

PROF. MARIO CAZZOLA (Orcid ID : 0000-0003-4895-9707)

Article type : Review

Long-acting muscarinic antagonists and small airways in asthma: which link?

Manuscript Acceptance Date: 03-Feb-2021

Mario Cazzola¹, Luigino Calzetta², Maria Gabriella Matera³

¹Department of Experimental Medicine, University of Rome "Tor Vergata", Rome, Italy

²Respiratory Disease and Lung Function Unit, Department of Medicine and Surgery, University of Parma, Parma, Italy

³Department of Experimental Medicine, University of Campania "Luigi Vanvitelli", Naples, Italy

Corresponding Author: Mario Cazzola, Department of Experimental Medicine, University of Rome Tor Vergata, Via Montpellier 1, Rome, 00133, Italy. E-mail: mario.cazzola@uniroma2.it

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/ALL.14766](https://doi.org/10.1111/ALL.14766)

This article is protected by copyright. All rights reserved

Short title: LAMAs and small airways in asthma

Keyword: Asthma; LAMAs; parasympathetic innervation; small airways

Abstract

Involvement of small airways, those of less than 2 mm in internal diameter, is present in all stages of asthma and contributes substantially to its pathophysiologic expression. Therefore, small airways are a potential target to achieve optimal asthma control. Airway tone, which is increased in asthma, is mainly controlled by the vagus nerve that releases acetylcholine (ACh) and activates muscarinic ACh receptors (mAChRs) post-synaptically on airway smooth muscle (ASM). In small airways, M₃ mAChRs are expressed, but there is no vagal innervation. Non-neuronal ACh released from the epithelial cells that may express choline acetyltransferase in response to inflammatory stimuli, as well as from other structural cells in the airways, including fibroblasts and mast cells, can activate mAChRs. By antagonizing M₃ mAChR, the contraction of the ASM is prevented and, potentially, local inflammation can be reduced and the progression of remodeling may be averted. In fact, ACh also contributes to inflammation and remodeling of the airways and regulates the growth of ASM. Several experimental studies have demonstrated the potential benefit derived from the use of mAChR antagonists, mainly long-acting mAChR antagonists (LAMAs), on small airways in asthma. However, there are several confounding factors that may cause a wrong estimation of the relationship between LAMAs and small airways in asthma. Further studies are needed to differentiate broncholytic and anti-inflammatory effects of LAMAs and to better understand the interaction between LAMAs and corticosteroids, also in the context of a triple therapy that includes a β_2 -AR agonist, at different levels of the bronchial tree.

Abbreviations: AC, adenylyl cyclase; ACh, acetylcholine; ASM, airway smooth muscle; BAL, bronchoalveolar lavage; β_2 -AR, β_2 -adrenoceptor; cAMP, cyclic adenosine monophosphate; ChAT, choline acetyltransferase; COPD, chronic obstructive pulmonary disease; EFS, electrical field stimulation; ERK, extracellular signal-regulated kinase; FEF_{25-75%}, forced expiratory flow at 25-75% of the vital capacity; FEV₁, forced expiratory volume in 1 second; ICS, inhaled corticosteroid; IL, interleukin; LABA, long-acting β_2 -AR agonist; LAMA, long-acting mAChR antagonist; mAChR, muscarinic ACh receptor; OVA, ovalbumin; PKA, protein kinase A; TGF- β 1, transforming growth factor- β 1.

Introduction

Small airways are usually defined as those airways of less than 2 mm in internal diameter and located beyond the 7th-8th generation of airways [1]. They represent more than 98% of the cross-sectional area of the lung but only less than 10% of the total lung volume [2], and end with the respiratory acinar zone (generations 17th-23rd) involved in gas exchange [1]. Small airways differ from large airways, having no cartilage, a relatively greater proportion of smooth muscle and fewer mucus-secreting goblet cells in the epithelial layer [3]. Because of the lack of cartilage that could support their structure, they are easily collapsible during forced expiration or smooth muscle contraction [1]. Physiological features of small airway obstruction include their premature closure with air trapping and regional heterogeneity and exaggerated volume dependence of airflow limitation [4].

Small airways in asthma

Small airway involvement is present in all stages of asthmatic disease [2] and affects 50-60% of individuals suffering from this disease [5]. Several specific abnormalities of small airways in asthma, such as cellular infiltration of bronchiolar walls, alveoli, and perivascular spaces [6, 7], goblet cell hyperplasia of the epithelium [7], collections of mucus and inflammatory cells obstructing the airway lumen [8], smooth muscle thickening, and submucosal remodeling [10], have been described.

Small airways exhibit greater resistance in patients with moderate-to-severe asthma, but they normally dilate in response to deep inspiration especially in the presence of airflow obstruction documented by spirometry [11]. This functional response suggests that in asthmatic patients it is also appropriate to focus on small airways in order to achieve effective improvements since it is likely that small airways remain sensitive to bronchoprotective therapies.

Indeed, as highlighted by an Interasma and World Allergy Organization document endorsed by Allergic Rhinitis and its Impact on Asthma and Global Allergy and Asthma European Network, the role of small airways in asthma is increasingly viewed as a potential target to optimise disease control [12]. Actually, dynamic hyperinflation resulting from small airway inflammation, which is highly prevalent in moderate-to-severe asthma

and is related to important patient outcomes associated to his/her daily life such as poorer asthma control and quality of life, and increased exacerbation risk, is an important goal for asthma treatment [13].

Acetylcholine and parasympathetic nerves in asthmatic small airways

Contractile and relaxant innervation of airway smooth muscle

The parasympathetic nerves carried in the vagus nerve are tonically active at rest, producing a stable and easily reversible basic tone of airway smooth muscle (ASM) [14]. Their responses can be modulated by activation of vagal afferents after a mechanical or chemical stimulus in the airway [15]. There is no direct sympathetic innervation of ASM, although the airway vascular system receives sympathetic innervation. However, sympathetic input to the parasympathetic ganglia has been documented and, moreover, there are β_2 -adrenoceptors (ARs) that are densely located on ASM [14, 16].

