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Review

How Far Are We from Research That Is Independent of the Use of Animal Models? A Comparative Analysis between Animal and 3D/On-a-Chip Models for the Study of Respiratory Diseases

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Abstract: Over the last ten years, with the progress of in vitro culture methods, it has been possible to build increasingly reliable models to effectively mimic in vivo ones. The translational methodological approach that combined biotechnology and biomedical engineering has produced remarkable results, such as the development of ex vivo 3D culture models, the construction of on-a-chip organoids, and the construction of complex systems capable of bypassing the static nature of the two-dimensional cultural models that have been typical of in vitro studies conducted to date. However, nowadays, there is still reluctance to completely abandon the animal model as an essential reference or as an integrated step for the validation of a model or a proposed study. This is due to the partially correct conviction of the impossibility of reproducing, in vitro or ex vivo, the complexity of pathological models or the spatial communication between different cytotypes, as well as, more generally, the lack of systems capable of mimicking the dynamism of a complex in vivo system. In this study, we will compare different methodological approaches in the study of the three most common types of respiratory diseases: chronic obstructive pulmonary disease (COPD), asthma, and lung carcinomas. The purpose of this comparative study is to evaluate the most current methodological approaches to understand how far research is from being independent from animal models. Animal studies are generally considered necessary, but are still questioned because of the ethics and the cost–benefit ratio involved.

Keywords: human anatomy; bioengineering; tissue engineering; biomaterials

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1. Macroscopical and Microscopical Structure of the Bronchial Tree and Airway Epithelium

The respiratory system organs are located in three different regions of the human body: splanchnocranium (external nose, nasal cavity, and nasopharynx); cervical region (larynx and cervical trachea), and thoracic cavity (thoracic tract of the trachea, bronchial tree, pleura, and lungs). The upper respiratory tract includes the external nose, the nasal cavity, the paranasal sinuses, and the pharynx. The lower respiratory tract is located in the thorax and includes the larynx, the trachea, the bronchial tree, and the lungs (covered by pleura, a serous membrane).

The nasal cavities are two symmetrical ducts that can be divided into an antero-inferior part, located in the cartilaginous portion of the external nose (nasal vestibule), and a more extensive posterior part, the proper nasal cavity, into which the paranasal sinuses open. The nasal cavities communicate posteriorly with the nasopharynx through the nasal choanae. The nasal cavities are mainly lined with respiratory mucosa, except for the vault (roof), which is covered by olfactory mucosa. The nasal mucosa presents a ciliated pseudostratified

epithelium (respiratory epithelium) underneath which a basement membrane separates it from the lamina propria. The lining epithelium is mainly characterized by:

- Columnar cells with vibrating cilia;
- Goblet cells, intercalated in the epithelium and responsible for the production of mucus;
- Basal cells.

The lamina propria of the respiratory mucosa also includes numerous seromucous glands, the secretions of which constantly cover the nasal mucosa and contain bactericidal agents such as lysozyme, beta-defensins, lactoferrin, and IgA. The nasal mucosa represents a physical barrier against various irritants (chemical, physical, bacterial, and viral) that hail from the outside. Furthermore, at this level, the air is preheated, humidified, and purified (the mucus retains dust, while the movement of the cilia facilitates its expulsion towards the pharynx). This structural module is also repeated in the nasopharynx and larynx (except for the vocal cords). The trachea lies between the esophagus and the sternum and consists anterolaterally of C-shaped incomplete cartilaginous rings and posteriorly of a fibromuscular wall. In proximity to the pulmonary hila, at the carina level, the trachea bifurcates into the right and left main bronchi. The trachea consists of four histological layers: mucosa, submucosa, musculocartilaginous layer, and adventitia. The mucosa consists of a respiratory epithelium and a lamina propria. The lining epithelium is a pseudostratified ciliated layer, while the lamina propria is formed by a connective tissue rich in elastic fibers and is crossed by the excretory ducts of the tracheal glands. The adenomeres of the tracheal glands are located in the submucosal tunica. Around the submucosa, the hyaline cartilage layer forms the support rings, which provide a strong but flexible structure that keeps the airways open and resists external stresses. The outermost layer of the trachea is the adventitia, formed by dense connective tissue rich in elastic fibers.

The structure of the lower airways also gradually changes craniocaudally.

(1) In the bronchi, the mucosa consists of a pseudostratified ciliated epithelium with intercalated goblet, brush, neuroendocrine (Kultchitsky's), and basal cells. Brush cells are characterized by the presence of microvilli on the cell surface, and their function is still unclear. One of the current hypotheses is that they could be intermediate elements that then differentiate into ciliated cells or cells able to regulate mucus clearance. Kulchitsky's cells participate in the local control of mucus production and cell differentiation (they drive basal cells to differentiate into goblet or columnar cells as needed). In the lamina propria, there are bronchial glands.

The submucosa is mainly composed of a thickening of elastic fibers. Cartilage is present in the form of rings that tend to gradually fragment.

(2) In the bronchioles, the epithelium becomes a simple ciliated cuboidal epithelium with intercalated club cells (formerly known as Clara cells) that are more prevalent in the terminal bronchioles. In physiological conditions, club cells are believed to secrete the primary components of the extracellular substance lining the respiratory bronchioles, as well as to regulate the rheological properties of the mucus by rendering it richer in serum and, therefore, more fluid in the distal segments of the respiratory tract. The submucosa is absent in the bronchioles. Smooth muscle fiber cells (also present at the bronchial level) are prevalent at the bronchiolar level, where they regulate the flow of air entering and leaving the alveoli, thus determining the mechanisms of bronchodilation and bronchoconstriction controlled by the autonomic nervous system.

