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Advanced *in vitro* lung-on-chip platforms for inhalation assays: From prospect to pipeline

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ABSTRACT

With rapid advances in micro-fabrication processes and the availability of biologically-relevant lung cells, the development of *lung-on-chip* platforms is offering novel avenues for more realistic inhalation assays in pharmaceutical research, and thereby an opportunity to depart from traditional *in vitro* lung assays. As advanced models capturing the cellular pulmonary make-up at an air-liquid interface (ALI), *lung-on-chips* emulate both morphological features and biological functionality of the airway barrier with the ability to integrate respiratory breathing motions and ensuing tissue strains. Such *in vitro* systems allow importantly to mimic more realistic physiological respiratory flow conditions, with the opportunity to integrate physically-relevant transport determinants of aerosol inhalation therapy, i.e. recapitulating the pathway from airborne flight to deposition on the airway lumen. In this short opinion, we discuss such points and describe how these attributes are paving new avenues for exploring improved drug carrier designs (e.g. shape, size, etc.) and targeting strategies (e.g. conductive vs. respiratory regions) amongst other. We argue that while technical challenges still lie along the way in rendering *in vitro lung-on-chip* platforms more widespread across the general pharmaceutical research community, significant momentum is steadily underway in accelerating the prospect of establishing these as *in vitro* "gold standards".

1. Introduction

Respiratory diseases are a global leading cause of disability and mortality, with lifestyle and environmental factors contributing to their growing prevalence and association with rising healthcare and economic burden [1]. Chronic Obstructive Pulmonary Disease (COPD) alone, with 200 million patients suffering from moderate to severe forms of it [2] is the third leading cause of death worldwide. In parallel, lung cancer (i.e. the most common worldwide), acute respiratory distress syndrome (ARDS) [3], idiopathic pulmonary fibrosis (IPF) as well as infectious diseases (e.g. pneumonia, tuberculosis) are all either fatal diseases or pathologies exhibiting high mortality rates. Despite an unmet need in treating respiratory diseases, few new compounds that are safe show efficacy and have eventually emerged as new therapeutic options. The success rate for the approval of novel inhalation medicine in the past forty years or so has been meager at best, whereby treatments approved have mainly consisted in improvements on existing classes of drug (e.g. long-acting β_2-agonists, long-acting muscarinic antagonists, safer inhaled corticosteroids and longer acting antibiotics). A widely acknowledged issue lies in the discrepancy between the performance of therapeutic candidate molecules in preclinical animal models and their disproportionately high failure rate for safety and/or efficacy issues upon reaching the stage of clinical trials [1].

To address the disconnect between the predictive capacity of *in vivo* animal models and delivering novel therapeutics for inhalation therapy, several alternative *in vitro* technologies capable of mimicking more accurately respiratory human physiology *in vivo* have been explored. In recent years, *organ-on-chips* (OOC) have been emerging as potential alternatives to animal testing [4]. OOCs are platforms that provide cells with an *in vitro* environment that attempts to more closely resemble

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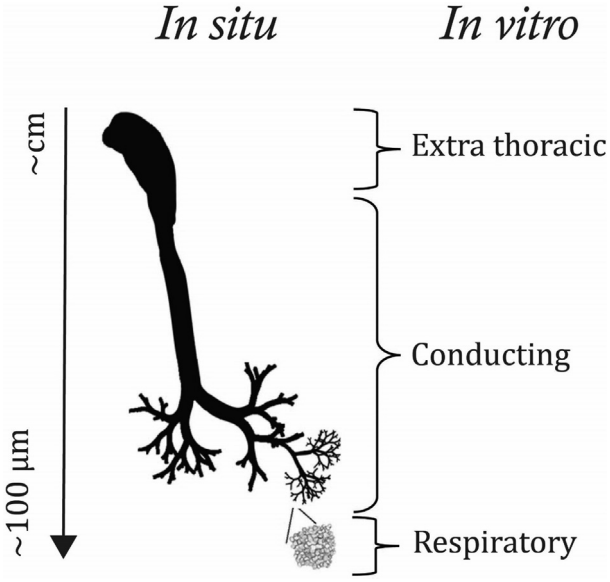