Acetylcholine (ACh) is the neurotransmitter of the parasympathetic nervous system [14, 16, 17]. It is released from the vagus and activates muscarinic ACh receptors (mAChRs) located post-synaptically on ASM, where M_2 and M_3 mAChRs represent the major population of mAChRs. ASM contraction is mediated primarily through activation of the G_q -coupled M_3 mAChRs, which are also the predominant receptors mediating mucus secretion and are involved in the dilation of airway blood vessels [14, 16]. Activation of M_3 mAChR is followed by activation of phospholipase C that releases inositol 1,4,5-triphosphate (IP_3) and thereby increases the levels of intracellular calcium. Also M_2 mAChRs, which couple to $G_{\alpha o/i}$ protein, are highly expressed on ASM and their activation counteracts β_2 -adrenoceptor (β_2 -AR) mediated relaxation due to phosphorylation of G_s protein, with consequent inhibition of adenylyl cyclase (AC) and reduced cyclic adenosine monophosphate (cAMP) generation and accumulation [18] (figure 1). Their role in ASM contraction is negligible [19], but M_2 mAChRs are also located in pre-junctional membranes of neuromuscular junctions of ASM where they regulate negative feedback to reduce ACh release in parasympathetic ganglia and nerve terminals [14]. M_2 mAChRs are even widely expressed by airway fibroblasts [18].

The mAChRs are found predominantly in the bronchus, but their density decreases from segmental to subsegmental bronchi (figure 2) [20]. The presence of a cholinergic

innervation in human peripheral airways is still under discussion. According to some researchers, small airways do not appear to be innervated by extrinsic or intrinsic cholinergic neurons [21, 22]. However, M_3 mAChRs, but not M_2 mAChRs, are expressed and activated in small airways by the so-called non-neuronal ACh, which is released by epithelial cells that express choline acetyltransferase (ChAT) in response to inflammatory stimuli [22]. The high affinity choline transporter 1 mediates the uptake of choline into cells and ACh is synthesized from choline and acetyl-coenzyme A by ChAT. Newly synthesized ACh is transported by the vesicular ACh transporter into the vesicles, stored there and then released into the extracellular space. Cigarette smoking or inflammatory stimuli induce the release of ACh from the epithelial cells of the airways [23]. Released ACh stimulates mAChRs on epithelial cells and surrounding cells in an autocrine or paracrine manner [22] (figure 3). However, more recent research suggests that small airways are innervated to an extent similar to that of the large airways. In fact, it has been shown that atropine significantly reduced human small airway contractions following repeated electrical field stimulation (EFS) of precision-cut lung slices [24]. In addition, imaging studies have suggested that even small airways are innervated, as are larger airways [25, 26]. M_3 mAChRs but not M_2 mAChRs are absent in the lung parenchyma [20].

Conversely, β_2 -ARs increase in number along the airways and their density in the subsegmental bronchi and lung parenchyma is approximately twice that of the M_3 mAChRs in the same region (figure 2) [20]. Using human tissues, it was observed that mAChR antagonists were more effective in central airways whereas β_2 -AR agonists worked better on peripheral airways, but both classes of bronchodilators were able to induce relaxation of the smooth muscle of the entire bronchial tree [27, 28].

Regardless of the distribution of receptor targets in the lung, the weak sympathetic tone is unable to counteract the parasympathetic activity. However, there is a complicated cross-talk between parasympathetic and sympathetic pathways perhaps at pre-synaptic and certainly at post-synaptic level that regulates the basic tone of ASM [29-31] (figure 1). At post-synaptic level, β_2 -AR signaling reduces M_3 mAChR-mediated IP_3 production by several distinct mechanisms that can involve protein kinase A (PKA). Conversely, M_3 mAChR activation stimulates desensitization of the β_2 -AR in a protein kinase C-

dependent manner. Furthermore, post-junctional M_2 mAChR activation limits the signaling and bronchorelaxant effect of β_2 -ARs by antagonizing β_2 -AR/ G_s activation of AC, although, as already mentioned, activation of M_2 mAChRs expressed pre-synaptically on parasympathetic nerve endings exerts negative feedback on neuronal ACh release, thereby limiting bronchoconstriction. In small airways, it is likely that the cross-talk takes place only between β_2 -ARs and M_3 mAChRs, if it is true that at that level there are no M_2 mAChRs on smooth muscle [20] and the parasympathetic innervation is absent [31].

Acetylcholine and asthmatic airway smooth muscle

Current research suggests that ACh, acting through mAChRs, is a key molecule of asthma pathophysiology [18] (figure 3). In a mouse model of asthma, the lungs had significantly more ACh than controls [32]. ACh was secreted to the entire lung regardless of its central or peripheral location. Furthermore, the incubation of isolated human bronchi with IgE enhanced EFS-induced bronchial contraction and ACh release via a M_2 mAChR dysfunction, without affecting the responses to exogenously applied ACh [33]. There is also evidence that an IgE-activating antibody caused a strong contraction in sensitised human small airways [34]. Obviously, the increased constriction of airways was caused by the ACh-induced activation of M_3 mAChRs on ASM [14].