(3) Two types of epithelial cells compose the alveolar epithelium: type I and type II alveolar cells. Type I are large flat cells that comprise about 95% of the alveolar surface. Type II are small cuboidal cells with distinctive lamellar inclusions and apical microvilli which cover around 5% of the alveolar surface. Type II cells produce and secrete pulmonary surfactant. Besides these two structural elements, also located on the luminal surface of the alveolar space are alveolar macrophages, the most abundant innate immune cells in the distal lung parenchyma (Figure 1). They are the first to

encounter incoming pathogens and pollutants, and help orchestrate the initiation and resolution of the immune response in the lung [1].

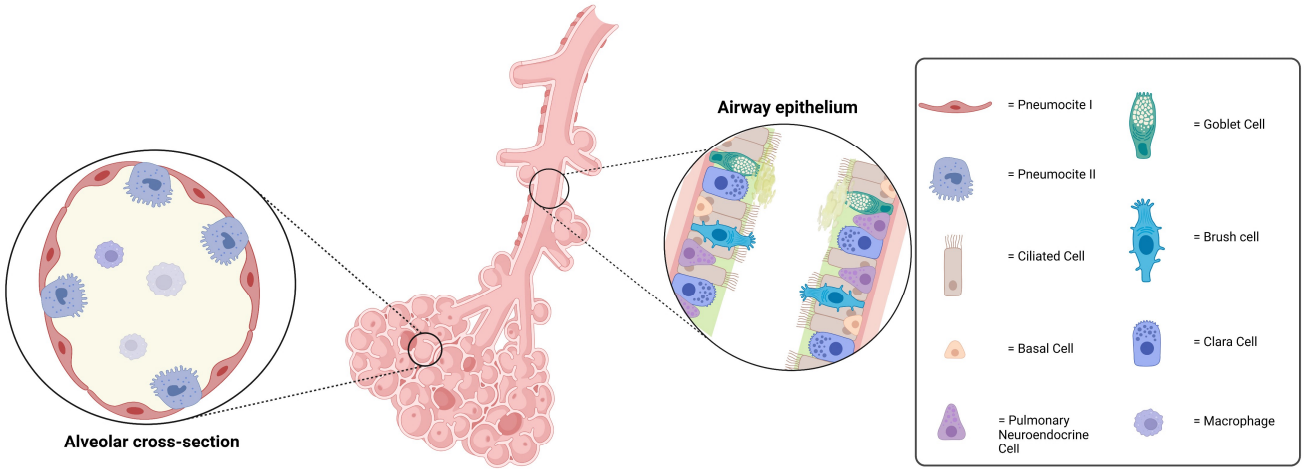

Figure 1. The airway epithelium is highly heterogeneous, and it is possible to find different sub-cell populations. In these cross-sections, it is possible to observe the bronchial epithelium and alveolar epithelium, with all the different cell types that populate them.

2. The Complexity of Methodological Approaches to Multifactorial Pathologies Such as COPD—The Reasons That Led Laboratory Animals to Be Considered the "Gold Standard"

Among respiratory diseases, COPD is undoubtedly one of the most widespread and most complex to study through reliable models that accurately mimic the morphological and pathogenetic features of the disease. COPD is estimated to be the third leading cause of death in the world, causing 3.93 million global deaths in 2019 [2,3]. COPD is a complex pathology consisting of four different types of clinical manifestations (emphysema, remodeling of the small airways, pulmonary hypertension, and chronic bronchitis), which may all be present in the same individual or not, and a functional impairment of the organ [4]. Even though main risk factors that can exacerbate the onset of the disease have now been identified, reproducing a reliable model of the disease in the laboratory is not simple. Although it is relatively simple to retrace the biomolecular damage induced downstream by some stress-producing agents, such as environmental pollutants and cigarette smoke, it is not so easy to establish univocal criteria that standardize the reference model, be it in vitro, ex vivo, or in vivo, due to the enormous clinical variability from patient to patient [5,6]. For this reason, the proposed animal models must consider the differences in interspecies development and anatomical variations, as well as the diverse responses of the organs to stressful agents [4,6].

Among the main animal models used in the study of chronic respiratory diseases, we currently include rodents (mice, rats, guinea pigs, etc.), rabbits, pigs, sheep, and non-human primates [7]. It is important to underline that each of the animal models used over the years has its advantages and disadvantages, as shown in the image below (Table 1). Briefly, some of the main advantages deriving from the use of specific animal models include the following: the use of rodents allows for a high genetic heterogeneity, a diversified response to stressful stimuli (consistent with the clinical manifestation of COPD), the onset of fibrosis following exposure to cigarette smoke (in rats), a moderate presence of goblet cells, a more represented ciliated epithelium (in hamsters and guinea pigs), and an immune response similar to that found in humans (in guinea pigs) [8]. Similarly, the use of other models also has several experimental advantages: the anatomical structure of rabbits' airways is more similar to that of humans compared to rodents, and similarly, their response to allergens is similar to that found in humans [9]. This in particular is highly relevant for the study of chronic respiratory diseases, and even more so in asthma, in which the release of

pro-inflammatory cytokines, increased levels of eosinophils, and alveolar obstruction in the advanced stages of a chronic inflammatory response are involved [10].

Table 1. Schematic representation of the main advantages/disadvantages of both methodological approaches to the study of airway diseases.

