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INVITED REVIEW

Use of human airway smooth muscle in vitro and ex vivo to investigate drugs for the treatment of chronic obstructive respiratory disorders

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Abstract

Isolated airway smooth muscle has been extensively investigated since 1840 to understand the pharmacology of airway diseases. There has often been poor predictability from murine experiments to drugs evaluated in patients with asthma or chronic obstructive pulmonary disease (COPD). However, the use of isolated human airways represents a sensible strategy to optimise the development of innovative molecules for the treatment of respiratory diseases. This review aims to provide updated evidence on the current uses of isolated human airways in validated in vitro methods to investigate drugs in development for the treatment of chronic obstructive respiratory disorders. This review also provides historical notes on the pioneering pharmacological research on isolated human airway tissues, the key differences between human and animal airways, as well as the pivotal differences between human medium bronchi and small airways. Experiments carried out with isolated human bronchial tissues in vitro and ex vivo replicate many of the main anatomical, pathophysiological, mechanical and immunological characteristics of patients with asthma or COPD. In vitro models of asthma and COPD using isolated human airways can provide information that is directly translatable into humans with obstructive lung diseases. Regardless of the technique used to investigate drugs for the treatment of chronic obstructive respiratory disorders (i.e., isolated organ bath systems, videomicroscopy and wire myography), the most limiting factors to produce high-quality and repeatable data remain closely tied to the manual skills of the researcher conducting experiments and the availability of suitable tissue.

Abbreviations: 5-LOX, 5-lipoxygenase; ASM, airway smooth muscle; COPD, chronic obstructive pulmonary disease; CSE, cigarette smoke extract; DI, deep inspiration; ECM, extracellular matrix; EFS, electrical field stimulation; EpiDRFs, epithelium-derived relaxing factors; eNANC, excitatory NANC; FEV_1, forced expiratory volume in 1 s; FEF_{25-75}, forced expiratory flow between 25% and 75% of vital capacity; FVC, forced vital capacity; GOLD, Global Initiative for Chronic Obstructive Lung Disease; iNANC, inhibitory NANC; KH, Krebs–Henseleit; mAb, monoclonal antibodies; MOI, multiplicity of infection; MLC, myosin light-chain; MLCP, MLC phosphatase; NF, nuclear factor; OCLN, occludin; PAH, polyaromatic hydrocarbon; PAL, persistent airflow limitation; PCLS, precision lung cut slices; PIV-3, parainfluenza virus type 3; PM, particulate matter; QS, quick stretch; SP, Substance P; PSS, physiological saline solution; uPAR, urokinase plasminogen activator receptor; WNT, wingless/integrase-1.

KEYWORDS
airway smooth muscle, asthma, COPD, epithelium damage, in vitro models, isolated human airways

1 | INTRODUCTION

Airway smooth muscle (ASM) has been extensively investigated in vitro and ex vivo to understand the pharmacology of asthma and chronic obstructive pulmonary disease (COPD). The term 'ex vivo' literally translates from Latin as 'out of the living', whereas the term 'in vitro' means 'in glass' as historically in vitro experiments were conducted in Petri dishes or tubes made of glass. In ex vivo studies, living tissue is directly taken from a living organism and it is immediately studied in an external environment with minimal alteration of natural conditions (Wang & Duan, 2019). On the other hand, in vitro experiments are generally performed on cells that are in culture outside their normal biological environment (Wang & Duan, 2019). However, ex vivo more conventionally means working with tissue samples taken from patients treated with a drug rather than in vitro using tissues removed from patients following surgery, where no drug was being investigated in vivo prior to resection (Gale et al., 2002).

Accessing MEDLINE with no time limits according to the following search query '(isolated AND human) AND (airway OR bronchi OR airway smooth muscle) AND pharmacology NOT review' resulted in more than 2800 records, with a publication peak of 144 papers in 2020 (Figure 1). According to SCOPUS, in the last 30 years the first three researchers producing original pharmacological evidence from isolated human airways were Naline E. (France), Advenier C. (France) and Calzetta L. (Italy). Detailed information regarding the search query is reported in the supporting information.

Asthma and COPD are increasingly relevant causes of morbidity, disability, and mortality worldwide, accounting together for an overall global prevalence of ≈14% (Adeloye et al., 2022; Innes Asher et al., 2020). Indeed, this worrying percentage fully justifies the search for new and improved therapies to treat these conditions. Unfortunately, the costs and time necessary for the development and approval of new effective drugs for the treatment of chronic obstructive respiratory disorders are increasing (Calzetta, Chetta, et al., 2023a) and apart from the introduction of some monoclonal antibodies (mAbs) for the treatment of severe asthma and COPD with type 2 inflammation, the cornerstone treatments used in the majority of patients with asthma or COPD still remains focused on pharmacological classes of drugs that have been around for decades, such as bronchodilators and corticosteroids (Bhatt et al., 2023; GINA, 2023a; GOLD, 2023). Certainly, mAbs have significantly changed the management of severe asthma, however their use is restricted to a small proportion of patients (GINA, 2023b).

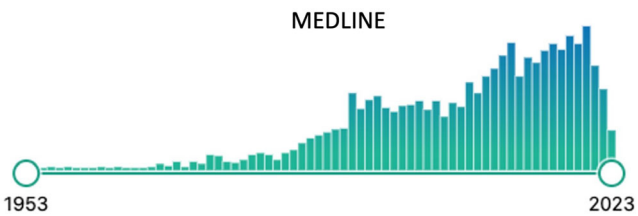

FIGURE 1 Documents by years in MEDLINE with no time limit according to the search query '(human AND isolated) AND (airway OR bronchi OR airway smooth muscle) AND pharmacology NOT review'.

There has been often poor predictability from murine experiments to the success of drugs subsequently evaluated in patients with asthma or COPD, and therefore the use of human tissues such as isolated airways represents a sensible strategy to optimise the development of innovative molecules for the treatment of respiratory diseases (Aun et al., 2017; Calzetta, Matera, et al., 2019a; Tanner & Single, 2020; Wright et al., 2008).

Therefore, the aim of this review, performed referring to the latest Concise Guide to Pharmacology (Alexander et al., 2021), was to provide updated evidence on the current uses of isolated human airways in validated in vitro methods to investigate drugs in the development of treatments for chronic obstructive respiratory disorders. This review also provides historical notes on pioneering pharmacological research on isolated human airways tissues, the key differences between human and animal airways, as well as the pivotal differences between human medium bronchi and small airways.

2 | HISTORICAL BACKGROUND

The first scientific report concerning experiments on the physiology of isolated airways is dated August 1840, when the British physician Charles James Blasius Williams described, in the Report of the 10th meeting of the British Association for the Advancement of Science, the results of 3 years of experiments on the contractility and sensibility to various stimuli of bronchi, that he called 'air-tubes', and lungs removed from animals (Williams, 1840). Williams described in detail results from experiments that were pioneering for modern respiratory pharmacology and he provided the basis for the treatment of chronic obstructive respiratory disorders. Williams definitely proved the 'muscular contractility of any part of the air-tubes below the larynx' and, among his elegant experiments, some are worthy of mention. Of note, at that time he proposed that several respiratory diseases, especially asthma, were related to an altered contractile response of the airways.

To record the airway contractility and relaxation, Williams used a Poiseuilles's haemadynamometer, the precursor of the modern isometric force transducers used to measure the ASM contractile tone in isolated organ bath systems.

He reported that canine isolated airways contracted in response to galvanic current, a transmural stimulation technique precursor of

the modern electrical field stimulation (EFS) activating the parasympathetic system, and that the extent of contractility was null or scarce in small airways and greater in larger bronchi as confirmed in more recent studies (Calzetta et al., 2020; Myers & Undem, 1996). Of note, Williams had unknowingly demonstrated the contractile activity of the cholinergic system on ASM and the fact that as we now recognise, small airways are not innervated by a functional parasympathetic neurons (Barnes, 2004; Barnes et al., 1983; Fryer & Jacoby, 1998; Koarai & Ichinose, 2018). Williams repeated similar experiments also in calves and rabbits, although he experienced more difficulty in detecting ASM contractility in isolated airways from rabbits, probably due to the low sensitivity of the dynamometer to measure the small change of pressure elicitable in the airways of smaller animals. He also recorded that galvanic current increases the release of mucus from isolated airways, a condition always related with parasympathetic activation and airway contractility (Calzetta, Ritondo, Zappa, Manzetti, et al., 2022e).

Observing the reaction of equine and canine airways to a scalpel, Williams also proposed that mechanical irritation may stimulate an airway contractile response, a condition similar to that induced in modern experiments in which mechanical stress is induced in isolated bronchial tissue via quick stretch (QS) (Faisy et al., 2011). He also reported the airway contractile response to irritating fluids in dogs and rabbits.

Among the experiments carried out by Williams, probably the most visionary are those in which he demonstrated that the airway contractility induced by galvanism in dogs and rabbits can be completely abolished by extracts obtained from *Atropa belladonna* and *Datura stramonium*, now known to contain the antimuscarinic agents atropine and scopolamine respectively (Fatur & Kreft, 2020). Notably, Williams provided the pharmacological rationale for the use of antimuscarinic agents as a cornerstone therapy for the treatment of COPD around 150 years before the first Global Initiative for Chronic Obstructive Lung Disease (GOLD) report was published (Rodriguez-Roisin, 2017).

A modern look at the William's investigations shows that his experiments were designed as translational research performed in experimental animals aimed to study the ASM responsiveness and to pharmacologically characterise the effect of bronchodilators to treat human asthma and COPD. The full report of experiments on the physiology of the lungs and air-tubes by Williams is available as supporting information.

3 | ANIMAL VERSUS HUMAN ISOLATED AIRWAYS

ASM plays a pivotal role in the pathophysiology of asthma and COPD as it contributes to the bronchoconstriction, airway hyperresponsiveness (AHR), airway remodelling, and inflammation that characterise these diseases, now recognised as two separate disorders characterised by different aetiology, symptoms, inflammatory profile, airway narrowing, response to therapy and time course (Cukic et al., 2012; Doeing & Solway, 2013). Although it is extensively recognised that ASM is a crucial target in the treatment of both of these chronic obstructive respiratory disorders, to date the pathophysiology of human airway narrowing that characterises asthma and COPD remains only partially understood (Bates, 2016; Hulsmann & de Jongste, 1993; Mauad & Dolhnikoff, 2008).

Therefore, in order to gain more insight into the underlying processes of asthma and COPD, many animal models have been developed. However, significant differences between commonly used experimental animal species (particularly mice) and humans exist (Hulsmann & de Jongste, 1993).

In the 2000s, a number of task forces attempted to standardise murine models of asthma and COPD (Brusselle et al., 2006; Kips et al., 2003). However, the same authors highlighted several major limitations of this approach, mainly related to the fact that these models mimic only specific traits of the diseases and the inflammatory profile resulting from these murine models does not overlap completely with systemic and tissue inflammation that characterise human asthma and COPD (Brusselle et al., 2006; Kips et al., 2003). However, in murine models of asthma and COPD, a certain level of small airway alteration can be detected, although the histological and inflammatory modifications only partially overlap those detectable in humans (Lam et al., 2023; Wright et al., 2008).

In this regard, a study comparing the genomic responses of mice and humans to inflammatory stimuli found limited overlap, indicating that murine models may not reliably predict human responses. Additionally, the controlled and often sterile laboratory environments in which mice are maintained may not accurately mirror the complex and dynamic conditions of human diseases (Junhee Seok et al., 2013). Effectively, several important publications highlighted the major limitations of murine models in accurately modelling human diseases and immune responses, with evidence suggesting that irreproducible preclinical results from animal models can lead to a massive waste of time and money in clinical trials (Check Hayden, 2014; Mestas & Hughes, 2004; Shay et al., 2013).

Other considerable physiological, anatomical, and immunological factors differentiate rodents from humans. Unlike humans, rodents breathe only from their nose, the submucosal glands are localised only at the level of the trachea, and pulmonary lobation and bronchial branching are substantially different, furthermore rodents have neither defined respiratory bronchioles nor distinct lobular architecture. Also, significant discrepancies in both innate and adaptive immunity have been reported between human and murine immune system (Mestas & Hughes, 2004). Finally, but no less important, there is a significant gap in lung volumes between humans and rodents, making technically challenging the accurate measurement of lung function in such small laboratory animals and requiring complex allometric calculations to ensure that experimental conditions in rodents are pharmacologically relevant in humans (Boxenbaum, 1982; Brusselle et al., 2006; Irvin & Bates, 2003). As a matter of fact, the mechanical properties and morphological characteristics of human ASM are similar, although not identical, to those of other larger animals such as dogs, pigs and sheep (Chin et al., 2010).

