inhaled fraction of 2.4% of the total aerosol, assuming that tidal volume is not significantly affected by the anaesthesia. Additionally, a severe condensation of the aerosol in the main chamber could be observed during nebulisation. The fraction of the aerosol condensed was estimated by recovering the condensed fraction and measuring the amount of radioactivity. The results showed that approximately 75% of the nebulised solution was indeed condensed in the walls of the chamber, and hence was not available to be breathed by the animals. Altogether, these results confirm that the maximal theoretical inhalable fraction per animal is close to 0.6%. For the Aeroneb nebuliser,

the inhaled fraction was similar to that obtained for the Ingeniatrics nebuliser, despite in this case the air flow was set to 100 mL/min and hence higher dose in the lungs could be expected. The low values obtained might be due to severe condensation, which was observed although in this case could not be measured due to the difficulties in recovering the condensed fraction.
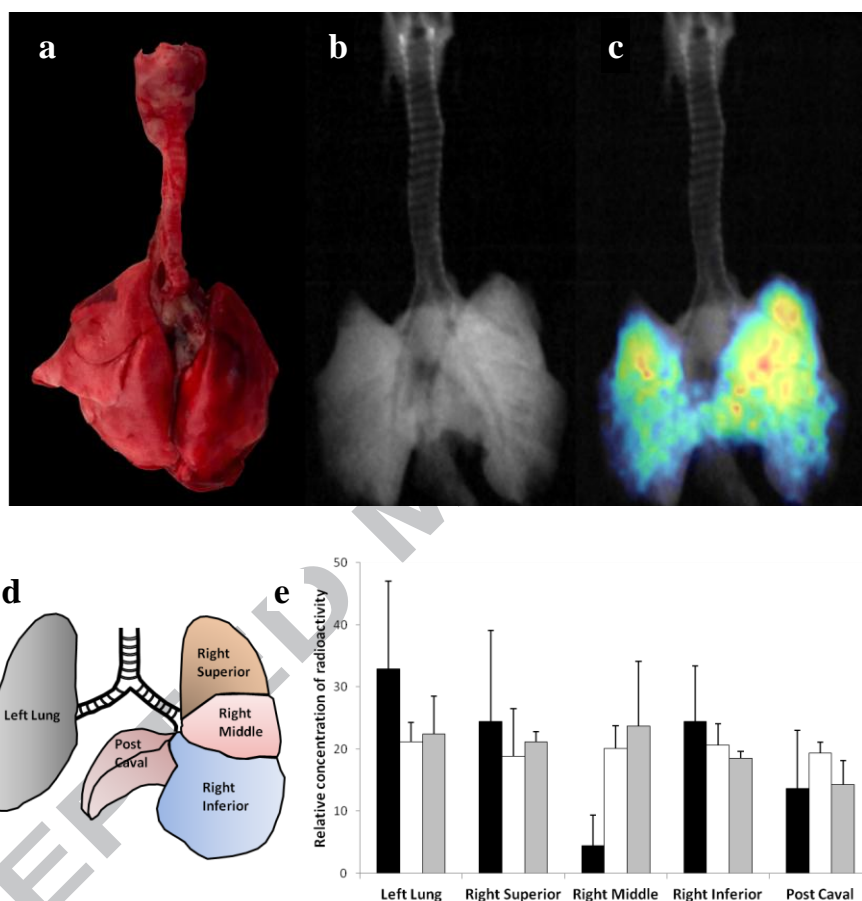

Figure 4. (a) Photograph of the harvested, fixed lungs of a rat after exposure to inhalation of [^{18}F]FDG using the Aeroneb nebuliser; (b) Coronal CT image of the harvested lungs; (c) coronal PET-CT *ex vivo* image of the harvested lungs; (d) schematic illustration of the different lung lobes; (e) relative concentration of radioactivity in each lobe after endotraqueal insufflation using the Penn-Century Aerosolizer (black bars), inhalation using Ingeniatrics nebuliser (white bars), and inhalation using Aeroneb nebuliser (grey bars).

All together, our results suggest that the administration tool used for the delivery of aerosols in the pre-clinical setting has a huge impact both in the dose deposited in the lungs and in the regional distribution within the lungs. This fact, which has been widely explored in the clinical

setting using nuclear imaging techniques (Eberl et al. 2001; Brand et al. 2008; Behr et al. 2009), has remained mostly unexplored in the pre-clinical setting. Taking into account that preclinical studies always precede clinical trials, our results suggest that studies to investigate both parameters should be routinely conducted for a proper interpretation of the results.

4. CONCLUSIONS

The work reported here shows that Positron Emission Tomography is a very useful tool for the evaluation of the regional distribution of aerosols in the lungs. In our case, radiolabelling of the aerosol using $[^{18}F]FDG$ enabled the investigation of the regional distribution within the lungs, while dissection and gamma counting enabled the determination of the fraction of the dose actually deposited in the lungs after administration using endotracheal insufflation and two different nebulisers. While endotracheal insufflations resulted in ca. 85% of the aerosols reaching the lungs, uniform distribution could not be achieved, with most of the dose deposited in the upper lobes and with high inter-subject variability. On the contrary, nebulisation using the two different nebulizers resulted in a homogeneous distribution within the lungs, although only <0.1% of the aerosol was actually deposited in the lungs. Both the fraction of the aerosol reaching the lungs and the regional distribution may have a huge impact in putative therapeutic or toxicologic studies. Hence, our results confirm that thorough investigation of aerosol generators is paramount in the pre-clinical context, and that nuclear imaging techniques may offer a valid alternative.

REFERENCES

Ariel, A., A. Altraja, et al. (2018). "Inhaled therapies in patients with moderate COPD in clinical practice: Current thinking." *International Journal of COPD* **13**: 45-56.