Models	Advantages	Disadvantages	References
Animal models	• Genetic variability and diversified stress response • Possibility of highlighting the onset of fibrotic processes following inhalation of cigarette smoke (rats, guinea pigs) • Possibility to study the response to allergens and the inflammatory response in a responsive model (rabbit and guinea pigs) • Ability to study the processes leading to tumor metastasis in dynamic in vivo models	• Poor genetic similarity with humans • High operator-dependent variability • High anatomical and histological differences of the individual models compared to humans • Relatively high maintenance costs • Ethical issues • Different composition of mucus	[8,9,11–15]
2D cultures models	• Easy development, maintenance and monitoring procedures • Relatively lower maintenance costs	• Inability to recreate the complexity of structures and mimic the functions of the organ-specific human environment in vivo	[16,17]
In vivo/ex vivo 3D culture models	• Preserved cytoarchitecture • Relatively lower costs • High scalability (possibility of integrating fluidics and sensors to the system capable of implementing its reliability and data output) • In co-colture models, it is possible to investigate the role of immune system on respiratory diseases	• Absence of a standard reference • Difficult to find human samples for the creation of 3D models • Ethical limitations	[18–20]

Rabbits are an excellent model for hematopoietic stem cell transplantation tests as these cells can differentiate into different cell lines, including endothelial cells, myocytes, neurons, and epithelial cells [21]. For example, studies conducted by Yuhgetsu et al. allowed for an evaluation of the effects of an autologous transplant of bone marrow mononuclear cells (BMnCs) [22], intended to mitigate the effects of pulmonary emphysema in rabbits [21]. The experiment, which reported significant improvements in the symptomatology of the animals affected by emphysema, allowed the researchers to clarify how the BMnCs were involved in the mitigation of the inflammatory response, in the reduction in MMP-2 expression, and in the reduction in apoptosis in favor of enhancing alveolar proliferation [9,23]. In analyzing the advantages, however, we certainly cannot overlook the enormous limitations of animal models in general, and of each of them specifically. In the cost–benefit analysis, we often opt for rodents as a study model; however, the main limitations certainly include the difficulties encountered in the study of pulmonary mechanics. For cigarette smoke exposure tests, for example, it is necessary to compare lung function, the presence of any fibrosis, and the onset of emphysema in animals exposed to the stressor. Pulmonary mechanics studies must be conducted with the animal alive, sedated, and intubated to maximize reliability and prevent physiological organ deterioration post mortem. This is particularly complex for small rodents, such as mice or guinea pigs.

Furthermore, it is necessary to highlight that there is a lack of methodical standards that can guarantee true reproducibility between different studies [4]. Even though mice are considered a more reliable model than rats in terms of compliance variation, P-V (partial pressure–volume) curves, and genetic variability (which translates into a more heteroge-

neous response to stressful stimuli), it is equally true that often, the results presented in different studies by different groups sometimes differ, even when using the same rodent strains [11,12]. This can be explained by the methods used for exposing the mice to cigarette smoke, as well as the cigarette types used to conduct the experiments [13]. The immune component should also not be overlooked; while it is true that some animal models, such as rabbits, have an immune response to allergens similar to the one that occurs in humans, this is not the case for mice. In a relatively low-cost and often preferred model, the involvement of neutrophils in inflammation and tissue damage is reduced and limited to acute exposure to cigarette smoke (CS) only, contrarily to what occurs in humans. Mice are the most frequently preferred model, mostly due to the large plethora of specie-specific reagents, low costs, and genetic heterogeneity. On the contrary, rabbits are more similar to humans in their allergic immune responses, but on the other hand, rabbit models have various disadvantages, such as monopodial lung structure, low gene heterogenicity, and different mucous composition compared to that of humans [6]. Thus, there is an effective lack of a model that can be considered to perfectly fit with what the researchers need in order to mimic COPD [14].

The Complexity of Methodological Approaches to Multifactorial Pathologies Such as COPD—Alternative 3D Culture Methods

It is now well known that the reactivation of the epithelial–mesenchymal trophic unit (EMTU) plays a decisive role in the pathogenesis of COPD [24] (Figure 2). The changes implemented by the unbalanced relationship between the epithelium and the connective tissue, through remodeling and chronic inflammatory processes, are the basis of the pathology [25]. A better understanding of the mechanisms that regulate the reactivation of the EMTU is essential to identify the molecular processes occurring in COPD and to carry through possible preventive and/or therapeutic intervention strategies.

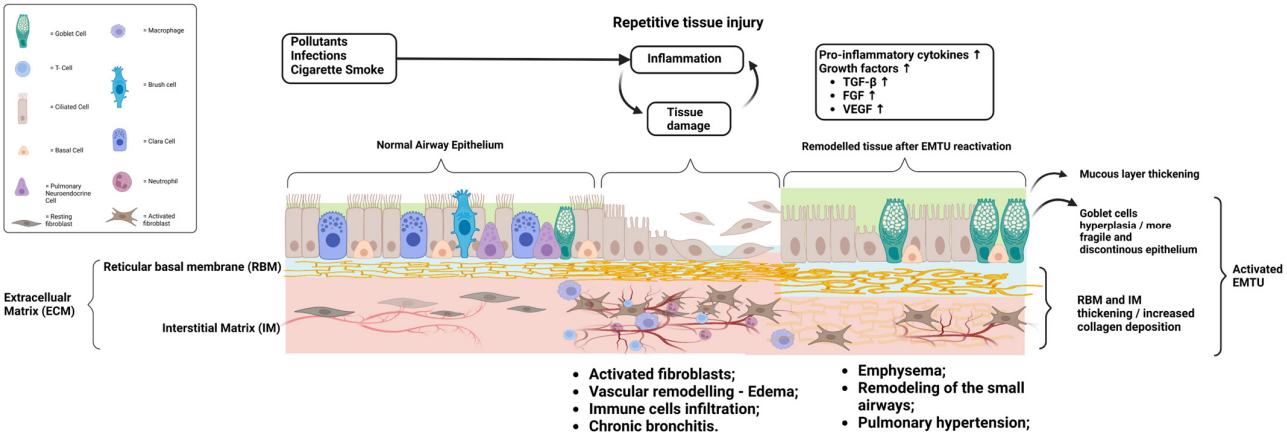

Figure 2. Representation of the epithelial–mesenchymal trophic unit (EMTU) reactivation process in the pathogenesis of COPD: the loss of balance between the epithelium and the connective tissue determines the remodeling of connective tissue with consequent deposition of collagen, activation of fibroblasts, and recruitment of cells of the immune compartment. The subsequent release of pro-inflammatory cytokines and chemokines at the epithelial level exacerbates the inflammatory process that is typical of the disease, which then becomes chronic.