Moreover, while in rodents cholinergic neurons innervate all the bronchial tree including peripheral airways, the distribution of functional parasympathetic innervation at the level of human small airways is sparse or even absent (Audrit et al., 2017; Barnes, 2004; Barnes et al., 1983; Canning, 2006; Garssen et al., 1993; Koarai & Ichinose, 2018; Struckmann et al., 2003). The airways of rodents are also directly innervated by sympathetic neurons, conversely in humans there is no evidence of functional direct sympathetic innervation of ASM (Barnes, 2012; Cazzola, Page, et al., 2012b; Kummer et al., 1992; Liu et al., 2020; Takachil et al., 1995). The human sympathetic system indirectly acts on the airways via circulating adrenaline and via an influence on parasympathetic ganglia (Cazzola, Page, et al., 2012b).

Along with the parasympathetic and sympathetic systems, the bronchial tone may be modulated by the non-adrenergic non-cholinergic system (NANC), via excitatory and inhibitory pathways called eNANC and iNANC, respectively (Cazzola, Page, et al., 2012b). Although the NANC systems were extensively investigated in late 1980s–1990s for the treatment of asthma, unfortunately it seems that NANC nerves have a scarce role in the modulation of human ASM tone, as no agents modulating eNANC and iNANC have been developed (Andersson & Grundström, 1987; Belvisi et al., 1992; Lammers et al., 1992; Widdicombe, 1998). Rather, it seems that NANC may have a certain role in fine-tuning the cholinergic system (Kistemaker & Prakash, 2019). However, the eNANC system may elicit neurogenic inflammation in human airways via **neurokinin A (NKA)** and **substance P (SP)** release (Calzetta, Luongo, et al., 2015b; Calzetta, Matera, & Cazzola, 2018a; Cazzola et al., 2017; Groneberg et al., 2004; Joos et al., 1995; Rogliani, Ritondo, Facciolo, et al., 2021b). On the other hand, the autonomic regulation of ASM tone through eNANC and iNANC is relevant in rodents (Lindén et al., 1993; Widdicombe, 1998).

Although the intimate regulation of ASM contractility mediated by the balance between myosin light-chain (MLC) kinase **(MLCK)** and MLC phosphatase (MLCP) is comparable among the species (Figure 2a), the upstream pathways modulating this balance is species-specific (Figure 2b,c). For these reasons there have been several recent calls to return to using guinea-pig tissues to study the pharmacology of airways in vitro as a better predictor of human airways (Adner et al., 2020; Tannu et al., 2010) as this species has been successfully used to predict the development of antimuscarinic agents (Alabaster, 1997), **β_2-adrenoceptor agonists** (Jack, 1991) and more recently **ensifentrine** (Boswell-Smith et al., 2006; Venkatasamy & Spina, 2016) as effective treatments for patients with asthma and COPD.

While murine models of asthma and COPD seem to have limited translational utility (Aun et al., 2017; Tanner & Single, 2020), it is noteworthy that most of the mAbs currently entering clinical trials or already approved for the treatment of chronic obstructive respiratory disorders are derived from humanised mice. In humanised mAbs, the only murine sequences retained in the antibodies are those directly involved in the antigen recognition in order to yield much reduced immunogenicity (Nixon et al., 2017). Moreover, recently animal models using humanised mice have been developed to preclinically investigate airway inflammation in asthma (Ito et al., 2018; Nunomura et al., 2022).

Large animal models of asthma and COPD have also been used in respiratory research beyond rodents. For example equines affected by

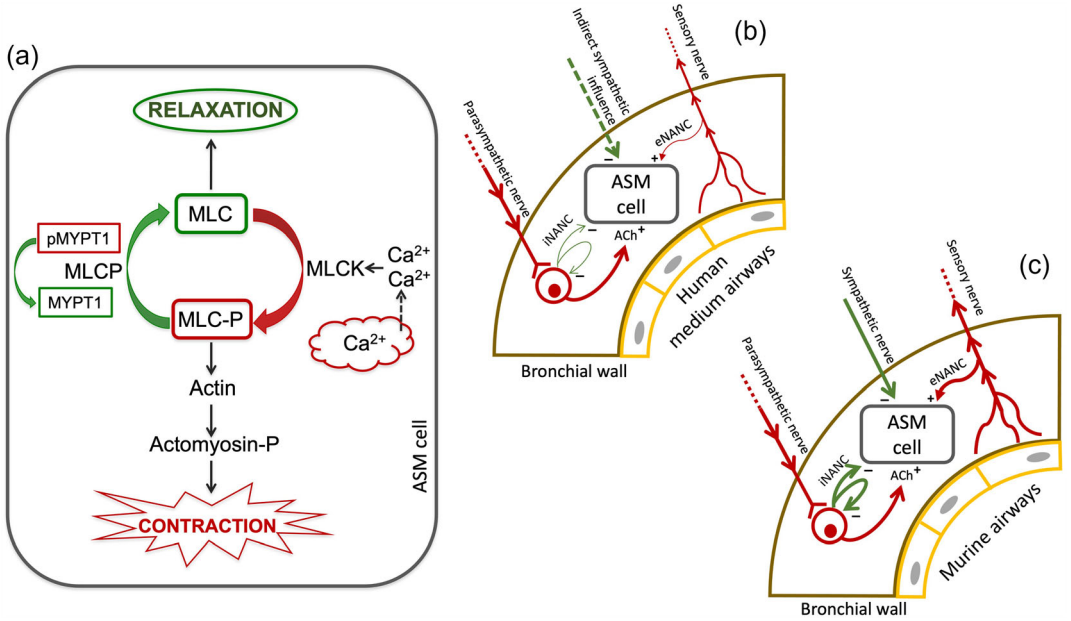

FIGURE 2 Intimate regulation of ASM contractility (a) and upstream pathways modulating the balance between bronchial contractility and relaxation in human medium bronchi (b) and murine airways (c). In (b) and (c) the thickness of arrows is directly proportional to the importance of the pathways. ACh: acetylcholine; ASM: airway smooth muscle; eNANC: excitatory non-adrenergic non-cholinergic system; iNANC: inhibitory non-adrenergic non-cholinergic system; MLC: myosin light-chain; MLCK: myosin light-chain kinase; MLCP: myosin light-chain; MYPT1: myosin phosphatase target subunit 1; -P: phosphorylated; pMYPT1: phospho myosin phosphatase target subunit 1.

heaves, currently improperly called equine asthma, may be a potential model of pathophysiological conditions overlapping between human asthma and COPD. In this respect, airways obtained from horses with heaves are characterised by persistent and not fully reversible airflow limitation, AHR, mucus hypersecretion, prevalent neutrophilic inflammation with concomitant signs of eosinophilic inflammation, allergic responses to aeroallergens, airway remodelling and emphysema in the more severe forms of disease (Bullone & Lavoie, 2017; Couëtil et al., 2016; Dixon et al., 2021; Hunter et al., 2020; Leclere et al., 2011). Indeed, this is a condition characterised by several features associated with asthma and several features associated with COPD, suggestive of asthma–COPD overlap (ACO) as already reported in humans (Miravitlles, 2017). Despite the anatomical, pathophysiological, and mechanistic differences with human airways, isolated equine bronchi may represent an alternative, although not a specific, model of human chronic obstructive respiratory disorders (Calzetta, Pistocchini, Ritondo, Cavalli, et al., 2022d; Calzetta, Rogliani, et al., 2019c; Calzetta, Rogliani, Pistocchini, et al., 2018d, 2018e).

Sheep models of asthma performed by immunisation with house dust mite (HDM), as well as some sheep models of COPD carried out inducing emphysema via segmental instillation of **lipopolysaccharide (LPS, also known as endotoxin**), have been also proposed; however, only a few drugs tested in these models have reached clinical development or final approval in humans to fully understand the predictability of this species to humans, although they have been used in asthma research for a number of years using Ascaris suum as an antigen (Bischof et al., 2003; Hewson et al., 2012; Van Der Velden et al., 2013; Van der Velden & Snibson, 2011; Woodrow et al., 2023).

Certainly, non-human primates are the best animal models of asthma and COPD but their use is restricted due to the costs and relevant ethical considerations (Bertho & Meurens, 2021; Coffman & Hessel, 2005; Plopper & Hyde, 2008). In the recent decades porcine models of asthma and COPD have also been proposed, representing an intermediate choice between murine and non-human primate models of chronic obstructive respiratory disorders (Bertho & Meurens, 2021). However, using pigs as models of asthma and COPD also has important limitations since these models are mainly focused on mucosal immunity, allergy, and trained immunity (Bertho & Meurens, 2021). Certainly, some important experiments have been carried out on isolated airways collected from pigs in the 2000s (LaPrad et al., 2008; Noble et al., 2007; Turner et al., 2002). However, porcine models remain expensive and this species is more challenging to be managed in an animal facility when compared to smaller laboratory animals (Walters & Prather, 2013).

Overall, considering the difficulties associated with studying small experimental animals, the poor predictive value of murine ASM contractile regulation with respect to human airways, and the costs and management of models in large animals, to date there is no ideal preclinical model in experimental animals for understanding chronic obstructive respiratory disorders (Aun et al., 2017; Brusselle et al., 2006; Canning, 2006; Kips et al., 2003; Van der Velden & Snibson, 2011). There remains therefore a need to, where possible, investigate human tissues to better understand human diseases and the use of isolated human airways represents a valid approach to investigate drugs for the treatment of chronic obstructive respiratory disorders (Black et al., 2012; Blume & Davies, 2013; Calzetta, Matera, et al., 2019a; Hulsmann & de Jongste, 1993; Persson, 2002; Roux et al., 1999; Selo et al., 2021). Effectively, several bench-to-bedside studies have demonstrated that results obtained from well-performed experiments on isolated human airways can be translated into the clinical setting (Calzetta, Aiello, Frizzelli, Bertorelli, & Chetta, 2022a; Calzetta, Matera, et al., 2019a; Calzetta, Pistocchini, et al., 2019b; Cazzola, Calzetta, Ora, et al., 2015a; Cazzola, Calzetta, Segreti, et al., 2015b; Rogliani et al., 2015; Rogliani, Ora, Girolami, et al., 2021a).

4 | HUMAN MEDIUM BRONCHI AND SMALL AIRWAYS

In vitro studies can be performed on fresh bronchial tissue obtained from surgical specimens or post-mortem. The vitality of isolated human airways during experiments to record ASM contractility in isolated organ bath systems or patching chambers may last up to 12 hrs and both medium bronchi (sub-segmental bronchi) and small airways (bronchioles) can be used (Bouyssou et al., 2010; Calzetta, Matera, et al., 2019a; Cazzola et al., 2014; Cazzola et al., 2016; Cazzola, Calzetta, Ora, et al., 2015a; Naline et al., 2007; Slack et al., 2013). Isolated tissue can be also cultured for up to 8 days for long-term subchronic incubation with specific agents and then the contractile responses can be evaluated in isolated organ bath systems or patching chambers (Naghshin et al., 2003), but during tissue culturing the airway contractility cannot be measured. Relevant structural and functional differences characterise the isolated airways originating from different anatomical parts of the bronchial tree.

Human medium bronchi originate after 4–6 generations from the trachea and are characterised by an inner diameter of ≈4 mm. They contain cartilage, ASM, and mucosal lining including pseudostratified ciliated columnar epithelium, mucus-producing goblet cells, mucus glands, and parasympathetic ganglia (Amador et al., 2022; Barnes, 2019; Calzetta, Ritondo, Zappa, Manzetti, et al., 2022e; Canning, 2006; Kim et al., 2011; Van Scott et al., 2013). Going further down the bronchial tree, the ratio between amount of cartilage and ASM mass decreases (Amador et al., 2022). At the level of human small airways, reached after 7–8 generations from the trachea and characterised by an inner diameter of <2 mm, there is no cartilage and the wall patency is due to the ASM and elastic fibres, the epithelium is simple cuboidal with club cells and few goblet cells, and no functional parasympathetic innervation is present (Amador et al., 2022; Barnes et al., 1983; Calzetta, Ritondo, Zappa, Manzetti, et al., 2022e; van den Bosch et al., 2021; Van Scott et al., 2013). Concerning the main receptors regulating the ASM contractile tone, the density of **M_3 muscarinic acetylcholine receptors** decreases from medium bronchi to small airways, conversely the expression of β_2-adrenoceptors increases along the bronchial tree. The ratio between

β_2-adrenoceptors and M_3 receptors is two-fold higher in bronchioles than in larger airways (Ikeda et al., 2012).