Fig. 1. The lungs encompass a complex multiscale organ with a cascade of airways covering a wide range of length scales (i.e. anatomy), from several centimeters to sub-millimeters (i.e. ~100 µm). In parallel, the organ covers a diverse cellular make-up that may be broadly categorized according to three main regions: extra-thoracic, conductive and respiratory. Translating pulmonary organ functions to relevant *in vitro* platforms comprising truthful physiological and biological functionalities requires limiting a model (e.g. microfluidics) to an isolated region of interest as a result of the multiscale challenge.

their native *in vivo* milieu. In such conditions, cells can maintain a notionally naïve phenotype or can be cued to differentiate into a phenotype in a controlled manner [5–7]. Although OOC models have already shown promising success in reproducing complex biological functions [8–11], such as in recapitulating lung edema formation or thrombosis [8], these systems and their wider use still remain in their infancy; their potential is only slowly starting to become more suitable for meaningful exploitation. In particular, the lungs comprise a highly-intricate organ exhibiting a multiscale challenge to replicate more realistically *in vitro* with so-called *lung-on-chip* platforms. This is especially critical when considering the transport and delivery of inhalation medicine, i.e. from mouth to airway deposition (Fig. 1), in conjunction with their effects on lung tissue including importantly translocation processes across the air-blood barrier. In this short opinion paper, we discuss in brief the current state of *lung-on-chips*, and more specifically leveraging such technologies as attractive tools for *in vitro* inhalation assays across the wider pharmaceutical research community.

2. From traditional *in vitro* models to *lung-on-chip* platforms

In vitro exposure assays for safety and efficacy assessment of given therapeutic drugs are still commonly conducted via instillations of a liquid suspension directly onto a cellular lung model. Broadly speaking, such approaches constitute the state-of-the-art whether simple epithelial monolayers (e.g. bronchial or alveolar) or rather co-/multi cell cultures (i.e. AECs, macrophages, dendritic cells, endothelium, etc.) are modeled. In mimicking more realistically the innate pulmonary environment, the adoption of transwell inserts has been increasingly popular in an effort to develop lung *in vitro* models, as "hardware" platforms, that enable lung cells to be cultured at an air-liquid interface (ALI). In past years, this has been visible within academic research communities as extensively discussed in a number of recent reviews [12,13] and is becoming more widely available commercially with companies providing ALI-based products to both the R&D sector and academic laboratories. Yet, historically, the use of instillation assays under submerged conditions, i.e. with cells grown at a liquid-liquid interface (LLI), has long acted as a "gold standard", i.e. considered as cell systems that mimic *in vitro* key events known to occur *in vivo*. One of the significant drawbacks of such *in vitro* pulmonary LLI assays lies in the ongoing lack of realism in replicating *in situ* inhaled delivery protocols. Delivering for example aerosolized toxic compounds (e.g. zinc oxide nanoparticles) at an ALI has been shown to elevate levels of secreted in pro-inflammatory markers compared with submerged cell cultures [14]; a point that has drawn attention in further advocating the need for ALI-based cytotoxicity assays [7]. For therapeutic research, such considerations apply most notably to the aerosolization of liquid droplets via a nebulizer or metered dose inhaler (MDI), or alternatively the administration of particulate matter (PM) via dry powder inhalers (DPI). The evaluation of realistic aerosol deposition outcomes in relation for example to efficacy or toxicity of a clinical inhalation therapy is still limited; a point we will return to further below.

Undeniably microfluidic systems, and more generally microfabrication technologies, have catalyzed important progress in devising advanced platforms that break away from traditional *in vitro* "hardware" [4,15]. This is especially true in the field of lung research as epitomized with the first *lung-on-chip* published nearly a decade ago [16,17], and the rapid development of some of the most advanced preclinical *in vitro* human models of lung pathologies [18], with therapeutic end points geared for example at pharmaco-kinetics and -dynamics. In particular, the field has seen a surge in the availability various microfluidic architecture designs. Though micro-devices typically feature three-dimensional (3D) geometries that enable (air-) flow through channels, lung cells (be it mono- or co-cultures) are typically grown at an ALI on a two-dimensional (2D) substrate, often times employing the same porous polyethylene terephthalate (PET) membranes used in commercially-available transwell inserts. While progress is now underway in devising *in vitro* platforms featuring cell cultures across the entire lumen (e.g. with hydrogels [19]), existing *lung-on-chip*s that employ for instance polydimethylsiloxane (PDMS) or biological membranes [20] are enabling for the first time new biological read-outs (e.g. creation of a lung edema following the disruption of the lung alveolar barrier via cytokines IL-2 or the stiffening of a membrane while cells form a confluent layer [21]). Such quantifications are brought in addition to the palette of biological endpoints available in more traditional assays, i.e. cell viability, gene expression, inflammatory cytokine responses and epithelial barrier properties amongst other. Furthermore, and of critical importance, unlike transwell and other traditional setups that are largely static when considering the exchange and/or collection (e.g. analytics of inflammation, etc.) of media, microfluidic platforms offer the integration of continuous perfusion from either the basal and/or apical side, depending on the application and the measurement endpoints.