Over the years, several in vitro and ex vivo models have been developed for the study of EMTU reactivation and the pathogenetic repercussions that this reactivation may have on COPD patients. These include: co-culture models using primary airway epithelial cells (PAECs), differentiated at an air–liquid interface (ALI); 3D culture models obtained from surgical resections, developed in ALI cultures as well; lung organoids; and supports designed to combine all of the core advantages of each of the aforementioned models (i.e., the lung-on-a-chip models).

The use of PAECs-ALI cultures has significant experimental advantages. With this model, primary cell lines are seeded on top of a coating of fibroblasts seeded inside a transwell and allowed to differentiate in an air–liquid interface (ALI). The air–liquid interface has the essential advantage of promoting the differentiation of epithelial cells into mucociliary cells, thus recreating and resembling the cytoarchitecture of the airways [18,26]. The transwell in which the co-culture coexists has a semipermeable membrane interposed between the connective and epithelial layers that favors the bi-directional passage of nutrients (from the basal to the apical side), as well as cytokines and mediators. Using this model, it is possible to carry out morphological analyses following stimulations with stressful agents that mimic the etiology of respiratory diseases. Thus, molecular analyses of the released mediators would clarify the relationship between the epithelium and the connective tissue, as well as the steps that lead to the reactivation of the EMTU and possible therapeutic strategies [26]. As described for PAECs, it is also possible to create outgrowth models derived from bronchial mucosa biopsies or surgical resections obtained from a donor. The biopsies are processed, cut into fragments of about 0.5 mm^2, and positioned on a transwell, similarly to the process described above. The biopsy fragment is then covered with a commercial extracellular matrix (such as Matrigel® or BME), and differentiation is favored in an air–liquid interface (ALI). The use of outgrowths starting from the respiratory mucosa of donors has significant advantages, largely comparable to those of PAECs-ALI cultures. The main difference from the previously described model involves the relationship between the connective tissue and the epithelium, which originate and differentiate from the precursor cells present in the biopsy fragment. In this model, the remodeling of the extracellular matrix by fibroblasts can be studied as well. By using synthetic matrices, as Matrigel® or CultrexTM—Basement Membrane Extracts (BME), it is possible to promote the maintenance and phenotypic differentiation of cultures, as well as to monitor the reabsorption and secretion of a new matrix and its remodeling in stimulated culture models. These cultures are also very resistant and suitable for studies involving prolonged exposures to etiological agents, such as cigarette smoke, environmental pollutants, and allergens. Another advantage of these cultures is the possibility of using them as study models for drug screening and drug testing (which will be discussed more extensively in the paragraph dedicated to the study of bronchial asthma). These models can also be employed to study the complex dynamics of leukocyte involvement in the context of chronic respiratory diseases using co-cultures, including immune system cells [27]. However, these models have several inherent limitations. The first of these undoubtedly concerns the availability of biopsies/surgical resections derived from donors; the approval of ethics committees often requires complex procedures, and finding suitable donors can be challenging. In this regard, the variability between individuals cannot be ignored. On the one hand, scientists who have worked with outgrowths know that samples from some donors may be more fibrotic than others and may respond unevenly to stimuli. On the other hand, the use of PAECs seems to be a valid alternative to address the issues discussed; however, both models (PAECs-ALI and outgrowth-ALI) have some limitations in studying the bronchial mucosa and its alterations in COPD. In such 3D cultures, and in standard and static culture conditions, it is difficult to realistically reproduce the sheer forces exerted by the air on the respiratory epithelium, and the in vitro reproduction of the alveolar epithelium is challenging. As briefly described in the previous paragraphs, two types of pneumocytes can be found at the alveolar level: AE1 and AE2 [28]. Unfortunately, in vitro AE2 pneumocytes tend to differentiate quickly into AE1, making it difficult to study them and to implement systems that are able to mimic the damage caused by COPD in the lower airways [29]. There are some commercial lines of stabilized AE2 which, combined with transwell and co-culture systems with fibroblasts and Rho-associated protein kinase (ROCK) inhibitors, may ensure good model stability [30,31]. It is also possible to generate AE2 from human induced pluripotent stem cells; however, this procedure is still very costly, time-consuming, and not resolutive of the intrinsic model limitations due to the incomplete phenotype that is generated [32]. Moreover, this approach eliminates the advantage of a

purely patient-derived model. In this regard, new methods aimed at expanding type 2 pneumocytes (AE2) have been developed through the use of organoids [19]. Organoids usually consist of a co-culture of cells with a high capacity for self-assembly, allowing for a realistic representation of cellular responses and interactions in vivo compared to traditional models. For this purpose, organoids can be cultured in vitro alone or in Matrigel or other extracellular matrix analogs, and can be derived from induced pluripotent stem cells (iPSCs) or adult cells and tissues, including tumor-like ones [33]. The phenotype of the AE2 seems to be able to be maintained using the authors' method for weeks, and, therefore, the model described by van Riet et al. appears to be a very promising model of an alveolar compartment affected by respiratory diseases. Researchers have investigated a possible methodological approach for the purification of AE2 pneumocytes (through a capture system based on an Ab-mediated capture that uses IgM anti-HTII-280 (mouse) and magnetic beads conjugated with an antibody anti-mouse and a subsequent analysis to compare the purified organoids from patients affected by the presence of a significant percentage of emphysematous tissue in two culture contexts. Firstly, the use of organoid-based cultures with an innovative organ on-a-chip (OOC) system is useful for investigating all the morpho-functional aspects of the alveolar microenvironment, including blood flow and strain caused by the pressure exerted by the flow on the lower airways [19]. The other is a transwell culture model similar to the one described in the previous paragraph. Both cultures of AE2 organoids, including those carried out on transwell and those carried out on chips, presented specific markers of the desired subtype (HTII-280, SP-C, SP-A, and SP-B) after one week despite the tendency of AE2 cultures to differentiate into AE1 in vitro [29]. This is explained by the authors as likely being due to the addition of a CHIR agonist WNT into the cultures [19]. In addition, the organoids were found to be able to withstand exposure to mechanical stresses, such as cyclic flows, in the on-a-chip model. The proposed model is, therefore, a dynamic system in which the factors that constitute the culture environment, such as the medium recirculation, or the mechanics produced by the pressure on the alveolar pneumocytes are also taken into consideration.