Neuronal plasticity and remodeling

As already mentioned, ACh increases bronchoconstriction by activating M_3 mAChRs on ASM, but it also contributes to inflammation and remodeling of the airways and regulates the growth of ASM [35]. In a mouse model of asthma, the increased ACh release was accompanied by an intense infiltration of eosinophils even in the absence of an acetylcholinesterase deficiency [32]. The quantitative distribution of ACh in the lung tissue was strongly associated with asthma severity, which implies that inflammatory cell levels potentially affect ACh levels in asthma pathology. Inflammatory cells such as macrophages, lymphocytes, and granulocytes, as well as structural cells in the airways, including fibroblasts and mast cells, are other non-neuronal ACh sources [20] and M_3 mAChRs are expressed in various types of inflammatory cells [22]. However, also β_2 -ARs are present on inflammatory cells such as mast cells, monocytes, eosinophils, T-

lymphocytes, and neutrophils [36] and a cross-talk between M_3 mAChRs and β_2 -ARs is possible even at this level.

Experimental evidence suggests that exposure to allergens in the first years of life causes an increase in ASM parasympathetic innervation and ACh release [37]. This involves an altered regulation of M_3 mAChRs with consequent dysregulation of the expression of the genes involved in the functional maturation of ASM that, in turn, causes the hypercontractile phenotype of ASM.

The increased neuronal activity may be the consequence of neuronal plasticity and remodeling likely driven by immune/inflammatory cells, such as eosinophils and mast cells, or by proteins that induce and promote impaired nerve growth and function, such as neurotrophins [38, 39]. Plasticity is manifested by an increase in the density of the parasympathetic ganglia, which results in an additional release of ACh. The increased signal activated by ACh causes a further increase in the density of the cholinergic nerves, thus creating a vicious circle.

Airway remodeling is a pathological feature observed in asthma [40]. It can affect both large and small airways and pathology limited to the small airways is uncommon [41]. Cycles of bronchoconstriction and mechanotransduction are one plausible mechanism for its origin [42].

In effect, even in the absence of inflammatory stimuli, mechanical compression alone is sufficient to induce an asthma-like molecular pattern in non-asthmatic airway epithelial cells [43]. The use of gene knockout mice combined with ovalbumin (OVA) challenge allowed to show that ACh contributes to allergen-induced remodeling and ASM mass via M_3 mAChRs and not via M_1 or M_2 mAChRs [44]. No stimulating role for M_3 mAChRs was observed in allergic inflammation, suggesting that the role of ACh in remodeling is independent of the allergic inflammatory response and may involve bronchoconstriction.

Bronchoconstriction without further inflammation induces remodeling of the airways in patients with asthma [45]. Fundamentally, the cholinergic and transforming growth factor- $\beta 1$ (TGF- $\beta 1$) pathways interact to induce the transition of myofibroblasts. In human lung fibroblasts, both carbachol and TGF- $\beta 1$ increased the expression of M_1 and M_3 mAChRs proteins and reduced M_2 mAChRs expression after 48 hours of exposure [46].

Moreover, activation of mAChRs provoked collagen synthesis [47], and induced the transition from fibroblasts to myofibroblasts through an increase in extracellular signal-regulated kinase (ERK)1/2 phosphorylation, the formation of Ras homolog family member A-guanosine-5'-triphosphate and a decrease in cAMP levels [36]. The distal lung is an important site of extracellular matrix remodeling in fatal asthma, with an imbalance between collagen I and III (increased collagen I and decreased collagen III content) and an increase in the fibronectin and matrix metalloproteinase content [48].

Remodeling induced by bronchoconstriction without further inflammation is of paramount importance when setting up treatment for asthmatic patients. In fact, although treatment of the inflammatory component of asthma is the first-line approach to disease control, bronchial hyperreactivity is often not normalized by inhaled corticosteroid (ICS) therapy, particularly in patients with more severe asthma, and additional therapy is required [45].

Rationale for using M₃ mAChR antagonists in asthma

The importance of airway tone alteration in patients with asthma is a concept that is becoming increasingly accepted. Airway tone is altered due to an increase in vagal activity caused by several mechanisms that focus primarily on the amplified expression and enhanced function of signal pathway molecules essential for the ASM contraction mediated by M₃ mAChRs and on the dysfunction of M₂ mAChRs which act as autoreceptors [39].

Targeting M₃ mAChRs has the potential to prevent harmful changes in gene expression and, thus, reverse the hypercontractility of ASM triggered by exposure to allergens in the first years of life [37]. However, this therapeutic approach has never been tested and, therefore, it remains only a hypothesis. Instead, the use of mAChR antagonists in asthmatic patients is realistic although the mechanisms of small airway contraction are still partially unknown.

An experimental study that examined small airway relaxation using lungs obtained from subjects undergoing pulmonary resection showed that a pre-treatment of pulmonary slices with tiotropium, 4-DAMP, which is a selective M₃ mAChR antagonist, or AF-DX116, which is a selective M₂ mAChR antagonist, inhibited carbachol-induced contraction of

these airways in a concentration-dependent manner [49]. AF-DX116, gallamine, which is another selective M₂ mAChR antagonist, and pertussis toxin, which is an inhibitor of the G_i protein, significantly increased isoproterenol-induced relaxation of small airways.

Collectively, these data suggest that mAChRs blockade in human small airways inhibits airway contraction via direct inhibition of contraction via M₃ mAChRs and by M₂ mAChRs-mediated inhibition of the relaxation response [49]. mAChR antagonists, by inhibiting the M₂ mAChRs coupled to the post-synaptic G_i protein, maintain the β₂-AR agonist-induced relaxation of ASM and support the activity of AC, although inhibition of the presynaptic M₂ mAChRs may increase the release of ACh into the synaptic space [30]. Neural regulation of pulmonary function occurs within the central nervous system and extends into the conducting airways, making differentiating between central and peripheral effects difficult [50].

Pharmacological effects of LAMAs in asthma also considering small airways

Several experimental studies, which used animal models or human tissues and/or cells, have shown that there are potential benefits in using mAChR antagonists, mainly long-acting mAChR antagonists (LAMAs), in asthma. In particular, they highlighted the effects of this class of bronchodilators on small airways.