Studying medium bronchi and small airways separately in vitro may have relevant translational implications. Of note, while subsegmental bronchi provide the greatest airway resistance along the bronchial tree, the contribution of bronchioles to airway resistance is very limited (Brooks, 1995; Thien, 2013). Indeed, the ASM tone detectable in medium isolated bronchi is related to the change in forced expiratory volume in 1 s (FEV_1) (Brown et al., 2006; Rogliani et al., 2015; Rogliani, Ritondo, Facciolo, et al., 2021b; Taube et al., 2006). On the other hand, the luminal area of small isolated airways is related to the forced expiratory flow between 25% and 75% of vital capacity (FEF_{25-75}), that is a predictive marker of peripheral obstruction leading to small airway disease (SAD) in the so-called 'silent zone' (Hogg et al., 2013; Kwon et al., 2020; Martin et al., 2013; Rogliani et al., 2015, 2023; Usmani et al., 2021) (Figure 3a,b).

5 | TISSUE COLLECTION, PREPARATION AND EQUIPMENT

Generally, isolated bronchi used to perform in vitro experiments are collected from patients undergoing lobectomy surgery for lung cancer who have consented to donate excised lung tissue that is not required for any diagnostic purpose. Macroscopically and microscopically normal regions of lungs are taken from uninvolved areas of neoplastic lesions, placed in Krebs–Henseleit (KH) buffer solution (NaCl, 119.0 mmol; KCl, 5.4 mmol; CaCl2, 2.5 mmol; KH2PO4 mmol, 1.2 mmol; MgSO4, 1.2 mmol; NaHCO3, 25.0 mmol; glucose, 11.7 mmol; pH 7.4), and transported to the laboratory from the nearby operating room. KH buffer solution is medicated with **indomethacin** (5 μM) to prevent altered ASM contractility related to the spontaneous generation of inflammatory mediators originating from the arachidonic pathway, and from histamine during tissue manipulation and microsurgery (Brink et al., 1980; Calzetta et al., 2011; Cuzzo & Lappin, 2022). It has been extensively demonstrated that the medication of KH buffer solution with indomethacin does not alter the ASM contractile response to agents active on bronchial tone (Brink et al., 1980; Naline et al., 2007).

In the laboratory, the airways are cut into rings with surgical scissors to produce medium isolated bronchial rings (thickness 1–2 mm) that are mounted into isolated, multi-channel organ bath systems. This is the most effective technique to prepare medium isolated bronchi to be studied in vitro in organ bath systems because it needs usually less than 100 mg of tissue, the tissue damage is reduced, and the ASM bundle is preserved (Calzetta, Aiello, et al., 2021a; Calzetta, Ora, et al., 2017a; Hulsmann & de Jongste, 1993; Ritondo et al., 2021). Several other techniques have been proposed to mount medium isolated airways into organ bath systems (Figure 4a). Isolated airways can be mounted as multi-chains of rings to elicit additive contractile effect of the rings in the chain preparation, but this approach is laborious, requires a lot of tissue, and each connecting knot may damage tissue and lead to mechanical instability and local inflammation (Hulsmann & de Jongste, 1993). Bronchial tissue can be also cut in spirals, an easy procedure that however can cause extensive tissue damage. The availability of modern sensitive isometric force transducers allows the use of bronchial strips, but in these preparations the ASM bundle is not always preserved (Hulsmann & de Jongste, 1993).

Usually, bronchial rings are transferred into a 10-ml organ bath system containing KH buffer solution (37°C) and continuously aerated with O_2/CO_2 (95:5%). Modern equipment ensures that bronchial rings are mounted on hooks, attached with thread to a stationary rod and the other end tied with thread to isometric force transducers characterised by a force range up to 25 g and a sensitivity of 1.5 $mV \cdot g^{-1}$ at 10 V. Airways are allowed to equilibrate by washing the tissue with fresh KH buffer solution and passive tension is determined by gentle stretching of tissue (0.5–1.0 g). The vitality of medium bronchi is tested via contractile responses induced by EFS at 25 Hz, a procedure

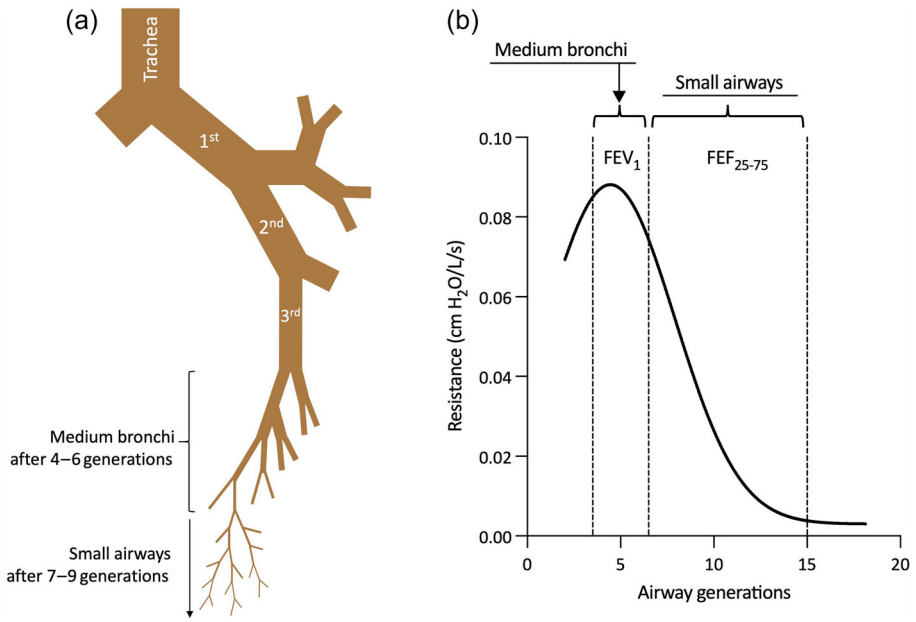

FIGURE 3 Schematic representation of the bronchial tree with airway generations (a) and airway resistance reporting the functional impact of airway generations on FEV_1 and FEF_{25-75} (b). FEF_{25-75}: forced expiratory flow between 25% and 75% of vital capacity; FEV_1: forced expiratory volume in 1 s.

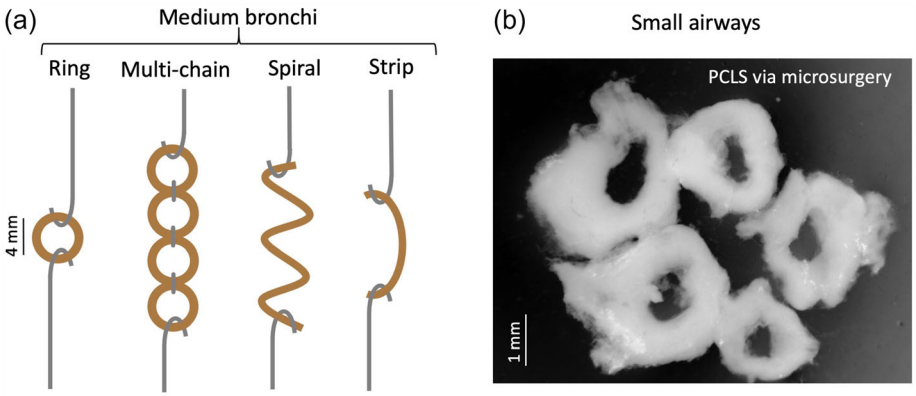

FIGURE 4 Schematic representation of the techniques used to mount medium isolated airways into organ bath systems (a) and small airways prepared as PCLS via microsurgery (b). PCLS: precision lung cut slice.

that also allows the bronchial rings to be correctly positioned between the hooks (Calzetta et al., 2020). When the passive contractile tone reaches the plateau, usually in 30–60 min, bronchial rings are washed three times with KH buffer solution and allowed to further equilibrate for 45 min.

Small isolated airways are prepared as precision lung cut slices (PCLS). Bronchioles (inner diameter ≈1 mm) are cut by a motorised vibratome equipped with ceramic blades and refined via microsurgery under a stereo microscope to obtain PCLS (thickness 250–500 μm) (Calzetta, Matera, et al., 2019a; Calzetta, Matera, Facciolo, et al., 2018b; Cazzola et al., 2016; Rogliani et al., 2020; Rogliani, Ritondo, Facciolo, et al., 2021b; Rogliani, Ritondo, Zerillo, et al., 2021c) (Figure 4b).

Alternatively, PCLS can be prepared by inflating bronchial tissue with 2.5% (w/v) warm ultralow melting point agarose. After that, the tissue is cooled, cut into 1 cm slices and then into 8 mm diameter cylindrical cores in which the airways are oriented longitudinally. The cores are then processed to ≈250 μm using a tissue slicer. Slices are transferred into tissue culture plate containing incubation buffer (Roswell Park Memorial Institute [RPMI] 1640 medium medicated with 100 $U \cdot ml^{-1}$ **penicillin**, 0.1 $mg \cdot ml^{-1}$ **streptomycin** and 4 mM **L-glutamine**) and incubated at 37°C. Slices are then washed with incubation buffer for 2–3 h and left overnight in a humidified incubator containing 5% CO_2 at 37°C on a rotating platform. The day after, slices are washed and returned to the incubator for 1 h before experiments (Koziol-White, 2022; Sanderson, 2011; Slack et al., 2013). The preparation of PCLS via inflation of warm agarose requires specific buffers such as the Hanks' balanced salt solution (HBSS) with Ca^{2+} and Mg^{2+} supplemented with HEPES and Dulbecco's modified Eagle medium (DMEM)/F-12 with antibiotics (Bai et al., 2016; Nayak et al., 2021).

The use of agarose to prepare PCLS is an effective, but complex, technique that includes several critical passages to inflate the airways with agarose and to adequately take away the agarose from the preparations. Indeed, processing small airways without the complications related to the use of confounding agarose gel to inflate the lung or complex parenchymal sections that also have numerous contracting elements may produce more reproducible results (Calzetta, Matera, et al., 2019a; Calzetta, Passeri, et al., 2014a; Van Lunteren & Moyer, 2001; Wohlsen et al., 2001).

PCLS are mounted into a visual imaging and patching chamber connected to a proportional integral derivative temperature controller with dual thermistor feedback containing KH buffer solution (37°C) and continuously aerated with O_2/CO_2 (95:5%). The contractility is assessed by a stereo microscope and a digital camera. Small airways are allowed to equilibrate by washing with fresh KH buffer solution until the luminal area is stable, usually 15–30 min. The vitality of small airways is assessed by **acetylcholine (ACh)** (0.3 μM) that produces an area reduction of at least 25% (Rogliani et al., 2020). After that, PCLS are washed three times with KH buffer solution and allowed to equilibrate for 45 min before experiments (Rogliani, Ritondo, Zerillo, et al., 2021c).

The contractility of small airways can also be studied via wire myography. Small airways with a length of ≈2 mm are mounted on two 40 μm steel wires in microvascular myographs in baths with physiological saline solution (PSS) heated to 37°C and aerated with O_2/CO_2 (95:5%). Preparations are allowed to equilibrate for 10 min thereafter and stretched to 2.4 kPa for optimal measurements. The vitality of the small airways is confirmed by a contractile response of at least 2 mN in response to a potassium-rich PSS (60 mM) (Kroigaard et al., 2012; Shaifta et al., 2018).

Another technique to investigate the mechanics of isolated airways is the use of perfused bronchial segments (Sparrow et al., 1992). In this method, segments of isolated airways ≈3.5 cm long are mounted into a specific isolated organ bath system, cannulated and perfused with KH buffer solution at 37°C, and maintained at a constant pressure head of 5 cmH_2O (Mitchell & Sparrow, 1989; Sparrow et al., 1992). Contractile or relaxant agents are injected into the lumen via the perfusate and the airway narrowing quantified by measuring flow or resistance in the isolated preparation (Mitchell & Sparrow, 1989; Sparrow et al., 1992). This technique offers the advantage of not exposing the tunica adventitia and cut edges of the isolated tissue to the treatments, thus replicating the exposure of inhaled agents via inhalation in vivo (Sparrow et al., 1992). However, this method mainly applies to airways explanted from large animals, such as pigs, due to the limited amount of isolated airways typically obtained from human donors, which may not be sufficient for perfused preparations, although this technique has also been used with rabbit airways for both in vitro and ex vivo investigations (Woisin et al., 2001).

More recently, a technique to measure the responsiveness of isolated human airways during tidal oscillation and deep inspiration (DI) has been described (Noble et al., 2011). Segments of isolated airways are mounted into a specific organ bath system in which side branches are cannulated and connected to a pressure transducer to measure contractility and a syringe pump oscillator to modulate the lumen pressure. Along with static measurement of contractility, this system allows reproducing ex vivo tidal oscillations (transmural pressure: 5–10 cmH_2O; frequency: 0.25 Hz) with intermittent DI (transmural pressure: peak at 30 cmH_2O). Detailed information on this system are available in the primary publication (Noble et al., 2011).