3. Asthma and Animal Models—The (im) Perfect Standard

Asthma is a chronic airway disease affecting almost 300 million people worldwide. It is characterized by several phenotypical expressions defined by clinical and physiological criteria. Asthmatic phenotypes include airflow obstruction, bronchial hyperresponsiveness (BHR), airway inflammation, and airway wall thickening [34,35]. This plethora of clinical phenotypic manifestations is strictly related to several mechanisms, and is continuously modulated by the complex network of innate and adaptive immune responses, which are dependent on the micro- and macro-environment [36]. The severity of asthma (moderate to acute), the response to drug therapy, the frequency of exacerbations, and the onset age are the most important criteria used to define the different clinical asthma events. The most frequent asthmatic phenotypes are environmental trigger-related (such as allergic, occupational, and menses-related asthma) and exercise-induced asthma [37]. Over the last few years, the complex clinical picture typical of this pathology has been studied in detail. However, a standard experimental model is difficult to develop due to the complexity and the plethora of etiological agents that are involved in the onset of this disease.

Traditionally, asthma has been studied using animal models. Sensitization of the animal to a particular antigen, followed by an evaluation of its effects on the airways, was carried out to further understand allergic responses [37]. Allergens used as triggers for asthmatic reactions included ovalbumin (OVA) [38], house dust mite (HDM) [39], cotton dust [40], and cockroach extracts [41]. Mice, rats, guinea pigs, rabbits, monkeys, sheep, and horses are, in general, the most often used animal models to study the onset of this chronic disease.

Mice are the most advantageous species for asthma research due to their fast reproducibility and ease of maintenance and use [42]. The animal model under investigation by Shneider and colleagues was found to exhibit a high level of sensitivity towards various

antigens, such as OVA [15], to which it is not typically exposed. Despite this, one of the main drawbacks of using mice as models is the inability to elicit a chronic response to allergens after sensitization.

Rats are often utilized as models for studying allergic airway disease due to their cost-effectiveness, which is comparable to that of mice, allowing for comprehensive research to be conducted. Additionally, their larger size and greater stability under anesthesia provide an advantage in measuring physiological outcomes such as the acute response to allergen inhalation [43]. However, a limitation shared by both rat and mouse models of asthma is the challenge of implementing protocols to induce the chronic changes in the airways that are typically associated with asthma.

Guinea pigs have been employed as a model for allergic airway responses for many years. Unlike murine models, guinea pigs can easily be sensitized to OVA, and they exhibit a response that resembles an asthmatic phenotype, as well as increased airway responsiveness [44]. Consequently, this animal is well-suited for studying hypersensitivity to chemical irritant factors. Moreover, the response of the guinea pig airway to pharmacological agonists has been shown to be similar to that of humans, making it a useful model for studying human airway sensitivity. Among the main limitations of their use as an experimental model are the low genetic variability; the presence of a soft palate, which makes any intratracheal procedures difficult; and, finally, axon reflex-controlled inflammation, must be considered.

Out of the many animal models available for studying anaphylactic responses, rabbits seem to be the most appropriate due to their similarity to humans in terms of the lungs being the primary target organ. The rabbit model also produces IgE as the primary anaphylactic antibody, which makes it an important tool for investigating asthma [45].

Transgenic mice have emerged as a popular animal model for investigating the underlying pathophysiological mechanisms of allergic asthma. By manipulating the expression of specific genes or molecules involved in key molecular pathways, researchers can gain insights into their roles in disease development [46]. For example, studies have examined the effects of altering the expression of IFN-inducible protein 10 (IP-10) in transgenic mice to elucidate its contribution to allergic diseases. In particular, investigations utilizing IP-10-deficient and IP-10-transgenic murine models of asthma have revealed a significant reduction in Th2-type allergic airway inflammation in IP-10-deficient mice compared to wild-type controls. Such transgenic mouse models provide valuable tools for investigation of the complex molecular mechanisms underlying allergic asthma [47]. The obtained results demonstrate that IP-10 upregulation in allergic pulmonary inflammation causes airway hyperreactivity [48]. A study was conducted in mice that lacked receptors for various interleukins and thymic stromal lymphopoietin (TSLP) to investigate their role in the development of allergic airway disease. The results showed that mice deficient in the IL-33 receptor and TSLP receptor had significantly reduced airway inflammation, IgE antibody levels, and airway hyperresponsiveness (AHR). However, there were minimal differences observed in mice lacking the IL-25 receptor or the IL-1 receptor compared to wild-type mice [49]. In another investigation, a transgenic mouse model with dual allergen-naïve traits displayed elevated production of Il-5 by mature T cells and overexpression of eotaxin 2 by lung epithelial cells, leading to various severe asthma-like pulmonary pathological features [50].

Although widely used, most animal models are not able to mimic human-specific features, resulting in studies with poor predictive value for human patients due to the great differences in the respiratory system structures of animal models and humans. For these reasons, the necessity of creating new in vitro and ex vivo experimental models has become evident, allowing scientists to study the mechanisms underlying the onset of the asthma phenotype in all its variability and complexity.