Animal models

In a guinea-pig model of asthma, antigen-induced hyperreactivity was completely blocked by tiotropium pre-treatment but only partially blocked by atropine pre-treatment [51]. Tiotropium blocked i.v. ACh-induced bronchoconstriction, but did not inhibit vagus-mediated bronchoconstriction in sensitized controls. This finding suggested that tiotropium does not inhibit hyperreactivity by blocking the effect on mAChRs of ACh released by the vagus. Rather, tiotropium may have acted through an anti-inflammatory mechanism, since it inhibited the antigen-induced accumulation of eosinophils in the lungs and around the nerves or, alternatively prevented airway hyperreactivity by blocking ACh released from non-neuronal sources such as epithelial cells or macrophages.

Extremely interesting, although still difficult to explain are the results of another experimental study which documented that while inflammatory cells and T2-type cytokine production were reduced in response to tiotropium in a chronic murine asthma model, these effects were reversed in a murine acute model [52]. In the acute model, in which the mice were immunized on days 0 and 7 by subcutaneous administration of OVA and challenged with intranasal OVA on days 21, 23, 25, and 27, and tiotropium was administered via the intranasal route 0.5 hour before the OVA challenge, the number of total cells, macrophages and eosinophils in bronchoalveolar lavage (BAL) fluids was significantly increased in the tiotropium-treated group compared to the control group. M_3 mAChRs were more expressed and M_2 mAChRs less expressed in the OVA group than in the naive group. In the chronic model, in which the mice were immunized with OVA once a week for 1 month and then underwent intranasal OVA challenges that started on day 31 and repeated twice a week for 3 months and tiotropium was administered twice a week for 3 months, the tiotropium group had significantly lower total cell counts, macrophages, eosinophils, and lymphocytes in BAL than the control group. Furthermore, in the acute asthma model, the tiotropium group showed increased levels of interleukin (IL)-4, IL-5, and IL-13 compared to the control group, whereas in the chronic asthma model, compared to the control group the IL-5 concentration in the BAL fluids was significantly lower and concentrations of IL-4 and IL-13 showed a trend towards a decrease in the tiotropium group. It was also observed that the tiotropium treatment for 3 months had a protective effect against airway remodeling parameters, including peribronchial fibrosis and smooth muscle thickening, by differential regulation of M_2 mAChRs and M_3 mAChRs expression. In fact, tiotropium treatment significantly reduced expression of the M_3 mAChRs and increased that of the M_2 mAChRs compared to the control group. Unfortunately, the underlying mechanism of an aggravation of inflammatory cells and cytokines in the acute asthma model was not studied. Consequently, it is difficult to explain why tiotropium increased the number inflammatory cells in BAL in the acute model. Nevertheless, the importance of this study is that it has suggested that tiotropium should be understood as a treatment for chronic asthma while in acute asthma it is even harmful. However, clinical studies confirming this hypothesis are absolutely necessary.

Tiotropium inhibited allergen-induced airway remodeling in a guinea pig model of ongoing asthma [53]. ASM, contractility and contractile protein expression were all partially or completely reduced. It has been suggested that the relaxation of ASM cells induced by tiotropium can prevent the production of extracellular matrix via the β -catenin signal [54]. β -Catenin is a dual-function protein involved in the regulation and coordination of cell-cell adhesion. The activation of the β -catenin signal has a regulatory function in the remodeling of the airways [55]. It is involved in the regulation of proliferation of ASM cells, epithelial-to-mesenchymal transition, differentiation of myofibroblasts, and production of extracellular matrix.

Glycopyrronium exerted a potent suppressive effect on lung inflammation and small airway remodeling induced by subchronic exposure to cigarette smoke in mice [56].

Human tissues and/or cells

Glycopyrronium was found to be significantly more effective in relaxing human bronchial rings passively sensitized with atopic serum than non-sensitized tissues [57]. The relaxing effect of glycopyrronium was also significantly more evident in the peripheral airways when they were passively sensitized.

Acridinium, a M_3 mAChR antagonist, dose-dependently inhibited the transition of human lung fibroblast to a myofibroblast contractile phenotype caused by carbachol and TGF- β 1 stimulation and also reduced fibroblast proliferation and migration [43]. Furthermore, acridinium pre-treatment prevented the upregulation of M_1 mAChRs and M_3 mAChRs, but not M_2 mAChRs downregulation induced by carbachol or TGF- β 1.

In addition to allergens, also cigarette smoke may induce inflammatory responses in human bronchial epithelial cells [23] and participate in lung remodeling [40]. In particular, cigarette smoke activates mAChRs in human bronchial fibroblasts by a non-neuronal cholinergic system, a mechanism that is involved in the upregulation of the myofibroblasts markers [58]. Treatment with glycopyrronium suppressed cigarette smoke extract-induced inflammatory responses in human bronchial epithelial cells and proliferation and collagen production in human lung fibroblasts [56]. It has been reported that another LAMA, acridinium reversed the cigarette smoke-induced myofibroblast

markers through inhibition of reactive oxygen species, generation, cAMP depletion, ERK1/2 phosphorylation and ChAT overexpression induced by cigarette smoke [57].

There is experimental documentation that LAMAs, such as glycopyrronium and tiotropium, reduced the release of ACh from isolated human bronchi when tested at low concentrations [28, 59]. Removal of the epithelium resulted in inhibition of this effect.

Experimental studies with LAMAs combined with corticosteroids and/or β_2 -AR agonists

ICSs are still the first-line anti-inflammatory therapy for persistent asthma of all severities [60]. Therefore, all this information generated in *in vitro* and *in vivo* studies raised the need to elucidate the potential interactions between corticosteroids and mAChRs antagonists. These interactions may provide the pharmacological rationale for the use of LAMA/ICS as dual combination or as a part of triple combination therapy that includes a β_2 -AR agonist for the treatment of patients suffering from asthma.