Usually, the contractile response of isolated airways can be recorded for up to 12 hrs (Bouyssou et al., 2010; Calzetta, Matera, et al., 2019a; Cazzola et al., 2014; Cazzola et al., 2016; Cazzola, Calzetta, Ora, et al., 2015a; Naline et al., 2007; Slack et al., 2013). However, in experiments requiring extended incubation periods with specific drugs or the evaluation of structural adaptations in bronchial tissue in response to chronic stimuli, medium bronchi and small airways can be cultured for up to 8 days (Naghshin et al., 2003). Isolated airways are placed in culture plates and washed three times with 4°C Connaught Medical Research Labs (CMRL) medium containing penicillin (10,000 $U{\cdot}ml^{-1}$) and streptomycin (10,000 $\mu g{\cdot}ml^{-1}$). After that, bronchial tissue is covered with CMRL medium at 4°C supplemented with 5% foetal bovine serum (FBS), bovine **insulin** (4 $mg{\cdot}ml^{-1}$), L-glutamine (10 $\mu L{\cdot}ml^{-1}$) and amphotericin B (250 $\mu g{\cdot}ml^{-1}$). Then, the culture plates are transferred into a humidified three gas incubator filled with a specific gas mixture (N_2 50%, O_2 45%, CO_2 5%) and gently agitated at 0.2 Hz at 37°C (Naghshin et al., 2003). The ASM contractility cannot be continuously monitored during the culturing period, but it can be assessed at specific time points or after 8 days by mounting preparations in isolated organ bath systems or patching chambers.

Generally, the study on isolated airways have been used to investigate the contractile mechanics of ASM. However, in the recent decades studies on isolated airways have gone beyond the investigation of contractile events. For example, at the end of experiments with human bronchial tissue, both medium bronchi and small airways, supernatants can be collected for assay via ELISA kits or sensitive multi-analyte profiling to detect specific analytes such as inflammatory mediators given the growing evidence that both ASM and the associated respiratory epithelium can release a range of inflammatory agents (Calzetta et al., 2020; Calzetta, Matera, & Cazzola, 2018a; Cazzola et al., 2016; Chesné et al., 2015; Dispenza et al., 2020).

Indeed, surgery is an effective procedure to collect large airway samples to study isolated ASM tissue in toto ex vivo. However, specific techniques may be used to source small tissue specimens useful to isolate ASM cells and then perform in vitro experiments. In this regard, since the early 2000s endobronchial biopsy via flexible bronchoscopy has been used to dissect ASM bundles from surrounding tissue and obtain cultured ASM cells from healthy, asthmatic and COPD donors (Burgess et al., 2003; Cazzola, Calzetta, et al., 2012a; Johnson et al., 2001; Zakarya et al., 2021). This procedure permitted characterisation of the increased proliferation of ASM cells in asthma (Burgess et al., 2003; Johnson et al., 2001). Further studies on biopsies allowed clarification in vitro of the crosstalk between ASM cells and mast cells and the investigation of the intracellular regulation of **phosphodiesterase** 4 (PDE4)/β_2-adrenoceptor signalling in asthma (Brightling et al., 2002; Kaur et al., 2012; Niimi et al., 2012).

The contractility of ASM can also be investigated in vitro in cultured ASM cells obtained by endobronchial biopsy. The contractility of ASM cells maintained adherent to the culture dish and presenting a well-defined outline is evaluated using digital time-lapse video microscopy. The cell length contraction/relaxation is expressed as a percentage reduction/enhancement in cell length compared to the maximal cell length reduction (Calzetta, Rogliani, et al., 2014b).

Also, other more complex methods, such as the optical magnetic twisting cytometry and Fourier-transform traction microscopy, have been developed to microscopically measure the stiffness and traction of single ASM cells in vitro in response to contractile and relaxing agents (An et al., 2006). In addition, mechanotransduction studies permit assessment of the ability of ASM cells to respond to mechanical stimuli. Normally, ASM stretched by the action of tidal breathing remains relaxed, whereas in asthmatic airways the contracted ASM can become stiff with reduced elastic properties (Fabry & Fredberg, 2007). Several factors are involved in the transition from normal to asthmatic ASM behaviour, including mechano-sensitive processes (An et al., 2007; Noble et al., 2014). These mechanotransduction events can be studied by a hybrid approach in which the contractility of ASM cell is modelled in silico so as to set a dynamic muscle load comparable to that experienced in vivo. Such a hybrid approach requires complex mathematical models of ASM behaviour able to capture the dynamic remodelling and the adaptation in response to the surrounding mechanical environment (Fabry & Fredberg, 2007; Silveira et al., 2005).

Indeed, mechanopharmacology studies can be useful to biomechanically assess in vitro the pathophysiology characteristics of ASM cells in asthma and COPD and to identify new drugs and specific targets. Several biomechanical models and techniques based on 2D and 3D environments have been described for these purposes (Krishnan et al., 2016). Of note, mechanopharmacological treatments seem to be effective in inducing synergistic relaxation of ASM cells (Wang et al., 2019).

6 | ASM CONTRACTILE MODELS

ASM tissue is characterised by specific viscoelastic and dynamic non-linear properties that are different to those detectable in cultured ASM cells (Ito et al., 2006). Moreover, when ASM cells are cultured on elastic membranes to study the response to contractile stimuli, their mechanical characteristics are biased by the compliance of the substratum leading to altered cell contractility that does not occur in vivo and in ASM tissue ex vivo (Puig-De-Morales et al., 2004). Some of the mechanical characteristics of ASM tissue can be related to the non-linear viscoelastic properties of the extracellular matrix (ECM) (Bates & Lauzon, 2005). However, the dynamic non-linear behaviour

of ASM tissue is also associated with the state of the contractile stress and originates from active polymerisation within the ASM cytoskeleton (Ito et al., 2006).

Detailed models can be used to describe the viscoelastic properties of ASM tissue, such as the following equation: $G^*(\omega) = G'(\omega) + jG''(\omega) = H\omega_n^{\beta} + j(G'\omega_n^{\beta} + R\omega)$, where $G^*(\omega)$ is the complex modulus spectra (also known as the instantaneous stress/strain ratio) that defines the deformation of a material, G' and G'' are the storage (elastic) and loss (frictional) moduli, respectively, ω is the circular frequency, $j = \sqrt{-1}$ that is the imaginary unit indicative of the out-of-phase behaviour, $\omega_n = \omega/\omega_0$ that is the normalised frequency with ω rad/s, G and H are the tissue damping and stiffness coefficients, respectively, and $\beta = 2/\pi^*\tan^{-1}(G/H)$ (Ito et al., 2006). Concerning the dynamic non-linearity, harmonic distortion has been identified according to the following equation: $K_d = \sqrt{P_{NI}/P_{TOT}}$, where K_d is the harmonic distortion index, P_{TOT} is the total output power and P_{NI} is the power at noninput frequencies; in a linear system, the K_d value is zero (Ito et al., 2006).

Along with these models, the adaptation of the Huxley model adequately describes the intrinsic mechanical properties of ASM tissue for small length changes between 10% and 20%, although it is based on the assumption from skeletal muscle that the contractile proteins are all aligned towards the direction of the overall force generation (Bates, 2015; Brook, 2014; Mijailovich et al., 2000; Politi et al., 2010). This small shortening causes a transient reduction in active ASM force related to a temporary detachment of bound cross-bridges caused by the relative motion of the actin and myosin fibres (Bates, 2015).

An ASM cell can be modelled as a rectangular area containing a regular grid of nodes (focal adhesions or dense plaques) connected by links (stress fibres or contractile filaments). Since in ASM cells the contractile force is proportional to the overlap between actin and myosin filaments, in this model the maximum overlap is assumed for each link at the moment of its formation providing a force equal to 1, regardless of the link length. If the link length changes due to mechanical stress, its force becomes <1 according to the following equation: $f(D) = (-D^2 + 2D + [m-1]^2 - 1)/(m-1)^2,\ 1-(m-1) \le D \le 81+(m-1)$, where $D = l'/l$ is the link distortion, l' is the current link length, l is the formation length and $m > 1$ represents a parameter controlling the shape of the force length curve. This is a parabolic function that approximates to the force-length relationship derived from the Huxley theory. This equation assumes that each contractile link follows classical force-length relationship with a well-defined optimal force and a well-defined optimal length (Silveira et al., 2005). Further detailed equations have been reported as stochastic models of cytoskeletal dynamics to describe the mechanotransduction properties regulating the length adaptation of ASM cells (Silveira et al., 2005).

When isolated airways are mounted into isolated organ bath system and receive passive tension during equilibration, a more sustained reduction in active force reduction can be detected following to a greater change in tissue length (Bates, 2015; Bates et al., 2009). Of note, the recovery of such a force reduction is much slower than that normally expected (Bates, 2015). This phenomenon can be explained by a more profound disruption than myosin cross-bridges merely becoming detached from their actin attachment sites and by the fact that the contractile filaments inside the ASM cells are oriented over a range of angles and not in parallel as in skeletal muscle (Bates, 2015; Walsmley & Murphy, 1987). An elegant, although rather complex, derivation of the Huxley model that is free of the assumptions about cross-bridge orientation has been postulated (Bates, 2015) to explain the impairment of ASM force by rapid stretch of large amplitude. Detailed information regarding this model under isometric condition are available in the primary publication (Bates, 2015).

The use of isometric force transducers to perform pharmacological experiments on isolated airways has been a common practice for decades. However, it is likely that ASM contracts semi-isotonically in vivo resulting in bronchial narrowing especially at the level of small airways given the absence of cartilage, which might reduce isotonic functioning. The rationale for performing experiments using isometric force transducers is related to the fact that the sensitivity of ASM tissue to contractile and relaxant agents is significantly greater when measured isometrically than isotonically, with a potency difference of around 0.5 logarithm (De Jongste et al., 1987). Probably isotonic responses elicit an incomplete activation of contractile filaments due to the concurrent shortening and thickening of the muscle, which might hinder the diffusion of the drugs and cause changes in the arrangement of the contractile elements, leading to impairment of optimal functioning (De Jongste et al., 1987).

Further evidence supports the isometric measure of ASM contractility because it has been demonstrated with a conceptual model that ASM may generate maximal force over a large length range due to length adaptation (Bossé et al., 2008). This model assumes that the number of contractile units that are arranged in series and parallel is a linear function of the adapted muscle cell length. Length adaptation is a rapid process occurring under static conditions in which ASM regains contractility after experiencing a force decrease elicited by length variation (Bossé et al., 2008). However, maladaptation of ASM could be elicited by an exaggerated constriction of the airways. Therefore, excessive passive tension should be avoided when isolated airways are mounted in isolated organ bath systems to prevent an excessive contractile response (Bossé et al., 2008).

Another model of length adaptation has been developed by mimicking ex vivo the sequence of contractile events that occur during an asthma attack. ASM tissue is submaximally contracted by carbachol and allowed to shorten to half of original length. After length adaptation, the contractile response to EFS was two-fold greater than that resulting from control ASM tissue that was not adapted to the short length (McParland et al., 2005). This evidence further highlights the need of applying a correct passive tension on isolated airways during the equilibration procedure to avoid altered ASM contractile responses in pharmacological experiments.

The bronchorelaxant response to bronchodilator agents and the dynamic mechanical lung environment interact, whereby a breathing airway in vivo may facilitate greater relaxation when compared with a static environment typical of experiments carried out in isolated bronchial rings that are often mechanically static (Ansell et al., 2014). Effectively, in isolated airways the oscillatory loads act synergistically

with pharmacologically mediated bronchodilation (Ansell et al., 2014). According to models resulting from experiments on perfused bronchial segments simulating breathing manoeuvres (Ansell et al., 2014), the outer ASM perimeter (P_{mo}) in isolated airways can be calculated according to the follwong equation: $P_{mo} = \sqrt{4}*\pi*(WA_i + lumen\_volume/airway\_length)$, where WA_i is the inner wall area, lumen volume is calculated at the trough of the pressure cycle, and the airway length is the length of the isolated airways mounted in the system. This equation assumes that WA_i is constant at all transmural pressure, that P_{mo} is circular, and that the lumen is cylindrical (Ansell et al., 2014).

The airway narrowing and bronchodilation in response to simulated tidal breathing with intermittent DI (Noble et al., 2011) have been modelled in isolated bronchi from asthmatic and COPD donors. The greater amount of ASM tissue in asthmatic airways contributes to increased airway narrowing under static conditions; conversely, the relaxant response to simulated DI is normal in asthmatic bronchi (Noble et al., 2013). Additional studies conducted on human ASM trachealis strips indicate that the mechanical characteristics of ASM in asthmatic and non-asthmatic individuals are similar, except for the increased passive stiffness and attenuated decline in force generation after an oscillatory perturbation (An et al., 2007). These findings indicate that the increased maximal airway narrowing, the bronchorelaxant response to DI and oscillatory perturbation in asthmatic airways are independent (An et al., 2007; Noble et al., 2013).