The realm of *lung-on-chips* provides tangible new opportunities to integrate critical anatomical, physiological and biological parameters *in vitro*, thereby contributing to more realistic models of (human) lung physiology [11]. Broadly speaking, their novelty may be categorized as spanning four novel characteristics that have remained until present beyond accessibility with traditional assays (Fig. 2). To begin, (i) morphological features of the lung anatomy can be emulated by designing for example ultra-thin and stretchable membranes [20,22,23] to mimic the lung basal membrane or via patterning of the design architecture [11] (e.g. airway tree, generational bifurcations, alveolar cavities, etc.). While such modeling is still far from true lungs [17,22,24], integrating elements of lung anatomy *in vitro* holds important ramifications when investigating aerosol transport and deposition, as discussed further in the next section. In parallel, (ii) microfluidic devices enable the integration of respiratory breathing motion via mechanical model [25] and/or blood-like flows over an endothelium layer on the basal side of the porous membrane. While still few examples of direct microfluidic *in vitro-in vivo* correlations are available, Huh et al. [21] revealed in a chip mimicking the lung alveolar-capillary interface that

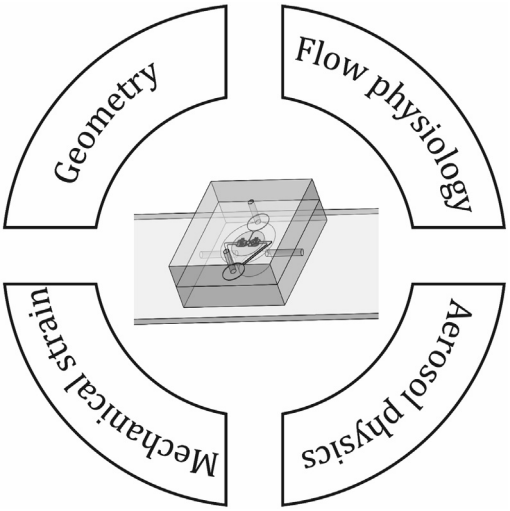

Fig. 2. *Lung-on-chip* platforms mimic the smallest functional biological units of the lungs, such as the lung alveolar barrier and/or the acinar airspace. They further enable to critically account for physical and physiological cues that go beyond traditional *in vitro* models at an air-liquid interface (ALI). The principal advantages include (i) mimicking elements of airway morphology (i.e. anatomy), (ii) incorporating the influence of mechanical strain due to breathing (i.e. stretching cells), (iii) implementing flow physiology resulting from representative respiratory airflows at the ALI as well as blood flows (on the basal side of the porous membrane) and (iv) mimic the basal membrane that supports the air-blood barrier. Importantly, *lung-on-chips* can deliver realistic aerosol transport assays that mimic the physics of inhaled particles (i.e. droplets or particulate matter), from airborne flight to wall deposition.

mechanical forces associated with breathing motions are critical in triggering inflammatory responses via cytokine (IL-2) release and in increasing vascular leakage leading to edema; such results corroborated with their *in vivo* experiments in rodents. The authors went on to identify potential new therapeutics which showed promising results both *in vitro* and *in vivo*. Lastly, (iv) *lung-on-chip* platforms of the second generation are now enabling to mimic a major component of the innate cellular microenvironment, i.e. the extra-cellular matrix (ECM) of the lungs. While inserts and PDMS membranes are usually coated with ECM proteins, the possibility to replace the PDMS membrane altogether with a thin, biological and importantly stretchable membrane made of proteins found in the lung ECM represents an important milestone, as recently demonstrated [20]. Taken together these four pillars are now offering a fifth avenue of relevance: the critical opportunity to integrate realistic aerosol transport determinants for *in vitro* inhalation assays.