The Building of Robust 2D/3D Cultural Models for the Investigation of Asthma

Single-layer cell culture is a simple and inexpensive research approach, but it cannot recreate the complex structure and function of the human organ-specific microenvironment in vivo [16]. Three-dimensional (3D) ALI models and three-dimensional spheroids based on hydrogels or grown on the inner surface of polymeric scaffolds [51] can better mimic the microenvironment, but still have disadvantages, including lack of perfusion, stress, and limited vascularization. Indeed, three-dimensional cultures of lung ALI can be representative of different cell types and more complex tissue-specific functions, but are unable to mimic organ-level pulmonary characteristics such as the cell–host immune response [52]. These models are not able to analyze the recruitment of circulating immune cells under conditions of active fluid flow, leading to unavoidable problems in modeling lung diseases. The combination of different scientific disciplines, such as biotechnology, medicine, and engineering, has helped in the development of new ex vivo models, such as human organ-on-a-chip (OOC) systems, which have the ability to successfully mimic many aspects of organ-level physiology [53]. The practical implications found by this interdisciplinary approach are not limited to the cell culture field. In fact, an interesting example is shown in the study conducted by Han Lim and colleagues, in which the authors questioned the actual distribution of drug particles administered by inhalation in bronchial asthmatic patients [54]. They highlight how predictive models, which analyze the diffusion of the particles, measure only the aerodynamics of the particles themselves, underlining a substantial discrepancy between these models and what occurs in vivo. Therefore, a 3D-printed model of the airways was created, and the distribution pattern of salbutamol sulfate was tested. This model provided results showing a reasonable in vitro–in vivo correlation, opening up possible practical applications in the field of personalized medicine. The main limitations of this study are the absence of investigations on formulations with different molecular weights and the absence of key physiological components, such as a capillary bed capable of modifying the temperature of the tissue and the presence of a mucous layer. These limitations, however, open the door for possible new implementations of highly scalable systems [54]. Another interesting example of possible applications of lung-on-chip (LOC) technologies was highlighted by the study conducted by Mermoud et al., in which the authors developed an innovative microimpedance tomography system (MITO) aimed at monitoring morpho-functional changes at the level of the alveolar–capillary barrier [55]. This system involved the creation of a LOC starting from 16HBE14o cells (2D cell cultural model implemented on a chip) which were placed on a polydimethylsiloxane (PDMS) membrane with 8 μm pores and pre-coated with 2.5 $\mu g/mL^{-1}$ of collagen 1. The basal slope was filled with a culture medium, and the sensors for measuring the microimpedance were placed in the center of the working electrodes (WE) to avoid zones of negative sensitivity. Consequently, the thus-designed chip allowed the research group to identify and quantify any damage to the alveolar membrane based on the impedance variations measured at constant intervals in the epithelial monolayer. To overcome the main limitations of OOCs, some research groups have developed integrated systems as close as possible to the concept of body-on-a-chip (BOC). Skardal et al. developed a chip connecting three types of different tissues—heart, liver, and lungs—to better investigate the interactions between the lungs and other organs [17]. The liver and cardiac organoids were connected by a circulatory perfusion system, and then the ALI culture of the pulmonary module (a culture that presented endothelial cells, fibroblasts, and epithelial cells) was also connected to the system. Using this design, through the chip and the integrated sensors (including electrodes for measuring trans-epithelial electrical resistance (TEER), different electrochemical sensors for the evaluation of variations of a wide range of analytes, and, finally, optical sensors for the morphological evaluation of tissues), they demonstrated that there was, indeed, a close interconnection between the examined organs. Following stimulation of the lung cultures with bleomycin, a compound known to induce fibrosis and inflammation at the level of the lung parenchyma, inflammation cascaded onto other organs, increasing the production of pro-inflammatory cytokines and various stress-related analytes. The authors offer an

important starting point and assistance in advancing the design of increasingly efficient and complex BOC models, which are integrated with solid sensors that allow for timely studies and analyses on the cause–effect relationships that underlie various respiratory diseases.

4. The Impact of Animal Models on the Study of Lung Carcinoma

We would like to underline the different methodological approaches to the study of neoplastic lung diseases. Lung cancer is one of the most common tumors worldwide, and it is, thus, essential to carefully study the mechanisms of carcinogenesis as well as tissue responses to novel drugs, starting from a reliable, reproducible, and faithful study model. Currently, the incidence of lung carcinoma is estimated at 60 cases per 100,000 people. Lung cancer is the leading cause of cancer in males, while in women, the incidence is lower, but this still reflects the increasing exposure to smoking around the world. The major contributor to the high incidence of lung cancer is undoubtedly tobacco smoke (in particular, cigarette smoke, which is more deeply inhaled), followed by air pollution (lung cancer is more frequent in industrialized urban areas) and exposure to carcinogenic substances [56]. Another important risk factor is the presence of scar areas from infarct lesions or previous tubercular processes where tumor cells may develop. The bronchial epithelium in such areas is replaced by cellular elements similar to those of the terminal bronchial or squamous epithelium, and cellular atypia may appear, from which a peripheral localization carcinoma may originate [57]. Histologically, all these neoplasms derive from the endodermis. The adjective "bronchogenic", often attached to these tumors, indicates their origin from the bronchi (although adenocarcinomas arise more often from the bronchioles) [58]. Lung carcinomas originate within a tumor microenvironment (TME) characterized by a high degree of vascularization and oxygenation. Many studies have suggested that the interaction between tumor cells and stroma is responsible for the development of lung carcinoma and tissue preferences for metastasis. In particular, metastasis reflects a phenomenon of tumor cell migration towards chemoattractants released from distant tissues or disseminated tumor cells that can survive in specific tissue microenvironments [59]. One consequence of the set of intrinsic responses of cancer cells to released cytokines is that different tumor types have preferences for the tissues in which they metastasize (tissue tropism). This implies that tumor heterogeneity is not only intrinsic to the tumor itself, but also extrinsic, with a clinical picture that may differ greatly from one patient to another [59]. It is clear that tissue heterogeneity is often identified as the main obstacle not only to the development of effective therapies, especially patient-specific target therapy, but also to the use of effective in vivo, in vitro, and ex vivo models for study and preclinical testing [60].