In isolated airways from COPD patients, the narrowing to contractile stimuli and the volume fraction of ECM within the ASM layer are increased, with no changes in the ASM thickness and a normal bronchorelaxant response to simulated DI (Cairncross et al., 2020). Studies on passive and active mechanical properties of human ASM measured by myography confirm that the ASM of COPD patients is characterised by increased contractility related to increased volume (Opazo Saez et al., 2000). These data suggest that, in the airways of COPD patients, the mechanical behaviour of the bronchial wall is altered and there is a concomitant shift in the structural composition of the ASM layer related to modification in both ASM and ECM (Cairncross et al., 2020; Opazo Saez et al., 2000).

Further studies report that the area and tone of ASM, but not the total wall area of the airways, are determinants for important dynamic properties of isolated bronchial segments from smoker COPD donors such as compliance, hysteresis, and collapsibility. Compliance (Cdyn, $\mu l \cdot cm \cdot H_2O^{-1}$) is calculated from the volume infused to inflate the airway from 0 to 15 cm H_2O, hysteresis (η, μl cm H_2O) is calculated as the area of the pressure–volume loop between 0 to 15 cm H_2O, and collapsibility (Pcol, cm H_2O) results from the pressure of the deflation limb where the distal pressure did not follow the proximal pressure, thus indicating closure of the airway lumen (Tiddens et al., 1999).

7 | IN VITRO STUDIES WITH ISOLATED AIRWAYS

Normal isolated bronchial tissue can be used for the classical pharmacological characterisation of agents active on the airways. Studies can be carried out with drugs and endogenous compounds, both agonists and antagonists, both small and large molecules, including the investigation of mAbs (Calzetta et al., 2011; Manson et al., 2020; Matera et al., 2011; Naline et al., 1989, 2007, 2018; Rogliani et al., 2015). Generally, the effect of agents active on ASM can be tested directly on the bronchial resting tone at baseline, the parasympathetic pathway via **carbachol (CCh)** and following EFS, the histaminergic pathway via **histamine**, and mechanical stress via QS (Calzetta et al., 2020; Calzetta, Pistocchini, et al., 2023b; Rogliani et al., 2020).

ACh released by parasympathetic nerves is the main neurotransmitter involved in regulating ASM tone in patients with COPD and the activity of the cholinergic system is increased in this disease (Yamada & Ichinose, 2018). CCh is a synthetic derivative of choline that acts as a M receptor agonist. Unlike ACh, CCh is resistant to hydrolysis by **acetylcholinesterase**, and thus, it is characterised by a long duration of action (Norel et al., 1993; Streichert & Sargent, 1992). Therefore, the pharmacological characterisation of bronchodilators for the treatment of COPD is generally performed on the ASM contractility induced by iatrogenic administration of CCh rather than using ACh.

However, the cholinergic system can also be investigated by eliciting the release of endogenous neuronal ACh. In isolated human medium airways, EFS activates parasympathetic ganglia in the bronchial wall that stimulate the post-ganglionic vagal neurons to release ACh. Released ACh acts on M_3 receptors expressed on ASM leading to the airway contractile response (Fryer & Jacoby, 1998; Schlepütz et al., 2011). The release of ACh is regulated by M_2 autoreceptors expressed at the level of pre-synaptic post-ganglionic neurons. M_2 receptors are also extensively expressed on ASM but they do not directly contribute to airway contractility. M_1 receptors are expressed on parasympathetic ganglia and facilitate cholinergic neurotransmission (Fryer & Jacoby, 1998; Schlepütz et al., 2011). Along with ACh, stimulating isolated human airways by EFS may induce the release of other neurotransmitters according to the frequency of firing. For example vagal, firing in human airways ranges from 2 to 50 Hz (Calzetta, Ora, et al., 2017a). Higher frequencies stimulate predominantly large myelinated A-fibres to release ACh whereas lower frequencies induce the activation of non-myelinated sensory C-fibres to release tachykinins such as NKA and SP; medium frequencies activate small myelinated B-fibres inducing the release of both ACh and tachykinins (Calzetta, Aiello, et al., 2021a; Calzetta, Cazzola, et al., 2015a; Calzetta, Luongo, et al., 2015b). Tachykinins seem to have a marginal role on the regulation of human ASM contractility, but are thought to contribute to airway neurogenic inflammation (Barnes, 2003; Belvisi et al., 1994). Considering the lack of functional parasympathetic innervation at the level of human small airways, there is neither a physiological nor pharmacological rationale to perform in vitro studies in EFS-stimulated small airways (Barnes, 2004; Barnes et al., 1983; Koarai & Ichinose, 2018).

Histamine is an important mediator regulating ASM contractility in asthmatic patients (Smuda & Bryce, 2011; Yamauchi & Ogasawara, 2019). The bronchoconstriction to histamine is mediated by **H_1 receptors** expressed on ASM (Yamauchi & Ogasawara, 2019).

Of note, histamine may also indirectly modulate the ASM tone in asthmatic patients via stimulation of vagal fibres and ACh release (Rogliani et al., 2020; Sly, 1982).

Airway hyperinflation induces mechanical stress on the bronchial wall leading to the release of reactive oxygen species (ROS) and inflammatory factors such as leukotrienes (LT) via **nitric oxide synthase** activation leading to contractile ASM response dependent on **Rho-kinase** (Faisy et al., 2011). Airway stretch also increases the responsiveness to ACh and expression of **matrix metallopeptidase 9 (MMP-9)** (Le Guen et al., 2015, 2016). Mechanical stress can be reproduced in vitro via bronchial QS of 0.25 and 0.50 mm. In the organ bath, calibrated thumbscrews enable the accurate measurement of the angular rotation of the threaded rod used to elicit QS. Considering that usually the pitch of the screw is 1.0 mm, a rapid (<200 ms) 90° and 180° rotation induces a QS of 0.25 and 0.5 mm on isolated airways, respectively. The resulting myogenic response to QS is amplitude-dependent and should be recorded for 10 min (Calzetta et al., 2020; Rabe, 1998).

In vitro studies on isolated airways allow the investigation of the primary pharmacological parameters to understand the actions of a drug, such as efficacy, assessed via maximal effect (E_{max}), and potency, defined as the concentration needed to produce 50% E_{max} (EC_{50} or IC_{50}). For a correct statistical analysis of the potency, EC_{50} and IC_{50} are transformed as the negative logarithm to base 10, namely pEC_{50} and pIC_{50} (Neubig et al., 2003). The Schild plot analysis can be used to calculate an important measure of the effectiveness of an antagonist, the so-called pA_2 value, the negative logarithm to base 10 of the molar concentration of an antagonist that makes it necessary to double the concentration of the agonist needed to elicit the original submaximal response obtained in the absence of antagonist (Neubig et al., 2003). Also, the onset of action ($t_{1/2}$), that is the time to attainment of 50% of E_{max}, and the duration of action of agonists and antagonists, can be evaluated in vitro with isolated bronchi (Calzetta, Matera, et al., 2019a; Cazzola et al., 2016; Haddad et al., 1999; Moulton & Fryer, 2011; Rogliani et al., 2015).

The study of drug interactions is also possible in isolated human airways and requires the assessment of E_{max} to be able to report the observed effect of investigated combined medications (Goldoni & Johansson, 2007). Unfortunately identifying E_{max} in clinical trials may be problematic due to the potential risk of serious adverse events when high doses of medications are administered in combination (Warren, 2019). On the other hand, full concentration-response curves (CRC) can be obtained from ex vivo studies and permit the adequate quantification of E_{max}. In this respect, experiments on isolated airways represent an optimal strategy to investigate the interaction between bronchodilators characterised by different mechanisms of action, also when combined with non-bronchodilator drugs such as anti-inflammatory compounds (Calzetta, Rogliani, Facciolo, et al., 2018c; Rogliani, Ora, Girolami, et al., 2021a). Synergistic interactions between drugs from different pharmacological classes can be analysed via several statistical approaches such as the Bliss Independence, Loewe, Highest Single Agent and Unified Theory (Chou, 2006; Di Veroli et al., 2016).

Along with experiments carried out on normal isolated human airways, in the last decades specific techniques have been developed and validated to reproduce in vitro other important characteristics of chronic obstructive respiratory disorders and better characterise the pharmacological impact of novel treatments as part of a bench-to-bedside approach.

7.1 | In vitro models of asthma

Although asthma and COPD are among the most common pulmonary diseases, the prevalence of asthma in patients suffering from lung cancer undergoing lobectomy is very low or even not reported (Dima et al., 2018; Dutkowska & Antczak, 2016; Tammemagi et al., 2003). Therefore, obtaining enough tissues from donors with asthma is not plausible to plan specific studies, as only a few experiments have been carried out on isolated bronchi from asthmatic patients (Bjorck et al., 1992; Goldie et al., 1986). However, some of the main characteristics of asthma and severe eosinophilic asthma can be reproduced in vitro using isolated airways from donors not suffering from chronic obstructive respiratory disorders.

7.1.1 | Passive sensitisation of human airways

Passive sensitisation is a technique validated in the 1990s to reproduce in vitro the heightened responsiveness of ASM that is a central abnormality in patients with asthma that induces enhanced sensitivity to a wide variety of stimuli (Rabe, 1998).

Passive sensitisation is elicited by incubating isolated airways from non-asthmatic donors overnight at room temperature in rotating tubes containing KH buffer solution in the presence of 10% vol^{-1} sensitising serum. Serum is prepared by centrifugation of whole blood obtained from patients suffering from atopic asthma (total IgE 1000 $U{\cdot}ml^{-1}$ specific against common aeroallergens) during an exacerbation as described elsewhere (Calzetta, Matera, Facciolo, et al., 2018b).

Passively sensitised bronchi are characterised by greater shortening velocity and increased isotonic shortening, increased myogenic contractile response to QS and EFS, increased potency and maximum effect in response to specific allergens (i.e., house dust mite) or non-specific contractile stimuli (i.e., histamine and carbachol) (Calzetta et al., 2020; Matera et al., 2011; Rabe, 1998). The allergen-induced AHR depends on the level of IgE in the sensitising serum, whereas the AHR induced by non-specific contractile agents is independent of the levels of IgE (Schmidt & Rabe, 2000). An unknown factor in the sensitising serum related to IgE, but not IgE itself, has also been shown to contribute to non-specific AHR observed in isolated airways (Schmidt & Rabe, 2000). Although the precise underlying mechanisms leading to the AHR induced by passive sensitisation are not yet fully understood (Mitchell et al., 1997; Rabe, 1998; Schmidt et al., 1999), it has been extensively demonstrated that this phenomenon is associated with reduced synthesis of cyclic adenosine monophosphate (cAMP) in ASM and increased release of endogenous non-neuronal

ACh (Calzetta et al., 2020; Rogliani et al., 2016, 2020; Rogliani, Ritondo, Zerillo, et al., 2021c).

Along with specific and non-specific AHR, passive sensitisation also elicits significant airway inflammation. After passive sensitisation, greater levels of interleukin **(IL)-4**, **IL-5**, **IL-6**, **IL-9**, **IL-13**, **tumour necrosis factor α (TNF-α)** and thymic stromal lymphopoietin **(TSLP)** are released by isolated airways. In these sensitised bronchi, neurogenic inflammation is also elicited, with increased release of NKA and SP (Rogliani, Ritondo, Zerillo, et al., 2021c). The reported airway inflammation induced by passive sensitisation demonstrates some overlap with that observed in patients with type 2 asthma (Lambrecht et al., 2019).

7.1.2 | Passive sensitisation plus platelet-activating factor (PAF) challenge

Resident eosinophils are normally present in the lungs and bronchi of healthy donors and they are phenotypically distinct from inflammatory eosinophils found in asthmatic airways (Mesnil et al., 2016). Although it has been reported that airway sensitisation may contribute to the adhesion of eosinophils to parasympathetic nerves with consequent release of major basic protein (MBP) and increased parasympathetic tone (Calzetta et al., 2020; Drake et al., 2018), to date no evidence exists that passive sensitisation activates resident eosinophils in bronchi from non-asthmatic donors. Conversely, it has been demonstrated that adding of **PAF** activates airway resident and blood eosinophils leading to significant narrowing of the bronchial lumen (Rabe et al., 1994; Schmidt et al., 2002). Effectively, PAF leads to the secretion of eosinophil peroxidase, generation of the superoxide anion, mobilisation of intracellular Ca^{2+}, and potentiation of the contractile response to histamine (Johnson et al., 1990, 1992; Kroegel et al., 1994; Rabe et al., 1994; Schmidt et al., 2002).