Asgharain, B., J. T. Kelly, et al. (2003). "Respiratory deposition and inhalability of monodisperse aerosols in Long-Evans rats." *Toxicological Sciences* **71**(1): 104-111.

Beck-Broichsitter, M., P. Kleimann, et al. (2012). "Nebulization performance of biodegradable sildenafil-loaded nanoparticles using the Aeroneb Pro: formulation aspects and nanoparticle stability to nebulization." *Int J Pharm* **422**(1-2): 398-408.

Beck-Broichsitter, M. and N. Oesterheld (2017). "Electrolyte type and nozzle composition affect the process of vibrating-membrane nebulization." *Eur J Pharm Biopharm* **119**: 11-16.

Behr, J., G. Zimmermann, et al. (2009). "Lung deposition of a liposomal cyclosporine A inhalation solution in patients after lung transplantation." *J Aerosol Med Pulm Drug Deliv* **22**(2): 121-130.

Brand, P., B. Hederer, et al. (2008). "Higher lung deposition with Respimat Soft Mist inhaler than HFA-MDI in COPD patients with poor technique." *Int J Chron Obstruct Pulmon Dis* **3**(4): 763-770.

Cosnier, F., S. Bau, et al. (2016). "Design and characterization of an inhalation system to expose rodents to nanoaerosols." *Aerosol and Air Quality Research* **16**(12): 2989-3000.

Degeorge, J. J., C. H. Ahn, et al. (1997). "Considerations of toxicology studies of respiratory drug products." *Regulatory Toxicology and Pharmacology* **25**(2): 189-193.

Dolovich, M. B. (2009). "18F-fluorodeoxyglucose positron emission tomographic imaging of pulmonary functions, pathology, and drug delivery." *Proceedings of the American Thoracic Society* **6**(5): 477-485.

Duret, C., N. Wauthoz, et al. (2012). "In vitro and in vivo evaluation of a dry powder endotracheal insufflator device for use in dose-dependent preclinical studies in mice." *European Journal of Pharmaceutics and Biopharmaceutics* **81**(3): 627-634.

Eberl, S., H. K. Chan, et al. (2001). "Aerosol deposition and clearance measurement: a novel technique using dynamic SPET." *Eur J Nucl Med* **28**(9): 1365-1372.

Fuhst, R., F. Runge, et al. (2013). "Toxicity profile of the GATA-3-specific DNAzyme hgd40 after inhalation exposure." *Pulmonary Pharmacology and Therapeutics* **26**(2): 281-289.

Garrastazu Pereira, G., A. J. Lawson, et al. (2016). "Loco-regional administration of nanomedicines for the treatment of lung cancer." *Drug Delivery* **23**(8): 2881-2896.

Ghazanfari, T., A. M. Elhissi, et al. (2007). "The influence of fluid physicochemical properties on vibrating-mesh nebulization." *Int J Pharm* **339**(1-2): 103-111.

Guillon, A., J. Montharu, et al. (2012). "Pulmonary delivery of dry powders to rats: Tolerability limits of an intra-tracheal administration model." *Int J Pharm* **434**(1-2): 481-487.

Heijerman, H., E. Westerman, et al. (2009). "Inhaled medication and inhalation devices for lung disease in patients with cystic fibrosis: A European consensus." *Journal of Cystic Fibrosis* **8**(5): 295-315.

Hoch, H. E. and S. J. Szefler (2016). "Intermittent steroid inhalation for the treatment of childhood asthma." *Expert Review of Clinical Immunology* **12**(2): 183-194.

Karathanasis, E., A. L. Ayyagari, et al. (2005). "Preparation of in vivo cleavable agglomerated liposomes suitable for modulated pulmonary drug delivery." *Journal of Controlled Release* **103**(1): 159-175.

Knoch, M. and M. Keller (2005). "The customised electronic nebuliser: a new category of liquid aerosol drug delivery systems." *Expert Opin Drug Deliv* **2**(2): 377-390.

Kwok, P. C. L. and H. K. Chan (2014). "Delivery of inhalation drugs to children for asthma and other respiratory diseases." *Advanced Drug Delivery Reviews* **73**: 83-88.

Lass, J. S., A. Sant, et al. (2006). "New advances in aerosolised drug delivery: vibrating membrane nebuliser technology." *Expert Opin Drug Deliv* **3**(5): 693-702.

Lizal, F., J. Jedelsky, et al. (2018). "Experimental methods for flow and aerosol measurements in human airways and their replicas." *Eur J Pharm Sci* **113**: 95-131.

Loira-Pastoriza, C., J. Todoroff, et al. (2014). "Delivery strategies for sustained drug release in the lungs." *Advanced Drug Delivery Reviews* **75**: 81-91.

Patton, J. S. and P. R. Byron (2007). "Inhaling medicines: delivering drugs to the body through the lungs." *Nat Rev Drug Discov* **6**(1): 67-74.

Strohl, K. P., A. J. Thomas, et al. (1997). "Ventilation and metabolism among rat strains." *J Appl Physiol (1985)* **82**(1): 317-323.

Tonnis, W. F., M. Bagerman, et al. (2014). "A novel aerosol generator for homogenous distribution of powder over the lungs after pulmonary administration to small laboratory animals." *Eur J Pharm Biopharm* **88**(3): 1056-1063.

Vaquero, J. J. and P. Kinahan (2015). "Positron Emission Tomography: Current Challenges and Opportunities for Technological Advances in Clinical and Preclinical Imaging Systems." *Annual Review of Biomedical Engineering* **17**: 385-414.

Zhang, G., A. David, et al. (2007). "Performance of the vibrating membrane aerosol generation device: Aeroneb Micropump Nebulizer." *J Aerosol Med* **20**(4): 408-416.