3. Strategies to mimic inhalation *in situ*

The past decades have gathered a plethora of supporting evidence, spanning *in vivo* [26–29] (e.g. gamma scintigraphy, MRI, synchrotron radiation CT), *in vitro* [24,30,31] (e.g. extra-thoracic and upper airway casts, microfluidic deep lung models, etc.) and *in silico* [32–35] (e.g. compartment models, trumpet models, computational fluid dynamics, etc.) approaches, that underlines how the interplay between inhalation maneuvers, via respiratory airflows along the airway tree, and aerosol physics (e.g. aerodynamic size, shape, etc.) significantly influences deposition outcomes in the lungs and ensuing efficiencies of inhalation therapy. Aerosol deposition endpoints (e.g. lung region, aerosol concentration, deposition heterogeneity, hot spots [36]) are intimately tied to the physical transport determinants of an aerosol, in particular through the leading aerosol deposition mechanisms [37–39]: convection (or drag), impaction, sedimentation and Brownian diffusion, as well as interception in the case of non-spherical elongated particulate matter (i.e. fibers [40,41]). Given that these governing mechanisms operate within a highly-intricate network of airways that spans a multitude of length scales (i.e. from centimeters in the extra-thoracic regions to sub-millimeter in the deep acinar regions, see Fig. 1), in conjunction with various gravitational orientations (e.g. apical vs. basal lung lobes) and distinct anatomical confinements (e.g. circular lumen versus alveolar cavities), determining reliably deposition outcomes in a quantitative manner remains challenging.

The prediction of inhaled aerosol deposition is rendered even more arduous in the absence of widely available high-resolution imaging modalities capable of precisely determining aerosol deposition patterns in human lungs; a critical step to corroborate both *in vitro* (and *in silico*) predictions with *in vivo* data. Not only are aerosol deposition data in humans limited (i.e. compared with more widely-available animal models) but existing imaging techniques for *in vivo* aerosol deposition assessment [42] are typically restricted to whole-lung deposition maps of 2D projections (e.g. gamma scintigraphy) partitioned according to coarse regions of interest (e.g. central versus peripheral lung regions). Establishing localized deposition predictions are further exacerbated when considering lungs in a diseased state or under inflamed conditions [43] (e.g. airway obstructions, emphysema, cystic fibrosis, etc.). This results as deposition outcomes are severely compromised compared with healthy airway trees; an outcome clearly visible in whole-lung gamma scintigraphy images. Indeed, much of the overarching paradigms in successful aerosol medicine are bound to airborne particles depositing within the least resistant, more accessible airways or lung lobes. Namely, fluid mechanics of internal (air) flows in a pipe predict that airway narrowing will resist flow passage for a given, fixed breathing effort. Such adverse outcomes accentuate how inhalation therapy is not only heterogeneous in nature but biased in yielding deposition where it is in fact the least needed; an ongoing challenge in devising efficient therapies.

Bearing in mind that the vast majority of the lung surface is contained within the acinar regions, *lung-on-chip* platforms are limited to mimic only a small representative segment of the lungs (Fig. 1), i.e. typically the smaller (e.g. bronchioles) or deep acinar airways. Nevertheless, such systems can replicate more faithfully the airborne delivery route (i.e. from mouth to lumen wall) and thus help improve screening assays in evaluating more precisely those particles that will eventually deposit (Fig. 3). In this context, Benam et al. [44] discuss opportunities for microfluidic airway models in COPD research and takes a detailed look in comparing *lung-on-chip* models against both static *in vitro* culture systems and animal models. The authors highlight amongst other the ability to study inhalation exposure to whole smoke under physiological breathing airflow compared with static *in vitro* cultures. Furthermore, *lung-on-chips* offer superior modeling of inhalation toxic-pathology studies and host–pathogen interactions as well as lung anatomy and route of exposure between humans and widely-used rodents. Such new *in vitro* strategies raise opportunities for improved design guidelines when deposition site is of utmost importance (e.g. conductive vs. respiratory regions, see Fig. 1), including for example in the context of topical (e.g. asthma, COPD, infectious diseases) versus systemic delivery [45,46] (e.g. immunization). While the biological endpoints quantified from *lung-on-chips* largely overlap with or replicate directly those obtained using more traditional *in vitro* models (Fig. 3), incorporating realistic transport outcomes is anticipated to help drug delivery targeting strategies (i.e. designing the carrier size, shape, etc.). Such approaches can help reduce what particles will ultimately be screened or lost during inhalation protocols (including those exhaled), thereby reducing unnecessary secondary effects while increasing the efficiency in delivery payloads to the originally intended airway sites.