Considering this evidence, a recent in vitro model of severe eosinophilic asthma has been validated in passively sensitised bronchi challenged with PAF (Calzetta, Pistocchini, et al., 2023b). Briefly, the day after the passive sensitisation procedure, sensitised airways are challenged for 45 min with PAF administered at 100 nM before beginning experiments. Challenging sensitised airways with PAF induces AHR to histamine and EFS with no changes on the ASM resting tone (Calzetta, Pistocchini, et al., 2023b).

7.1.3 | Passive sensitisation plus M receptor activation

Persistent airflow limitation (PAL) in asthma is defined as a post-bronchodilator FEV_1/forced vital capacity (FVC) less than the lower limit of normal at baseline, a condition representing a specific phenotype in asthmatic patients (GINA, 2023a; Kole et al., 2023). The bronchial tissue of asthmatic patients with PAL is characterised by inflammation and remodelling (Ferreira et al., 2018; Kozlik et al., 2020). Although airway remodelling cannot be replicated in experiments with isolated airways, the main mechanics and inflammatory characteristics of PAL can be reproduced ex vivo in passively sensitised bronchi. During the sensitisation procedure, isolated airways can be incubated overnight also in the presence of CCh administered at low dose (i.e., 1 μM) in order to activate M receptors and, thus, mimic altered activity and plasticity of parasympathetic pathway as well as tissue inflammatory response (Calzetta, Pistocchini, Cito, Ritondo, et al., 2022c; Gosens et al., 2006; Kistemaker & Prakash, 2019; Rogliani, Ritondo, Zerillo, et al., 2021c). Of note, it has been demonstrated that the stimulation of M receptors with other iatrogenic agonists such as methacholine, elicited persistent ventilation defects due to obstruction in specific lung regions of asthmatic patients (de Lange et al., 2007).

7.2 | In vitro models of COPD

COPD is the most prevalent comorbidity of patients with lung cancer (Dima et al., 2018; Dutkowska & Antczak, 2016; Tammemagi et al., 2003), and therefore, in vitro models of stable COPD can be approached by simply using bronchial tissue from COPD donors undergoing lobectomy for lung cancer (Rogliani et al., 2020; Rogliani, Ritondo, Facciolo, et al., 2021b).

In addition to tobacco smoking and exposure to environmental factors such as biomass fuel and air pollution, certain host-related factors, including genetic abnormalities, irregular lung development, and accelerated ageing, can contribute to the individual susceptibility to developing COPD (GOLD, 2023). Accordingly, accurate evaluation of patients' medical history and anamnesis, along with genetic evaluations of isolated airways collected from COPD donors, may be crucial for the development of improved in vitro COPD models that accurately reflect the heterogeneity observed among COPD patients in real-life settings.

7.2.1 | Acute LPS challenge

The airways of COPD patients are often chronically colonised by Gram-negative bacteria (Dacydchenko & Bova, 2007; Wilson, 2001). LPS is an important proinflammatory glycolipid component of the cell wall of Gram-negative bacteria that plays a key role in host-pathogen interactions with the innate immune system by binding with Toll-like receptor 4 (**TLR4**) (Gupta et al., 2015; Maldonado et al., 2016). Moreover, LPS is an active component of cigarette smoke (Hasday et al., 1999; Kharitonov & Sjöbring, 2007). Being a very potent pro-inflammatory substance, after inhalation LPS induces fever, chills and bronchoconstriction (Kharitonov & Sjöbring, 2007), as well as airway neutrophilia (Franciosi et al., 2013). Airway colonisation by Gram-negative bacteria is a distinguishing feature of patients affected by COPD. Thus, COPD patients are chronically exposed to LPS, which may well contribute to the accelerated decline in lung function, disease severity and deterioration, as well as COPD exacerbations (Gupta et al., 2015; Jagielo et al., 1996; Kharitonov & Sjöbring, 2007).

Challenging isolated human airways, bronchial epithelial cells, alveolar macrophages and lung fibroblasts with LPS is a technique extensively used to reproduce in vitro and ex vivo the detrimental conditions detectable in the airways of patients with stable COPD that are at higher risk of exacerbation due to the of the respiratory tract by Gram-negative bacteria (Beasley et al., 2012; Calzetta, Aiello, Frizzelli, Pistocchini, et al., 2022b; Calzetta, Luongo, et al., 2015b; Calzetta, Matera, & Cazzola, 2018a; Cazzola et al., 2011; Cazzola et al., 2017; Papi et al., 2006; Wedzicha & Seemungal, 2007). More specifically, the incubation of bronchial tissue with LPS significantly alters ASM contractility (Calzetta, Luongo, et al., 2015b; Calzetta, Pistocchini, Ritondo, Cavalli, et al., 2022d; Calzetta, Rogliani, Pistocchini, et al., 2018e; Cazzola et al., 2011; Cazzola et al., 2017), induces neutrophilic inflammation (Calzetta, Matera, & Cazzola, 2018a; Calzetta, Pistocchini, Ritondo, Cavalli, et al., 2022d; Cazzola et al., 2017) and NKA-driven neurogenic inflammation (Calzetta, Luongo, et al., 2015b; Calzetta, Matera, & Cazzola, 2018a; Calzetta, Pistocchini, Ritondo, Cavalli, et al., 2022d; Calzetta, Rogliani, et al., 2017b; Calzetta, Rogliani, Pistocchini, et al., 2018e) by activating capsaicin-sensitive sensory nerves (Calzetta, Luongo, et al., 2015b; Calzetta, Pistocchini, Ritondo, Cavalli, et al., 2022d; Calzetta, Rogliani, Pistocchini, et al., 2018e), affects the oxidant/antioxidant profile (Calzetta, Matera, & Cazzola, 2018a; Cazzola et al., 2017) and triggers protein prenylation (Cazzola et al., 2011). Certainly, these conditions are difficult to be standardised and to be detected primarily in isolated airways from COPD donors not challenged by specific stimuli such as LPS.

A potential criticism of exposing normal airways to LPS is that the acute inflammation elicited by LPS may only partially mimic COPD inflammation. However, inhaled LPS has been demonstrated to be effective in increasing airway neutrophil numbers in healthy subjects, a condition resembling the increase in airway neutrophil numbers occurring on a background in COPD patients whose airways are colonised by Gram-negative bacteria (Aul et al., 2012; Gupta et al., 2015; Kharitonov & Sjöbring, 2007). Accordingly, acute inhalation of LPS elicits a dose-dependent, acute neutrophilic response and release of inflammatory mediators typical of COPD in the airways of healthy volunteers that can be quantified in induced sputum (Aul et al., 2012; Kharitonov & Sjöbring, 2007; Leaker et al., 2013). Also, the fall in lung function after LPS challenge in healthy subjects mimics the increased airway contractility in COPD patients (Aul et al., 2012).

Therefore, the inflammatory response and ASM contractility typical of COPD can be mimicked in vitro via acute exposure (2 h) of airways to LPS administered at 300 $ng \cdot ml^{-1}$ (Calzetta, Luongo, et al., 2015b; Cazzola et al., 2011).

7.2.2 | Sub-acute LPS challenge

The overnight incubation of bronchial tissue collected from COPD donors with LPS administered at 100 $ng \cdot ml^{-1}$ is an effective in vitro model of COPD exacerbations (Calzetta, Matera, & Cazzola, 2018a; Cazzola et al., 2017). This model significantly affects the bronchial contractile responses to EFS due to airway desensitisation. Moreover, it increases the pro-oxidant activity, reduces the anti-oxidant capacity in the bronchial tissue, and elicits cytokine release and neurogenic inflammation (Calzetta, Matera, & Cazzola, 2018a; Cazzola et al., 2017).

7.2.3 | Overnight treatment with CSE

Cigarette smoke is the most commonly encountered risk factor for patients developing COPD (GOLD, 2023). Cigarette smoke contains a highly complex mixture of around 4800 chemical compounds, but it is not yet well established what the cellular mechanisms leading to smoking-related features of COPD are due to (Baum et al., 2003; Schamberger et al., 2014). Cigarette smoke extract (CSE) can be prepared by using research-grade cigarettes (3R4F) according to standardised procedures adapted for experiments with isolated bronchial tissue. The procedures require that smoke from 29 cigarettes is bubbled through 400 ml of KH buffer solution at puffing speed in a closed environment with limited airflow. After that, CSE is filtered through a 0.20-μm filter, aliquoted and stored at −20°C (van Rijt et al., 2012). The obtained CSE stock is then diluted 1:10 for the overnight treatment of bronchial tissue (van Rijt et al., 2012). CSE treatment increases cytokine expression and reduces nuclear expression of **sirtuin** and **histone deacetylase (HDAC)** (Calzetta, Pistocchini, Cito, Ritondo, et al., 2022c). CSE also induces mucus hypersecretion and mucin MUC5AC gene and protein overexpression (Cortijo et al., 2011; Di et al., 2012). Although the treatment with CSE elevates cytosolic Ca^{2+} in ASM leading to MLC phosphorylation, the effect of CSE on ASM contractility is scarce or even non-detectable (Chen et al., 2014; van Rijt et al., 2012; Yoon et al., 2011). Considering that mucus hypersecretion represents an early developmental phase of COPD, treating airways from COPD donors overnight with CSE 1:10 is a suitable in vitro model of early COPD according to the current definitions (Agustí et al., 2023; Agusti & Faner, 2018; Cosío et al., 2020; Lee & Han, 2023; Yang et al., 2021). On the other hand, the overnight incubation of airways from donors with normal lung function with CSE 1:10 is a relevant in vitro model of pre-COPD, based on current evidence of patients with no airway obstruction who are at risk of progression to COPD (Agustí et al., 2023; Han et al., 2021).

8 | AIRWAY CELLS AND MODELS OF ASTHMA, COPD AND VIRAL INFECTION

ASM bundles in endobronchial biopsy are isolated and cultured. After that, ASM cells (passage 3–7) from asthmatic patients are plated into six-well plates ($9.6 * 10^4$ cells per 2 ml DMEM, 10% (FBS)) and grown for 1 week. The growth is arrested for 48 h with a serum-deprived medium before starting pharmacological experiments by using these cells as an in vitro model of asthma (Brightling et al., 2005).

ASM cells isolated from the airways of COPD patients undergoing lung transplantation or lung resection for thoracic malignancy can be grown (passage 3–7) in RPMI 1640 plus 10% FBS and then used as an in vitro model of COPD (Chen et al., 2010; Zakarya et al., 2021).

Cultured ASM cells, including those originating from asthmatic and COPD patients, can also be exposed to rhinovirus (RV), respiratory syncytial virus (RSV), and parainfluenza virus type 3 (PIV-3) to study in vitro the effect of pharmacological treatments on viral-induced exacerbations (Elias et al., 1997; Oliver et al., 2006; Van Ly et al., 2013). Viral infection is performed by exposing cultured ASM cells to medium containing viruses and allowing the virus to adsorb to the ASM cells monolayers at a multiplicity of infection (MOI) of 1–3 plaque-performing units per cell for 1 h. The infecting medium is then washed 3 times with phosphate-buffered saline (PBS) and replaced with DMEM (0.1%–10% FBS). The medium can be harvested after 24 h post infection, following centrifugation to remove non-adherent ASM cells, for specific analyses. Negative controls are ASM cells either left uninfected or exposed to UV inactivated virus (Elias et al., 1997; Oliver et al., 2006; Van Ly et al., 2013).

9 | IN VITRO AND EX VIVO MODELS OF RESPIRATORY EPITHELIAL DAMAGE

9.1 | Epithelium and isolated airways

Respiratory epithelium has a pivotal role in modulating airway contractility and inflammation. Healthy bronchial epithelial cells are a predominant source of multiple endogenous epithelium-derived relaxing factors (EpiDRFs) leading to ASM relaxation (Calzetta, Rogliani, et al., 2014b; Gallos et al., 2013; Vanhoutte, 2013). It has been extensively documented that removing epithelium from the airways favours the contractile responsiveness of the surrounding ASM (Flavahan et al., 1985; Matera et al., 2011; Vanhoutte, 1989, 2013). However, if damaged or mechanically stressed, respiratory epithelium may also increase ASM proliferation and contractility (Lan et al., 2018; Vanhoutte, 2013). Moreover, when damaged, respiratory epithelium generates a large number of cytokines, including alarmins, thus contributing to the type 2 inflammatory response (Calvén et al., 2020; Chung & Barnes, 1999; Holgate et al., 2009; Lambrecht & Hammad, 2012; Nagarkar et al., 2012). Interestingly, the epithelium derived alarmins (i.e., TSLP, **IL-33** and **IL-25**) have been demonstrated to enhance mast cell-dependent contractility of isolated airways and have a role in the inflammatory cascade of both asthma and COPD (Belikova et al., 2023; Calzetta, Pietroiusti, et al., 2021b; Hong et al., 2020).