When comparing the ability of tiotropium and budesonide to inhibit allergen-induced airway remodeling, it was possible to document that the anti-remodeling effects of tiotropium were not substantially dissimilar to those of budesonide [61]. Similar to budesonide, tiotropium partially reduced the accumulation of eosinophils in the submucosal compartments of the cartilaginous and non-cartilaginous airways in guinea pigs sensitized to OVA and stimulated with OVA for 12 weeks. However, budesonide was more effective in preventing ASM thickening in the noncartilaginous airways and abrogated the increase in contractile protein accumulation, whereas the inhibitory effect of tiotropium was partial but tiotropium reduced the contractility of tracheal smooth muscle to a greater extent than did budesonide.

However, in another chronic asthma model, ASM thickening in noncartilaginous airways was reduced by both tiotropium and ciclesonide treatment [62]. No remodeling of the pulmonary microcirculation was observed. OVA-induced airway eosinophilia was reduced in a dose-dependent manner by both tiotropium and ciclesonide treatment, but this inhibitory effect was most marked in the submucosa of cartilaginous airways. Nevertheless, combined treatment with tiotropium and ciclesonide had profound anti-inflammatory effects compared to OVA-challenged animals, significantly inhibiting airway

eosinophilia in both cartilaginous and noncartilaginous airways. Furthermore, it inhibited OVA-induced ASM mass by 81%.

A study that pharmacologically investigated the interaction between beclomethasone and glycopyrronium on the human ASM tone was unable to detect synergistic interaction in non-sensitized airways, but the beclomethasone/glycopyrronium combination synergistically enhanced the relaxation of passively sensitized medium and small airways [57]. In small airways, the combination was able to increase the relaxation of ASM by 2.5-fold compared with mono-components. The synergistic interaction was associated with an increase of cAMP concentrations.

Interestingly, the combination of a LAMA with a long-acting β_2 -AR agonist (LABA) not only induces potent, significant and long-lasting relaxation of both isolated human medium bronchi and small airways pre-contracted by ACh, but also a significant increase in cAMP concentrations [28, 59].

When the interaction between glycopyrronium bromide, the ICS beclomethasone dipropionate, and the β_2 -AR agonist formoterol fumarate was assessed in isolated human airways, it was shown that this triple combination caused a very strong synergistic effect on relaxation of medium bronchi and middle to very strong synergy in small airways [63]. The synergy was related with the activation of intracellular glucocorticoid receptors and G_{sa} subunit G-protein of β_2 -ARs, leading to the modulation of cyclic AMP-dependent PKA pathway.

Effects of adding a LAMA on the small airways of asthmatic patients

The usefulness of a LAMA in improving the patency of small airways, even in the context of triple therapy, has been documented in asthmatic patients. In the IRIDIUM study, which evaluated a once-daily, single-inhaler ICS/LABA/LAMA combination, specifically, medium-dose and high-dose mometasone/indacaterol/glycopyrronium delivered via Breezhaler in patients with inadequately controlled asthma, it was observed an improvement in trough forced expiratory flow at 25-75% of the vital capacity ($FEF_{25-75\%}$), which is a potentially sensitive parameter for assessing peripheral airway function [64], of 69 ml and 41 ml after 26 and 52 weeks, respectively, when glycopyrronium was added to medium doses of mometasone plus indacaterol, and of 81 ml and 95 ml after 26 and 52

weeks, respectively, when glycopyrronium was added to high-dose of mometasone plus indacaterol [65]. Breezhaler is a conventional coarse-particle inhaler and delivers particles with a mass median aerodynamic diameter (MMAD) $>2.0\ \mu\text{m}$ [66], but only particles smaller than $2\ \mu\text{m}$, termed extra-fine particles, are able to penetrate the small airways or, at least, result in improved deposition in the small airways [67]. Nonetheless, the optimal particle size for an inhaled drug to reach and deposit into the small airways effectively has not been defined clearly [66], and, in any case, there is a normal distribution curve of particle size around the MMAD, so it is practically inevitable that a small number of extrafine particles that will reach the small airways, even with a coarse particle formulation [67].

Chronic dosing with triple therapy comprising olodaterol/tiotropium via the soft mist RespiMat inhaler added to extrafine hydrofluoroalkane-beclomethasone dipropionate was superior to dual therapy with ICS/LABA for effects on trough small airway outcomes in a small group of asthma patients who smoked [68]. The MMAD for the RespiMat is $\sim 2\ \mu\text{m}$ [66]. After chronic dosing, impulse oscillometry outcomes at trough, except for the resistance at 20Hz (R20), which represents the central airway resistance, were significantly improved with ICS/LABA/LAMA compared to ICS/LABA. Furthermore, results showed that FEF_{25-75} but not forced expiratory volume in 1 second (FEV_1) was also sensitive in detecting improved response at trough comparing treatments.

In another small group of patients, a single-inhaler triple therapy of an extrafine formulation of beclomethasone/formoterol/glycopyrronium (with a MMAD of $\sim 1.1\ \mu\text{m}$ [69]) induced ceiling bronchodilation at the level of small airways of severe asthmatic patients after 2 weeks of treatment [70]. Impulse oscillometry was performed to assess the small airways resistance at 5-19Hz (R5-19), and it was observed that on top of beclomethasone/formoterol/glycopyrronium, salbutamol did not significantly improve R5-19Hz either at peak or at trough.