Here, we open a short remark in emphasizing that an additional, yet crucial, objective in devising attractive *lung-on-chip* platforms is to reproduce an *in vivo*-like cellular environment, in which lung epithelial cells can maintain their native phenotype; this is in stark contrast to the limitations of working solely with cell lines for example. In such context, both the effect and translocation of inhaled drugs on and across a

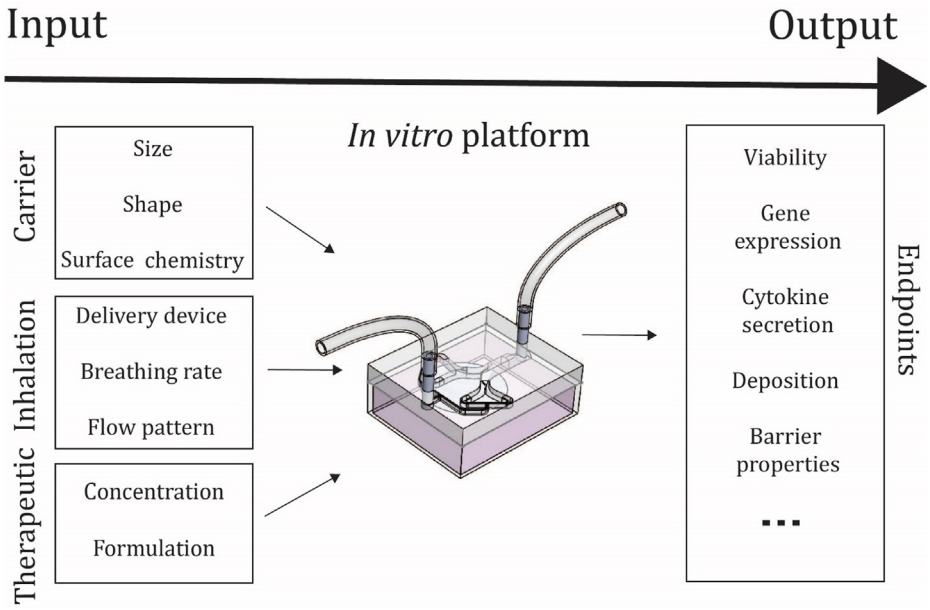

Fig. 3. Schematic of the *in vitro* pipeline for inhalation assays with advanced microfluidic platforms. Input parameter screens include exploring the carrier design (e.g. particle size, shape, etc.), the inhalation protocol (e.g. flow and breathing rate) as well as the drug or compound (e.g. composition and formulation). In analogy to traditional *in vitro* models (e.g. transwell inserts), output parameters of interest feature well-established biological endpoints (e.g. cytokine secretion, viability, gene expression, etc.) and barrier properties (e.g. permeability, TEER, etc.) as well as a new opportunity to characterize directly the outcomes of aerosol deposition (e.g. location and concentration).

healthy or diseased air-blood barrier is a key element of the drug discovery process. In particular, a recent study has demonstrated that both type I and type II lung alveolar epithelial cells obtained from patients could be jointly cultured and maintained on chip for several days [22].