The role of bronchial epithelium in respiratory pharmacology can be investigated by performing the same experiments in epithelium-intact and epithelium-denuded isolated bronchi. The comparison between results obtained from epithelium-intact and epithelium-denuded bronchi allows a better understanding as to whether the epithelium may have a role in the response (i.e., contractility and inflammation) of isolated airways. Moreover, ultrastructural analysis via transmission electron microscopy can be performed to evaluate the subcellular characteristics of epithelial cells (alterations of cell membranes, mitochondria, presence of intracellular electrodense bodies, presence and ultrastructural alterations of cilia, lysosomes) as well as the basal lamina (layers alignment, thickness and alterations). The epithelium can be mechanically removed from isolated airways using a cotton-tipped applicator gently rubbed for 5 sec on the luminal surface (Cazzola et al., 2016; Matera et al., 2011; Reinheimer et al., 1997).

Experiments can also be carried out directly on primary bronchial epithelial cells that are harvested by gently scraping the luminal airway surface with a convex scalpel blade #10 (Fulcher et al., 2005; Matera et al., 2011). Collected epithelial cells are then pooled in PBS, centrifuged at 500 g for 5 min at 4°C and then directly used for experiments. Eventually, airway epithelial cells can be resuspended, cultured with 1:1 mixture of LHC-9 and RPMI 1640 medium in a volume of 10^6 cells·ml^{-1} and maintained at 37°C in a 5% CO_2 humidified incubator (Cazzola et al., 2016; Fulcher et al., 2005).

Both epithelium removal and cell harvesting are effective procedures confirmed by histology that do not penetrate the basal membrane and leave the lamina propria intact (Fulcher et al., 2005; Matera et al., 2011; Reinheimer et al., 1996).

9.2 | Passive sensitisation

Bronchial epithelial cells can be passively sensitised to reproduce important characteristics of asthmatic respiratory epithelium in vitro (Hsieh et al., 2005). Epithelial cells are incubated overnight at 37°C in a 5% CO_2 humidified incubator with 1:1 mixture of LHC-9 and RPMI 1640 in the presence of 2% vol^{-1} sensitising serum from an asthmatic patient (total IgE 1000 U·ml^{-1} specific against common aeroallergens) during exacerbation (Calzetta, Matera, Facciolo, et al., 2018b; Calzetta, Rogliani, et al., 2014b).

9.3 | Acute LPS challenge

The airways of both asthmatic and COPD patients are characterised by loss of barrier integrity contributing to the high incidence of bronchial infections, exacerbations, hospitalisation, and mortality (Kricker et al., 2021). Therefore, the clinical relevance of airway epithelial integrity in asthma and COPD is unquestioned and one consequence of airway epithelial barrier failure is an unhindered access of external particles to various cells of the immune system, producing an inappropriate inflammatory response, a recognised hallmark of several lung disorders (Knight & Holgate, 2003; Wittekindt, 2017).

For decades it has been well recognised that airway respiratory epithelium is more than just a barrier. Indeed, it represents a physical barrier that protects capsaicin-sensitive sensory nerves and ASM from exposure to irritants and pathogenic organisms. However, epithelial cells also release mediators that inhibit bronchospasm by relaxing the underlying ASM via EpiDRFs. In the same manner, airway epithelium

also has a central role in modulating the inflammatory response by interacting with innate and adaptive immunity (Kato & Schleimer, 2007; Tam et al., 2011).

It is well-known that in respiratory tissue LPS induces the synthesis of respiratory epithelium-derived cytokines, namely TSLP, IL-25, and IL-33 (Fu et al., 2021; Liao et al., 2015). In bronchial epithelial cells, LPS also induces the expression of an active and inducible 5-**lipoxygenase (5-LO**X) pathway generating the LT family of bronchoconstrictor and pro-inflammatory mediators, cytokines such as IL-6, **IL-8** and TNF-α (Aitken et al., 2021; Hulina-Tomašković et al., 2019, 2022; Jame et al., 2007).

Concerning epithelial integrity, LPS impairs the structure of the respiratory epithelium, leading to dislocation of adherens junction (AJ) protein E-cadherin at cell–cell contacts and down-expression of both adherens junction and tight junction (TJ) proteins Claudin-2 and occludin (OCLN). Moreover, LPS induces airway epithelial barrier dysfunction by changing the ionic and macromolecular permeability, and it modifies cytoskeleton organisation in bronchial epithelial cells (D'Anna et al., 2017; Li et al., 2021; Ma et al., 2018). Effectively, the LPS-induced lung inflammation seems to be linked to increased airway epithelial permeability via epithelial MLCK, **protein-kinase C (PKC)** and **extracellular regulated kinase 1/2 (ERK1/2)** activation (Eutamene et al., 2005; Serikov et al., 2004).

LPS is also responsible for enhanced methacholine-evoked contractility due to epithelial alterations. Thus, the integrity of the epithelium plays a pivotal role in regulating AHR (Ismailoglu et al., 2004; Secher et al., 2012).

With respect to structural and ultrastructural alterations, exposure to LPS increases the number of surface epithelial cells per length of basal lamina. Moreover, in LPS-exposed epithelium, atypical epithelial cells containing numerous basal bodies, few cilia, and few apical secretory granules can be found, conditions leading to increased amounts of intraepithelial mucosubstances, secretory cell hyperplasia, and excess luminal mucus in the airways (Harkema & Hotchkiss, 1992).

Therefore, epithelium-intact bronchi, epithelium-denuded bronchi, and respiratory epithelial cells harvested from isolated airways can be challenged with LPS 300 ng·ml^{-1} for 2 h to directly and indirectly investigate in vitro and ex vivo the impact of epithelial damage in respiratory pharmacology (Calzetta, Luongo, et al., 2015b; Cazzola et al., 2011).

9.4 | Overnight treatment with CSE

CSE has several detrimental effects on the respiratory epithelium. CSE impairs expression of E-cadherin, zonula occludent (ZO)-1, and OCLN via **epidermal growth factor receptor (EGFR)** activation in primary respiratory epithelial cells from COPD donors (Heijink et al., 2012; Milara et al., 2013). Moreover, CSE induces the so called epithelial to mesenchymal transition (EMT) via urokinase plasminogen activator receptor (uPAR)-dependent phosphatidylinositol (PI) 3-Akt activation (Wang et al., 2013, 2015) and elicits altered regulation of wingless/integrase-1 (WNT) ligands in airway epithelial cells from COPD patients (Aghapour et al., 2018). Overall, these effects of CSE lead to impairment of transepithelial resistance (Aghapour et al., 2018).

As already reported for acute LPS challenge, epithelium-intact bronchi, epithelium-denuded bronchi, and respiratory epithelial cells harvested from isolated airways can be treated overnight with CSE 1:10 to directly and indirectly investigate in vitro and ex vivo the impact of smoke on epithelium damage in respiratory pharmacology (Heijink et al., 2016).

9.5 | Exposure to pollutants

The exposure of respiratory epithelial cells to air pollution induces respiratory epithelial barrier dysfunction via several mechanisms such as increased oxidative stress, exaggerated cytokine responses, and impaired host defence (Aghapour et al., 2022). Moreover, the dysfunction of the airway epithelial barrier may enhance susceptibility to respiratory infections leading to increased risk of exacerbations (Aghapour et al., 2022). Overall, these detrimental conditions contribute to the development and worsening of asthma and COPD (Aghapour et al., 2022). Several models of pollutant exposure have been proposed to investigate the role of air pollution in asthma and COPD (Albano et al., 2022). Among these, the exposure to fine particles or particulate matter ≤2.5 μm ($PM_{2.5}$, i.e., vehicle exhaust) and coarse particles or PM 2.5–10 μm (PM_{10}, i.e., dust) have been reported to have a pivotal role on airway epithelial barrier dysfunction in asthma and COPD (Aghapour et al., 2022; Albano et al., 2022; Calzetta, Aiello, et al., 2021a). $PM_{2.5}$ and PM_{10} are among the most studied outdoor pollutants affecting the integrity of the respiratory epithelium (Comunian et al., 2020; Goossens et al., 2021). Moreover, urban PM increases airway inflammatory responses via activation of **mitogen-activated protein kinase (MAPK)** and downstream nuclear factor (NF)-κB signalling pathways; urban PM also induces ROS release (Lee et al., 2020). $PM_{2.5}$ is related to traffic air pollution and, along with black carbon, it carries sulphate, nitrate, ammonium, silicon, and phase polyaromatic hydrocarbon (PAH). It is particularly harmful because it reaches small airways (Comunian et al., 2020; Goossens et al., 2021). PM_{10} accumulates at the level of conducting airways and it promotes airway infection and induces inflammasome activation; the co-exposure with LPS attenuates the expression of E-cadherin and claudin 1 (Aghapour et al., 2022; Goossens et al., 2021; Kim et al., 2019).

Therefore, epithelium-intact bronchi, epithelium-denuded bronchi, and respiratory epithelial cells harvested from isolated airways can be treated overnight with $PM_{2.5}$ and PM_{10} both administered at 50 μg·ml^{-1} to directly and indirectly investigate in vitro and ex vivo the impact of pollutants exposure in respiratory pharmacology (Calzetta, Aiello, et al., 2021a; Lee et al., 2020; Pozzi et al., 2003).

TABLE 1 Summary of in vitro and ex vivo models from human airways.

Tissue characteristics	Tissue treatments	Disease models	Pharmacological evaluation of ASM contractility	Note	Main references
Normal isolated bronchial tissue	/	/	Classical pharmacological parameters (i.e., E_{max}, EC_{50}, IC_{50}, pEC_{50}, pIC_{50}, pA_2 and $t_{1/2}$), drug interaction (i.e., synergy, antagonism and additivity)	Pharmacological pathways can be also evaluated	(Calzetta et al., 2011, Calzetta et al., 2020, Calzetta, Pistocchini, et al., 2023b, Manson et al., 2020, Matera et al., 2011, Naline et al., 1989, 2007, Naline et al., 2018, Rogliani et al., 2015, 2020)
Isolated bronchial tissue from asthmatic donors	/	Ex vivo model of asthma	/	Scarce tissue availability	(Bjorck et al., 1992)
Normal isolated bronchial tissue	Passive sensitisation (overnight incubation with 10% vol^{-1} sensitising serum) in epithelium-intact and/or epithelium-denuded bronchi	Ex vivo model of asthma	AHR, classical pharmacological parameters (i.e., E_{max}, EC_{50}, IC_{50}, pEC_{50}, pIC_{50}, pA_2 and $t_{1/2}$), drug interaction (i.e., synergy, antagonism and additivity)	Inflammatory profile, alarmins (TSLP, IL-33 and IL-25) release, pharmacological pathways, and ultrastructural analysis via transmission electron microscope can be also evaluated	(Calzetta et al., 2020; Calzetta, Matera, Facciolo, et al., 2018b; Matera et al., 2011; Mitchell et al., 1997; Rabe, 1998; Rogliani et al., 2016, 2020; Rogliani, Ritondo, Zerillo, et al., 2021c; Schmidt et al., 1999; Schmidt & Rabe, 2000)
Normal isolated bronchial tissue	Passive sensitisation plus PAF challenge (overnight incubation with 10% vol^{-1} sensitising serum and then 45 min challenge with PAF 100 nM) in epithelium-intact and/or epithelium-denuded bronchi	Ex vivo model of severe eosinophilic asthma	AHR, classical pharmacological parameters (i.e., E_{max}, EC_{50}, IC_{50}, pEC_{50}, pIC_{50}, pA_2 and $t_{1/2}$), drug interaction (i.e. synergy, antagonism, additivity)	Inflammatory profile, alarmins (TSLP, IL-33 and IL-25) release, pharmacological pathways, and ultrastructural analysis via transmission electron microscope can be also evaluated	(Calzetta, Pistocchini, et al., 2023b; Johnson et al., 1990, 1992; Kroegel et al., 1994; Rabe et al., 1994; Schmidt et al., 2002)
Normal isolated bronchial tissue	Passive sensitisation plus activation of M receptors (concomitant overnight incubation with 10% vol^{-1} sensitising serum and CCh 1 μM) in epithelium-intact and/or epithelium-denuded bronchi	Ex vivo model of PAL	AHR, classical pharmacological parameters (i.e., E_{max}, EC_{50}, IC_{50}, pEC_{50}, pIC_{50}, pA_2 and $t_{1/2}$), drug interaction (i.e., synergy, antagonism and additivity)	Inflammatory profile, alarmins (TSLP, IL-33 and IL-25) release, parasympathetic plasticity, pharmacological pathways, and ultrastructural analysis via transmission electron microscope can be also evaluated	(Calzetta, Pistocchini, Cito, Ritondo, et al., 2022c; Ferreira et al., 2018; Gosens et al., 2006; Kistemaker & Prakash, 2019; Kozlik et al., 2020; Rogliani, Ritondo, Zerillo, et al., 2021c)
Isolated bronchial tissue from COPD donors	/	Ex vivo model of stable COPD	Classical pharmacological parameters (i.e., E_{max}, EC_{50}, IC_{50}, pEC_{50}, pIC_{50}, pA_2 and $t_{1/2}$), drug interaction (i.e.,	Inflammatory profile, alarmins (TSLP, IL-33, IL-25) release, pharmacological pathways, and ultrastructural analysis via transmission	(Rogliani et al., 2020, Rogliani, Ritondo, Facciolo, et al., 2021b)