This triple combination was examined in the TRIMARAN and TRIGGER trials, which were double-blind, 52-week studies comparing medium-strength (TRIMARAN, beclomethasone $100\ \mu\text{g}$) and high-strength (TRIGGER, beclomethasone $200\ \mu\text{g}$) extrafine beclomethasone/formoterol/glycopyrronium with the respective extrafine beclomethasone/formoterol strengths in adults with uncontrolled asthma and a history

of ≥ 1 exacerbation [71]. Both studies showed significant improvements in pulmonary function and the frequency of exacerbations in patients with asthma. Unfortunately, although this triple combination contains an extrafine formulation with a MMAD of <2.0 μm , it is not yet known how this triple combination acted on small airways during these trials.

There is no doubt that improvement in inhaler technology is critical to improve the aerosolization performance of drug particles and their delivery efficiency to peripheral regions of lungs. Data is accumulating to show that medications that also target the small airways show clinical efficacy that is superior to that of conventional asthma medications with larger particle size, but more robust evidence generated by randomized controlled trials is needed [72]. Lung deposition studies have shown that small-particle aerosols achieve better pulmonary deposition as well as effective penetration into the peripheral lung [73]. The ability to deliver small particles allows aerosols to treat the small airways in asthma.

Unanswered questions

There are still open questions to which it is difficult to give an answer because of several confounding factors that may cause a wrong estimation of the relationship between LAMAs and small airways in asthma. In particular, we still do not know whether a prolonged treatment and/or a chronic pathological condition can modify the access of the drug to mAChRs, and how these receptors respond to the drug. Besides, it should be clarified whether the effect of LAMAs on small airways of asthmatic patients is limited to a broncholytic action or involves also an anti-inflammatory activity. Furthermore, it is important to assess whether the assumption of a sex-related responsiveness to the pharmacological treatment of chronic obstructive pulmonary disease (COPD) [74] applies also to the use of LAMAs in asthma in general, and its impact on small airways in particular.

An old study showed that chronic exposure to atropine, which is a nonselective mAChR antagonist, resulted in increased airway M_3 mAChR density that was associated with enhanced ASM contraction in rabbits [75], which might be a reason for the rebound hyperresponsiveness observed after chronic inhalation of mAChR antagonists [76, 77].

On the other hand, there is evidence that the mAChR density expressed by peripheral blood lymphocytes was increased in patients with allergic rhinitis [78] and also in asthmatic patients [79] when compared with healthy controls. The increase was related to the degree of methacholine-induced bronchial hyperresponsiveness. It is not yet known whether this effect also occurs in small airways.

In the asthmatic subject, airway obstruction and bronchial hyperreactivity usually affect the central parts of the lungs while the inflammation can be distributed more peripherally [80]. This implies the absolute need to differentiate broncholytic effects of LAMAs from their anti-inflammatory effects, and to better understand the interaction between LAMAs and corticosteroids also in the context of a triple therapy that includes a β_2 -AR agonist, at different levels of the bronchial tree.

Some data indicate that female COPD patients may be more responsive to mAChR antagonists (in terms of FEV₁) than male COPD patients [81], but similar documentation in asthmatics is still lacking. It has been shown that small airway involvement in asthma, as reflected by methacholine-induced air trapping on CT scan, was significantly greater in males than females while bronchial exhaled nitric oxide, which may reflect small airway inflammation, was greater in females than males [82]. This indicates that males show a small airway involvement that is due to reduced patency of these airways, whereas in females the involvement is mainly due to small airway inflammation. Nevertheless, a greater pulmonary gene expression for M₃ mAChRs compared to M₂ mAChRs has been found in the female lungs than male ones [81]. At least potentially, this sexual dimorphism in the expression of mAChRs in the airways can modulate different therapeutic responses to LAMAs based on sex. There is, therefore, a need to evaluate the sex based therapeutic responsiveness to LAMAs, also focusing attention on the effects on small airways.

Conclusion

Experimental and clinical evidence of LAMAs efficacy in asthmatic patients is currently available, which suggests that there is a role for this class of bronchodilators in asthma, and also a link between small airways and potential benefit from LAMAs in this disease. However, as appropriately highlighted by Gosens and Gross [18], further studies are

needed to clarify in more detail the role of cholinergic control in the pathophysiology of asthma, and in our opinion also in the control of small airways. In particular, the areas that require further investigation are neuronal plasticity in asthma and its contribution to hyperreactivity and remodeling of the airways, the mechanisms underlying the cholinergic control of inflammation, mainly T2-type inflammation, and airway remodeling, and the remodeling induced by bronchoconstriction. In particular, with regards to LAMAs, the areas that need to be explored in depth are their real anti-inflammatory efficacy in asthma, whether there is a role for them in T2-type inflammation, and also their influence on neuronal plasticity and airway remodeling induced by bronchoconstriction.

LAMAs should never be used as monotherapy in asthmatic patients and, anyway, there is scarce evidence showing that ICS/LABA can be replaced by ICS/LAMA. Therefore, LAMAs must always be included in the context of a triple therapy [39]. However, LAMAs can have a role in the treatable traits approach that has been proposed as a new paradigm also for asthma management [83]. We strongly believe that small airway disease must be considered a treatable trait and, consequently, triple therapy should start earlier than currently recommended by Global Initiative for Asthma strategy [60] to reduce lung hyperinflation, which means improved asthma control [13], and also the possibility of influencing neuronal plasticity [39].

Obviously, the addition of a LAMA must always be considered for the treatment of smokers with asthma who have more severe obstructive impairments than light and never smokers with similar ICS dose [84], also because of the greater small airway involvement [85]. Furthermore, waiting for understand if there is a sex-related responsiveness of LAMAs in asthma, the simultaneous administration of a LAMA with a corticosteroid plus a LABA may be an empirical approach to overcome the sexual differences in the prevailing pathological impact of asthma on small airways and in M₃ mAChR expression.