Although, over the years, they have provided important evidence on pathology, traditional study models are not able to replicate heterogeneity or the TME. Among in vivo models, there are patient-specific animal models, such as patient-derived xenografts and genetically modified models [61]. These are certainly more complex models that have evolved from subcutaneously xenografted tumor models to orthotopic tumor models by surgically implanting tumor grafts or injecting tumor cells into the lung. However, the former does not allow for the study of tumor migration, while the latter generates artefacts due to the premature escape of tumor cells in suspension, probably due to the anatomy and ventilation mechanisms of the lung itself [62]. Furthermore, tumors derived from the inoculation of tumor cells in suspension can differ significantly in size due to several factors, such as the variation in the amount of the cell suspension and differences in the injection site [63]. Indeed, differences in tissue disruption caused by injection could lead to differences in the settlement of the inoculated cells, and, thus, in the shape of the tumor [64]. Patient-derived xenograft models represent an evolution from their predecessors. They rely on immunocompromised mice subjected to clonal selection at the time of human tumor tissue grafting, leading, however, to genetic and phenotypic divergence from the progenitor tumor. Genetically modified mouse models also have their limitations; although it is possible to study tumorigenesis and the efficacy of anti-tumor agents (targeted anti-cancer agents), they still lead to the early death of the animal, thus preventing the study of tumor

progression. Furthermore, the economic impact (in vivo animal models are expensive) and the relatively low efficiency of this type of model must be considered.

Is There an Alternative to Animal Models for the Study of Lung Carcinoma?

The development of three-dimensional (3D) cultures, which represent a fundamental tool for studying the biological activities, cellular changes, and regulatory mechanisms of architectural changes in normal and neoplastic tissues, is a relatively novel evolution in the field of in vitro research. These culture models more accurately reproduce certain aspects of cell physiology, migration, and response to drug treatment. However, the process of tumorigenesis relies on the interaction between tumor cells and the microenvironment, which consists of stromal cells, endothelial cells, and immune system cells [65,66]. Recently, three-dimensional multicellular spheroids (MCS) have emerged as interesting and reliable in vitro models of cancer. Spheroids are structures that reproduce cellular responses and interactions in vivo which have been used by several studies in the context of lung cancer and, more generally, lung tissue. Spheroids are easily reproducible and versatile. They can be developed from primary bronchial epithelial cells cultured in Matrigel to mimic the context of the extracellular matrix and simulate the structure of lung acini (similarly to what was previously described for the COPD cultural models). In addition, spheroids can be obtained from healthy human tissue samples and inoculated with lung progenitor cells for the study of various lung diseases. Another advantage of spheroids is that they can be used to reproduce the tumor microenvironment by co-culturing with stromal cells, such as fibroblasts. Moreover, when they are of sufficient size, MCS have a hypoxic and necrotic core similar to that of solid tumors in vivo, which plays a key role in anti-cancer drug resistance [67]. This means that what is commonly considered an intrinsic limitation of these cultures for the study of other diseases becomes a substantial advantage in a tumoral context. More recently, human tumor tissue organoids have emerged as a representative and low-cost study model that can characterize both tumor heterogeneity and TME interactions. Organoids are three-dimensional multicellular microtissues designed to accurately copy the complex structures and functions of human organs [68]. In an interesting study by Chen and colleagues, embryonic stem cells were used to create lung organoids that were grown for up to 170 days. After this time frame, the gene expression of the organoids matched the profile found in vivo in the human lung at the end of the second trimester of gestation. This protocol was then employed by the same study group to model the organoids based on the inclusion of mutated genes, or even the elimination of genes, to recreate disease models. The principle of gene elimination and gene editing could, for example, help in identifying the mechanisms that regulate malignant transformation and metastasis of lung cancer. Moreover, since the same lung organoid can be cultured for months, any long-term changes can be monitored in real time. Although the time and costs associated with maintaining organoids are two of the most important limitations of these models to date, a study by Sachs et al. demonstrated how optimization of traditional organoid models could lead to the creation of a standard for studying and screening drugs in vitro. Their research group developed an organoid model obtained from lung tissue collected from non-small cell lung cancer (NSCLC) patients undergoing surgery. The resulting organoids were composed of properly polarized and pseudostratified airway epithelium containing basal, muciparous, and ciliated cells, and were named airway organoids (AOs). Subsequently, AOs were used for drug screening, with individual organoids varying significantly in their respective responses to treatment, as is in line with their mutational profile. The same protocol was also applied to biopsies of metastatic NSCLC, demonstrating that mutant tumor AOs from primary and metastatic NSCLC can reproduce the tumor's microscopic anatomy and mutation status, and can be tested for in vitro drug screening [20].

Advances in bioengineering have led to the development of innovative organ-on-a-chip models that can simulate human organ function more accurately than traditional animal models. These models include the extracellular matrix and various cell types that enable cell-to-cell communication [53]. For example, Huh and colleagues created an

organ-on-a-chip model that replicated the human lung and ventilation mechanisms, and also allowed for the study of angiogenesis mechanisms and the infiltration of tumor cells. Cancer-on-a-chip models have been used to test drug toxicity using primary tumor cells derived from patients. The benefits of these models include the ability to integrate various cytotypes to reproduce the tumor microenvironment, measure experimental parameters and specific biomarkers, and expose the chip to carcinogens while measuring predictive markers. However, limitations exist regarding the use of organoids, such as the current inability to study the long-term effects of specific treatments or accurately reflect the metabolism of drugs in vivo. Nevertheless, these bioengineering advancements offer promising potential for reducing production costs, using the models on a larger scale, and providing a possible alternative to traditional animal models for studying tumoral diseases (Figure 3).