Returning to the current paradigms of *in vitro* lung assays, efforts have been made to move beyond instilling liquid suspensions directly onto cell cultures and instead deliver aerosols via direct spraying [47–49]. More recently, *in vitro* designs have included for example the development of the Pharmaceutical Aerosol Deposition Device on Cell Cultures [50] (PADDOCC) for DPIs as well as the air-liquid interface cell exposure (ALICE) setup for nebulized suspensions [51]. Namely, the open cell culture design, characteristic to insert cultures but atypical for microfluidic systems, has been implemented more recently in a number of *lung-on-chip* assays [20,22,23] in an effort to integrate the use of aerosol deposition systems (Fig. 4). While these studies underline the importance of aerosolization at an ALI towards mimicking more truthfully the fate of aerosols depositing on the airway lumen, they come short of replicating the "journey" taken by inhaled aerosols within the lungs (Fig. 1). Such journey is known to result in important aerosol screening "losses" along the airway pathway; a direct result of sedimentation and impaction amongst other. This deposition outcome is emphasized for example in the ICRP deposition curves [52] that are anticipated to reflect to some extent the fate of any inhaled dose. Such aerosol screening phenomenon raises in turn the question of how to appropriately calibrate or adapt deposition results in isolated *lung-on-chip* models, as the entire pathway from mouth to model is absent from the delivery route. Despite such shortcomings, isolated *lung-on-chip* platforms that incorporate physiological airflows and aerosol transport physics (Fig. 2) provide essential steps in uncovering how critical particle size is towards successful localized airway deposition. This was underlined for example in recent microfluidic acinar airway models [24] whereby targeting efficiently aerosols to the deepest regions (i.e. half of all alveoli are located in the last bifurcating generation) remains exceedingly challenging. In particular, we raise the prospect of leveraging *lung-on-chips* for dosimetry applications, as originally motivated with setups such as the PADDOCC, ALICE as well as commercially-available systems such as VITROCELL and Cultex. *In vitro* assays including how effectively an administered dose following an inhalation maneuver (e.g. single metered dose) is deposited with a real inhaler and subsequently absorbed or locally active in the lungs represents appealing new opportunities for exploration in pulmonary drug delivery. In particular, once the dose of inhaled drugs reaching the lower airways

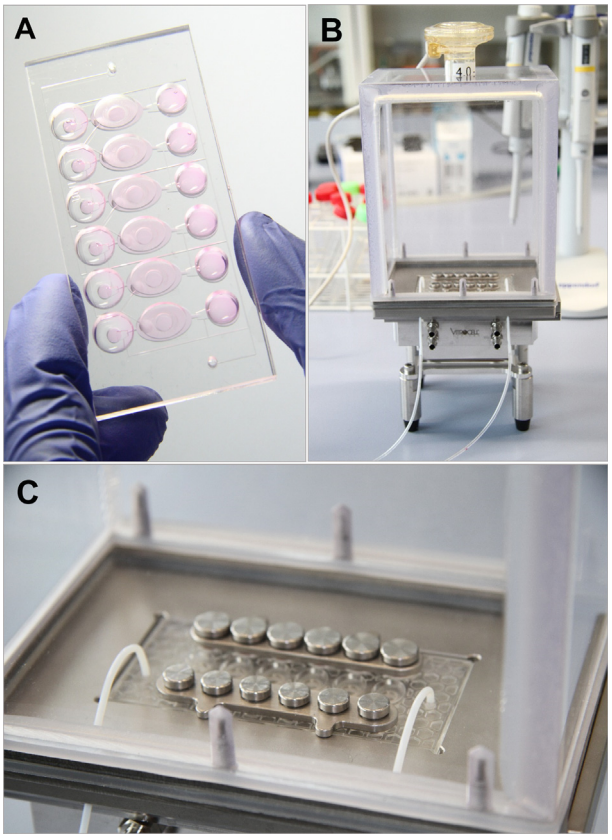

Fig. 4. (A) Prototype of a *lung-on-chip* design featuring six wells, following the work of Stucki et al. [23], mimicking the lung alveolar barrier whereby cells are cultured directly at an air-liquid interface (ALI). (B) and (C) The *lung-on-chip* design is integrated with an *in vitro* inhalation system for liquid aerosol deposition. The setup allows for aerosol exposure and breathing concurrently, under controlled temperature conditions. Additionally, such setup can be equipped with a Quartz Crystal Microbalance (QCM) for online particle counting.

is determined, the ensuing effects of drugs at the air-blood barrier, either for efficacy or safety purposes, can be assessed.