Conflict of interest statement

MC has participated as a speaker and/or advisor in scientific meetings and courses under the sponsorship of Abdi Ibrahim, Almirall, AstraZeneca, Biofutura, Boehringer Ingelheim, Chiesi Farmaceutici, Cipla, GlaxoSmithKline, Lallemand, Menarini Group, Mundipharma,

Novartis, Pfizer, Verona Pharma, and Zambon and has been a consultant to ABC Farmaceutici, Chiesi Farmaceutici, Edmond Pharma, Lallemand, Novartis, Ockham Biotech, Verona Pharma, and Zambon.

LC has participated as advisor in scientific meetings under the sponsorship of Boehringer Ingelheim and Novartis, received nonfinancial support from AstraZeneca, a research grant partially funded by Chiesi Farmaceutici, Boehringer Ingelheim, Novartis, and Almirall, and is or has been a consultant to ABC Farmaceutici, Edmond Pharma, Ockham Biotech, Verona Pharma, and Zambon.

MGM has participated as a speaker and/or advisor in scientific meetings and courses under the sponsorship of ABC Farmaceutici, Almirall, AstraZeneca, Boehringer Ingelheim, Chiesi Farmaceutici, GlaxoSmithKline, and Novartis and has been a consultant to ABC Farmaceutici, GlaxoSmithKline, and Chiesi Farmaceutici.

Author contributions

All authors coordinated the work, designed the structure of the review, and reviewed the literature. MC wrote the manuscript, and prepared the figure. LC and MGM critically reviewed and finalized the manuscript.

References

1. Carr TF, Altisheh R, Zitt M. Small airways disease and severe asthma. *World Allergy Organ J.* 2017;**10**(1):20.
2. Bjermer L. The role of small airway disease in asthma. *Curr Opin Pulm Med.* 2014;**20**(1):23-30.
3. Sturton G, Persson C, Barnes PJ. Small airways: an important but neglected target in the treatment of obstructive airway diseases. *Trends Pharmacol Sci.* 2008;**29**(7):340-345.
4. Burgel PR. The role of small airways in obstructive airway diseases. *Eur Respir Rev.* 2011;**20**(119):23-33.
5. Usmani OS, Singh D, Spinola M, Bizzi A, Barnes PJ. The prevalence of small airways disease in adult asthma: A systematic literature review. *Respir Med.* 2016;**116**:19-27.

6. Carroll N, Cooke C, James A. The distribution of eosinophils and lymphocytes in the large and small airways of asthmatics. *Eur Respir J*. 1997;**10**(2):292-300.
7. de Magalhães Simões S, dos Santos MA, da Silva Oliveira M, et al. Inflammatory cell mapping of the respiratory tract in fatal asthma. *Clin Exp Allergy*. 2005;**35**(5):602-11.
8. Aikawa T, Shimura S, Sasaki H, Ebina M, Takishima T. Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack. *Chest*. 1992;**101**(4):916-921.
9. Kuyper LM, Paré PD, Hogg JC, et al. Characterization of airway plugging in fatal asthma. *Am J Med*. 2003;**115**(1):6-11.
10. de Medeiros Matsushita M, da Silva LF, dos Santos MA, et al. Airway proteoglycans are differentially altered in fatal asthma. *J Pathol*. 2005;**207**(1):102-110.
11. Foy BH, Natarajan S, Munawar A, et al. Characterising the role of small airways in severe asthma using low frequency forced oscillations: A combined computational and clinical approach. *Respir Med*. 2020;**170**:106022.
12. Braidó F, Scichilone N, Lavorini F, et al. Manifesto on small airway involvement and management in asthma and chronic obstructive pulmonary disease: an Interasma (Global Asthma Association - GAA) and World Allergy Organization (WAO) document endorsed by Allergic Rhinitis and its Impact on Asthma (ARIA) and Global Allergy and Asthma European Network (GA²LEN). *Asthma Res Pract*. 2016;**2**:12.
13. van der Meer AN, de Jong K, Hoekstra-Kuik A, Bel EH, Ten Brinke A. Dynamic hyperinflation impairs daily life activity in asthma. *Eur Respir J*. 2019;**53**(4):1801500.
14. Cazzola M, Page CP, Calzetta L, Matera MG. Pharmacology and therapeutics of bronchodilators. *Pharmacol Rev*. 2012;**64**(3):450-504.
15. Canning BJ. Reflex regulation of airway smooth muscle tone. *J Appl Physiol* (1985). 2006;**101**(3):971-985.
16. Matera MG, Page CP, Calzetta L, Rogliani P, Cazzola M. Pharmacology and therapeutics of bronchodilators revisited. *Pharmacol Rev*. 2020;**72**(1):218-252.
17. Meurs H, Dekkers BG, Maarsingh H, Halayko AJ, Zaagsma J, Gosens R. Muscarinic receptors on airway mesenchymal cells: novel findings for an ancient target. *Pulm Pharmacol Ther*. 2013;**26**(1):145-155.
18. Gosens R, Gross N. The mode of action of anticholinergics in asthma. *Eur Respir J*. 2018;**52**(4):1701247.