(Continues)

TABLE 1 (Continued)

Tissue characteristics	Tissue treatments	Disease models	Pharmacological evaluation of ASM contractility	Note	Main references
			synergy, antagonism and additivity)	electron microscope can be also evaluated	
Normal isolated bronchial tissue	Acute LPS challenge (incubation 2 h with LPS 300 ng·ml^{-1}) in epithelium-intact and/or epithelium-denuded bronchi	Ex vivo model of COPD	AHR, classical pharmacological parameters (i.e., E_{max}, EC_{50}, IC_{50}, pEC_{50}, pIC_{50}, pA_2 and $t_{1/2}$), drug interaction (i.e., synergy, antagonism and additivity)	Inflammatory profile, alarmins (TSLP, IL-33 and IL-25) release, pharmacological pathways, and ultrastructural analysis via transmission electron microscope can be also evaluated	(Calzetta, Aiello, Frizzelli, Pistocchini, et al., 2022b, Calzetta, Luongo, et al., 2015b, Calzetta, Matera, & Cazzola, 2018a, Cazzola et al., 2011, Cazzola et al., 2017)
Isolated bronchial tissue from COPD donors	Sub-acute LPS challenge (overnight incubation with LPS 100 ng/ml)	Ex vivo model of COPD exacerbation	Airway desensitisation, drug interaction (i.e. synergy, antagonism, additivity)	Inflammatory profile, oxidative profile, and pharmacological pathways can be also evaluated	(Calzetta, Matera, & Cazzola, 2018a; Cazzola et al., 2017)
Normal isolated bronchial tissue	CSE treatment (overnight incubation with CSE 1:10) in epithelium-intact and/or epithelium-denuded bronchi	Ex vivo model of pre-COPD	Classical pharmacological parameters (i.e., E_{max}, EC_{50}, IC_{50}, pEC_{50}, pIC_{50}, pA_2 and $t_{1/2}$), drug interaction (i.e. synergy, antagonism, additivity)	Inflammatory profile, oxidative profile, mucus hypersecretion, alarmins (TSLP, IL-33 and IL-25) release, pharmacological pathways and ultrastructural analysis via transmission electron microscope can be also evaluated	(Agustí et al., 2023; Agusti & Faner, 2018; Chen et al., 2014; Cortijo et al., 2011; Cosío et al., 2020; Di et al., 2012; Lee & Han, 2023; van Rijt et al., 2012; Yang et al., 2021; Yoon et al., 2011).
Isolated bronchial tissue from COPD donors	CSE treatment (overnight incubation with CSE 1:10) in epithelium-intact and/or epithelium-denuded bronchi	Ex vivo model of early COPD	Classical pharmacological parameters (i.e., E_{max}, EC_{50}, IC_{50}, pEC_{50}, pIC_{50}, pA_2 and $t_{1/2}$), drug interaction (i.e., synergy, antagonism and additivity)	Inflammatory profile, oxidative profile, mucus hypersecretion, alarmins (TSLP, IL-33 and IL-25) release, pharmacological pathways and ultrastructural analysis via transmission electron microscope can be also evaluated	(Cortijo et al.; Yoon et al., 2011; Di et al., 2012; van Rijt et al., 2012; Chen et al., 2014; Han et al., 2021; Agustí et al., 2023)
Cultured ASM cells from asthmatic donors undergoing endobronchial biopsy	/	In vitro model of asthma	Mechanotransduction, stiffness, traction, viscoelastic characteristic, length adaptation	Inflammatory profile and cell migration can be also evaluated	(Brightling et al., 2005; Fabry & Fredberg, 2007; Silveira et al., 2005)
Cultured ASM cells from COPD donors undergoing lung transplantation or lung resection	/	In vitro model of COPD	Mechanotransduction, stiffness, traction, viscoelastic characteristic length adaptation	Inflammatory profile and cell migration can be also evaluated	(Chen et al., 2010; Fabry & Fredberg, 2007; Silveira et al., 2005; Zakarya et al., 2021)

TABLE 1 (Continued)

Tissue characteristics	Tissue treatments	Disease models	Pharmacological evaluation of ASM contractility	Note	Main references
Cultured ASM cells, including cells from asthmatic and COPD donors	Exposure to respiratory virus	In vitro model of viral infection	/	Inflammatory profile, receptor desensitisation	(Elias et al., 1997; Oliver et al., 2006; Van Ly et al., 2013)
Primary bronchial epithelial cells from normal isolated bronchial tissue	Harvested cells (scraping the luminal airway surface with a convex scalpel blade #10) undergoing passive sensitisation procedure (overnight incubation with 2% vol^{-1} sensitising serum)	In vitro model of asthma	/	Inflammatory profile, alarmins (TSLP, IL-33 and IL-25) release, and pharmacological pathways can be evaluated	(Calzetta, Matera, Facciolo, et al., 2018b; Calzetta, Rogliani, et al., 2014b; Fulcher et al., 2005; Matera et al., 2011)
Primary bronchial epithelial cells from normal isolated bronchial tissue	Harvested cells (scraping the luminal airway surface with a convex scalpel blade #10) undergoing acute LPS challenge (incubation 2 h with LPS 300 $ng{\cdot}ml^{-1}$)	In vitro model of COPD	/	Inflammatory profile, alarmins (TSLP, IL-33 and IL-25) release, and pharmacological pathways can be evaluated	(Calzetta, Luongo, et al., 2015b; Cazzola et al., 2011; Fulcher et al., 2005; Matera et al., 2011)
Primary bronchial epithelial cells from normal isolated bronchial tissue	Harvested cells (scraping the luminal airway surface with a convex scalpel blade #10) undergoing CSE treatment (overnight incubation with CSE 1:10)	In vitro model of pre-COPD	/	Inflammatory profile, alarmins (TSLP, IL-33 and IL-25) release, and pharmacological pathways can be evaluated	(Agustí et al., 2023; Fulcher et al., 2005; Han et al., 2021; Matera et al., 2011)
Primary bronchial epithelial cells from isolated bronchial tissue from COPD donors	Harvested cells (scraping the luminal airway surface with a convex scalpel blade #10) undergoing CSE treatment (overnight incubation with CSE 1:10)	In vitro model of early COPD	/	Inflammatory profile, alarmins (TSLP, IL-33 and IL-25) release, and pharmacological pathways can be evaluated	(Agustí et al., 2023; Agusti & Faner, 2018; Cosío et al., 2020; Fulcher et al., 2005; Lee & Han, 2023; Matera et al., 2011; Yang et al., 2021)
Normal isolated bronchial tissue used for in vitro models of asthma and COPD	Exposure to PM (overnight incubation with $PM_{2.5}$ and PM_{10} 50 $\mu g{\cdot}ml^{-1}$) in epithelium-intact and/or epithelium-denuded bronchi	Ex vivo model of pollutants exposure	AHR, classical pharmacological parameters (i.e., E_{max}, EC_{50}, IC_{50}, pEC_{50}, pIC_{50}, pA_2 and $t_{1/2}$), drug interaction (i.e., synergy, antagonism and additivity)	Inflammatory profile, oxidative profile, alarmins (TSLP, IL-33 and IL-25) release, pharmacological pathways and ultrastructural analysis via transmission electron microscope can be also evaluated	(Aghapour et al., 2022; Albano et al., 2022; Calzetta, Aiello, et al., 2021a; Lee et al., 2020; Pozzi et al., 2003)
Primary bronchial epithelial cells from normal isolated bronchial	Harvested cells (scraping the luminal airway surface with a convex scalpel	In vitro model of pollutants exposure	/	Inflammatory profile, alarmins (TSLP, IL-33 IL-25) release, and pharmacological	(Calzetta, Aiello, et al., 2021a; Fulcher et al., 2005; Lee et al., 2020; Matera

(Continues)

TABLE 1 (Continued)

Tissue characteristics	Tissue treatments	Disease models	Pharmacological evaluation of ASM contractility	Note	Main references
tissue used for in vitro models of asthma and COPD	blade #10) undergoing exposure to PM (overnight incubation with $PM_{2.5}$ and PM_{10} 50 μg/ml)			pathways can be evaluated	et al., 2011; Pozzi et al., 2003)

Note: /: data not available.

Abbreviations: AHR: airway hyperresponsiveness; COPD: chronic obstructive pulmonary disease; CSE: Cigarette smoke extract; EC_{50} or IC_{50}: concentration needed to produce 50% E_{max} (EC_{50} or IC_{50}); E_{max}: maximal effect; IL: interleukin; LPS: lipopolysaccharide; M receptors: muscarinic acetylcholine receptors; pA_2: the negative logarithm to base 10 of the molar concentration of an antagonist that makes it necessary to double the concentration of the agonist needed to elicit the original submaximal response obtained in the absence of antagonist; PAF: platelet-activating factor; PAL: persistent airflow limitation; pEC_{50}: negative logarithm to base 10 of EC_{50}; pIC_{50}: negative logarithm to base 10 of IC_{50}; PM: particulate matter; $t_{1/2}$: onset of action; TSLP: thymic stromal lymphopoietin.

10 | CONCLUSIONS

Experiments carried out with isolated human bronchial tissues in vitro and ex vivo replicate many of the main anatomical, pathophysiological, mechanical, and immunological characteristics of patients with asthma or COPD. Moreover, the effect of drugs active on the bronchial tree at the level of both medium bronchi and small airways can be tested directly on the relevant target tissue and independently of the systemic influences of in vivo preparations.

However, the use of isolated human airways does have some limitations. The duration of treatment can only be tested maximally in sub-chronic experimental settings, a certain level of tissue inflammation may be induced by tissue manipulation and cutting during the preparation of the tissues, and bronchial tissue from asthmatic patients is scarcely available.

Any drug active on the airways, including those under development for the treatment of asthma and COPD, may be tested in vitro in both medium bronchi and small airways (Table 1) and experiments carried out on isolated human bronchial tissues allow the full pharmacological characterisation of novel compounds, both agonists and antagonists. Furthermore, the characterisation of the inflammatory fingerprint and the anti-inflammatory profile of drugs at the level of relevant target tissue can also be investigated with the methodologies discussed above. It is also possible to assess the impact of mucolytics and anti-oxidant agents, as well as that of mAbs, on isolated human airways. A large body of evidence indicates that in vitro models of asthma and COPD using isolated human airways can provide information that is directly translatable into humans with obstructive lung diseases and can provide accurate qualitative and quantitative assessment of drug interactions. Indeed, well performed experiments in isolated human bronchial tissue may guide the strategic development of new compounds under investigation, and optimise the current therapeutic strategies in asthma and COPD.

Ultimately, regardless of the technique used to investigate drugs for the treatment of chronic obstructive respiratory disorders, such as bronchial rings in isolated organ bath systems, videomicroscopy with visual imaging and a patching chamber or the use of a wire myograph, the limiting factors to producing high-quality and repeatable data from experiments on isolated human airways remain closely tied to the manual skills of the researcher conducting experiments and the availability of suitable tissue.

10.1 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Christopoulos et al., 2021; Alexander, Fabbro et al., 2021a, 2021b).

AUTHOR CONTRIBUTIONS

Luigino Calzetta: Conceptualization (lead); data curation (lead); funding acquisition (lead); methodology (lead); project administration (lead); resources (lead); software (lead); supervision (lead); visualization (lead); writing—original draft (lead); writing—review and editing (lead). **Clive Page:** Conceptualization (equal); data curation (equal); methodology (equal); project administration (equal); visualization (equal); writing—original draft (equal); writing—review and editing (equal). **Maria Gabriella Matera:** Methodology (equal); visualization (equal); writing—original draft (equal); writing—review and editing (equal). **Mario Cazzola:** Methodology (equal); visualization (equal); writing—original draft (equal); writing—review and editing (equal). **Paola Rogliani:** Conceptualization (equal); data curation (equal); funding acquisition (equal); methodology (equal); project administration (equal); resources (equal); software (equal); supervision (equal); visualization (equal); writing—original draf (equal)t; writing—review and editing (equal).

CONFLICT OF INTEREST STATEMENT

All the authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

N/A-Review.

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