4. Towards end user applications

A pertinent advantage of the more established *in vitro* models (i.e. transwell inserts, etc.) lies in the robustness and easiness of their handling due to the relative simplicity of such designs. This includes amongst other the packaging of multiple inserts on plates as well as the static nature of such assays (e.g. lack of complex moving parts). These attributes remain important factors to consider when attempting to translate some of the aforementioned microfluidic *in vitro* platforms for wider use across the scientific community. Although advanced microfluidic-based devices incorporate critical physiological cues (e.g. airflow, strain, etc.) and relevant aerosol exposure characteristics (e.g. particle physics, open microfluidic design, etc.) that ultimately influence biological endpoints under more realistic conditions (e.g. models that resemble more closely drug and molecule dispersal in the human body as well as underlying pathological and toxicological mechanisms), they require undeniably specific training of the end users. These systems are more complex to handle due to their additional characteristics (e.g. breathing movements, flow, etc.). In turn, the design of *lung-on-chip* systems should strive to reach a compromise between the recapitulation of the *in vivo* complexity and the ease of use for the end user, and most importantly the read-out of the specific biological question posed. Ultimately, the designs and robustness of these microdevices must be such that the end users routinely running the standard biological assays (Fig. 3) are enticed to switch and choose these new available options [53].

The above considerations are important if *lung-on-chips*, and more generally *organ-on-chips*, are considered as realistic options for medium (MTS) or high-throughput screening (HTS) applications; a topic that has drawn attention in recent reviews [54,55]. At present, standard methods for HTS (e.g. well plate readers) enable automated screening endpoints of more than 100,000 drug candidates per day [56]. In contrast, however, *in vitro* tissue models are still far from becoming "high throughput" methods. Nevertheless, *organ-on-chips* technologies are pushing for increasing the experimental throughput as highlighted in various studies [57–61]. In lung research specifically, efforts are still very much in their infancy. Most notably, Stucki et al. [23] have recently mimicked several critical features of the alveolar microenvironment including breathing motion at an ALI as well as recapitulating an air-blood barrier, whereby six parallel experiments are simultaneously conducted on a single chip (Fig. 4). As examples, target identification may be carried in simple *in vitro* assays, provided the phenotype of the cells can be maintained, whereas translocation assays of molecules across the alveolar barrier may need cyclic mechanical strain characteristic of breathing motions.

More generally, the path to scale *organ-on-chip* platforms is tightly connected to the added value of the model and the biological question to be answered. In particular, recent discussions on *organ-on-chips* altogether have alluded to the hurdles to overcome for clinical translation of such devices regarding materials, cellular fidelity, multiplexing, sensing, scalability and validation [62]. This is indeed critical, in particular for *lung-on-chips*, when considering their strategic incorporation towards physiologically-based pharmacokinetic (PBPK)/pharmacodynamic models (PD) in drug development; a topic that has been discussed when considering organ-organ interactions [63]. While beyond our present scope, we raise the prospect and attractiveness of *lung-* and *organ-on-chips* for example in, but not limited to, the validation phase of a selected group of identified drugs, rather than in the initial large-scale screening phase [54,64]. Taking advantage of microfluidic-based *in vitro* models may be attractive in advanced research steps, whereby the value of mimicking more closely the *in situ* physiological environment becomes increasingly significant. Such endeavors are also part of a broader discussion in considering the revision of Good Cell Culture Practice (GCCP) guidance [65].

5. Outlook

Lung-on-chips have triggered important advances in realizing novel *in vitro* models that strive to mimic more closely the human pulmonary environment. We have argued in this opinion paper that such platforms offer tremendous potential in laying a pipeline for advanced *in vitro* inhalation assays that go far beyond current capabilities with simple transwell inserts of lung cell cultures at an ALI. Yet, their widespread adoption and standard use in the broader research community remain to be established as such novel methods are still not ready for regulatory purposes despite rapid progress; a point recently highlighted in the "Guidance Document on Good *In Vitro* Method Practices (GIVIMP)" [66]. Whether *lung-on-chips* will eventually lead up in delivering new *in vitro* "gold standard" models in pulmonary pharmaceutical research is a point to be monitored over the coming years. The most significant questions are whether *lung-on-chips* can address specific biological questions, and reflect the targeted endpoint as highlighted in the recent review of Hittinger and colleagues [12]. Hence, it is likely that the simpler, more robust designs will prevail while offering the prospect of increased biological complexity with new read-outs unavailable with standard transwell systems. In addressing both physical aerosol transport determinants and cellular barrier functions, *lung-on-chips* have the potential to become a game changer in the respiratory field.

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Declaration of Competing Interest

CM Lehr is co-founder, scientific advisor and shareholder of PharmBioTec GmbH, Saarbrücken, Germany. OT Guenat and N Hobi are shareholders of the start-up AlveoliX AG.

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