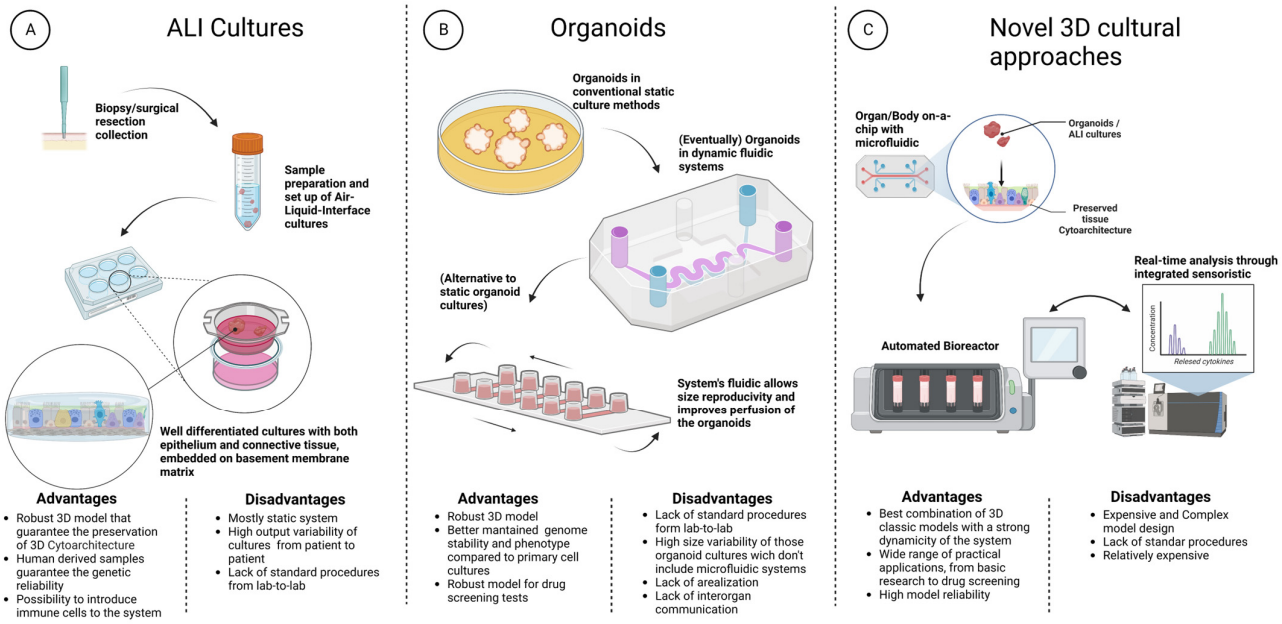

Figure 3. Schematic representation of the most frequently used models for the in vitro study of respiratory diseases. Each panel presents a short workflow of each procedure and the consequent advantages/disadvantages of each methodological approach. (**A**) Air-liquid interface culture models developed from bioptic fragments grown on the bottom of transwell inserts. Well-differentiated epithelial cells recreate the cytoarchitecture of the airways. (**B**) Organoids developed from adult cells, tissue, or induced pluripotent stem cells (iPSCs) grown in static culture methods or fluid dynamic systems. The fluidic system allows the real representation of in vivo environment and cellular responses. (**C**) On-a-chip models are grown on an automated dynamic system simulates the real physiology of the organ. A complex sensors system allows real-time analysis of a full spectrum of analytes.

5. Conclusions

To date, in different fields of basic and applied research, the animal model is commonly considered the gold standard for the validation of morpho-functional studies, pharmacological screening, and studies of pathologies using complex models. However, in a context in which several alternative strategies have been implemented (i.e., the introduction and improvement of alternative and complex models such as organoids, "alveolar lung-on-a-chip", outgrowths capable of reproducing the 3D structure of the respiratory mucosa and bioreactors that mimicry dynamic in vivo systems), the animal model, with all its ethical and practical limitations, seems to be less and less imperative (Figure 3). Within the most modern "organ-on-a-chip" technology, it is often possible to integrate sensors capable of analyzing a vast plethora of metabolites in a very short time frame, reducing the experimental gap between the experiment and data collection [17]. This also allows for the

creation of complex and realistic systems of the physiological and pathological model of reference. The main limits of such modern experimental techniques can, therefore, be found in the current lack of reference quality standards and of methods shared by various working groups. Up to now, there has been no univocal standard either in the production of the cultures used (whether they are organoids or outgrowths) or in chips capable of mimicking the complexity of the reference organ. Moreover, the integration of liquid and gaseous fluidics systems, designed to mimic the dynamism of the circulatory stream and the air pressure peaks on the respiratory mucosa, are not habitually taken into consideration as indispensable in the utilized study models. Thus, the complexity of the systems is reduced, as is the reliability of the in vivo model.

Currently, the scientific community is often led to think that the animal model is essential for quality research. The enormous progress made in this field tends to suggest the opposite, thanks to an increasingly transversal and multidisciplinary approach. To conclude, the definitive discontinuation of the animal model for the study of pathologies and clinical diagnostic approaches to respiratory diseases depends on the will of the scientific community, on the recent engineering and biotechnological advances, and on the desire to redirect funds and resources towards research groups with more heterogeneous and transversal backgrounds.

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Abbreviations

3D	Three-dimensional
AHR	Airway Hyperresponsiveness
ALI	Air–Liquid Interface
Aos	Airway Organoids
BHR	Bronchial Hyperresponsiveness
BME	Basement Membrane Extract
BMnCs	Bone marrow Mononuclear Cells
BOC	Body-On-a-Chip
COPD	Chronic Obstructive Pulmonary Disease
CS	Cigarette Smoke
EMTU	Epithelial–Mesenchymal Trophic Unit
HDM	House Dust Mite
IP-10	IFN-inducible protein 10
IPSCs	Induced Pluripotent Stem Cells
LOC	Lung-On-a-Chip
MCS	Multicellular Spheroids
MITO	Microimpedance Tomography
NSCLC	Non-Small Cell Lung Cancer
OOC	Organ-On-a-Chip
OVA	Ovalbumin
P-V	Pressure–Volume
PAECs	Primary Airway Epithelial Cells
PDMS	Polydimethylsiloxane

ROCK	Rho-associated protein kinase
TEER	Trans-Epithelial Electrical Resistance
TME	Tumor microenvironment
TSLP	Thymic stromal lymphopoietin
WE	Working Electrodes

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