19. Fisher JT, Vincent SG, Gomeza J, Yamada M, Wess J. Loss of vagally mediated bradycardia and bronchoconstriction in mice lacking M₂ or M₃ muscarinic acetylcholine receptors. *FASEB J*. 2004;**18**(6):711-713.
20. Ikeda T, Anisuzzaman AS, Yoshiki H, et al. Regional quantification of muscarinic acetylcholine receptors and β -adrenoceptors in human airways. *Br J Pharmacol*. 2012;**166**(6):1804-1814.
21. Barnes PJ. Distribution of receptor targets in the lung. *Proc Am Thorac Soc*. 2004;**1**(4):345-351.
22. Koarai A, Ichinose M. Possible involvement of acetylcholine-mediated inflammation in airway diseases. *Allergol Int*. 2018;**67**(4):460-466.
23. Profita M, Bonanno A, Montalbano AM, et al. Cigarette smoke extract activates human bronchial epithelial cells affecting non-neuronal cholinergic system signalling in vitro. *Life Sci*. 2011;**89**(1-2):36-43.
24. Maarsingh H, Bidan CM, Brook BS, et al. Small airway hyperresponsiveness in COPD: relationship between structure and function in lung slices. *Am J Physiol Lung Cell Mol Physiol*. 2019;**316**(3):L537-L546.
25. Scott GD, Blum ED, Fryer AD, Jacoby DB. Tissue optical clearing, three-dimensional imaging, and computer morphometry in whole mouse lungs and human airways. *Am J Respir Cell Mol Biol*. 2014;**51**(1):43-55.
26. West PW, Canning BJ, Merlo-Pich E, Woodcock AA, Smith JA. Morphologic characterization of nerves in whole-mount airway biopsies. *Am J Respir Crit Care Med*. 2015;**192**(1):30-39.
27. Cazzola M, Calzetta L, Page CP, et al. Pharmacological characterization of the interaction between acridinium bromide and formoterol fumarate on human isolated bronchi. *Eur J Pharmacol*. 2014;**745**:135-143.
28. Cazzola M, Calzetta L, Puxeddu E, et al. Pharmacological characterisation of the interaction between glycopyrronium bromide and indacaterol fumarate in human isolated bronchi, small airways and bronchial epithelial cells. *Respir Res*. 2016;**17**(1):70.
29. Cazzola M, Molimard M. The scientific rationale for combining long-acting β_2 -agonists and muscarinic antagonists in COPD. *Pulm Pharmacol Ther*. 2010;**23**(4):257-267.

30. Calzetta L, Matera MG, Cazzola M. Pharmacological interaction between LABAs and LAMAs in the airways: optimizing synergy. *Eur J Pharmacol*. 2015;**761**:168-173.
31. Calzetta L, Matera MG, Cazzola M. Pharmacological mechanisms leading to synergy in fixed-dose dual bronchodilator therapy. *Curr Opin Pharmacol*. 2018;**40**:95-103.
32. Matsuda T, Suzuki Y, Fujisawa T, et al. Imaging mass spectrometry to visualise increased acetylcholine in lungs of asthma model mice. *Anal Bioanal Chem*. 2020;**412**(18):4327-4341.
33. Ichinose M, Miura M, Tomaki M, Oyake T, Kageyama N, Ikarashi Y, et al. Incubation with IgE increases cholinergic neurotransmission in human airways in vitro. *Am J Respir Crit Care Med*. 1996;**154**(5):1272-1276.
34. Wohlsen A, Martin C, Vollmer E, Branscheid D, Magnussen H, Becker WM, et al. The early allergic response in small airways of human precision-cut lung slices. *Eur Respir J*. 2003;**21**(6):1024-1032.
35. Kistemaker LE, Gosens R. Acetylcholine beyond bronchoconstriction: roles in inflammation and remodeling. *Trends Pharmacol Sci*. 2015;**36**(3):164-171.
36. Johnson M. Effects of β_2 -agonists on resident and infiltrating inflammatory cells. *J Allergy Clin Immunol*. 2002;**110**(6 Suppl):S282-S290.
37. Patel KR, Bai Y, Trieu KG, Barrios J, Ai X. Targeting acetylcholine receptor M3 prevents the progression of airway hyperreactivity in a mouse model of childhood asthma. *FASEB J*. 2017;**31**(10):4335-4346.
38. Kistemaker LEM, Prakash YS. Airway innervation and plasticity in asthma. *Physiology (Bethesda)*. 2019;**34**(4):283-298.
39. Cazzola M, Rogliani P, Matera MG. The latest on the role of LAMAs in asthma. *J Allergy Clin Immunol*. 2020;**146**(6):1288-1291.
40. Hough KP, Curtiss ML, Blain TJ, et al. Airway remodeling in asthma. *Front Med (Lausanne)*. 2020;**7**:191.
41. Elliot JG, Jones RL, Abramson MJ, et al. Distribution of airway smooth muscle remodelling in asthma: relation to airway inflammation. *Respirology*. 2015;**20**(1):66-72.
42. Pascoe CD, Green FHY, Elliot JG, James AL, Noble PB, Donovan GM. Airway remodelling with spatial correlations: Implications for asthma pathogenesis. *Respir Physiol Neurobiol*. 2020;**279**:103469.

43. Kılıç A, Ameli A, Park JA, et al. Mechanical forces induce an asthma gene signature in healthy airway epithelial cells. *Sci Rep*. 2020;**10**(1):966.
44. Kistemaker LE, Bos ST, Mudde WM, et al. Muscarinic M₃ receptors contribute to allergen-induced airway remodeling in mice. *Am J Respir Cell Mol Biol*. 2014;**50**(4):690-698.
45. Grainge CL, Lau LC, Ward JA, et al. Effect of bronchoconstriction on airway remodeling in asthma. *N Engl J Med*. 2011;**364**(21):2006-2015.
46. Milara J, Serrano A, Peiró T, et al. Aclidinium inhibits human lung fibroblast to myofibroblast transition. *Thorax*. 2012;**67**(3):229-237
47. Haag S, Matthiesen S, Juergens UR, Racké K. Muscarinic receptors mediate stimulation of collagen synthesis in human lung fibroblasts. *Eur Respir J*. 2008;**32**(3):555-62.
48. Dolhnikoff M, da Silva LF, de Araujo BB, et al. The outer wall of small airways is a major site of remodeling in fatal asthma. *J Allergy Clin Immunol*. 2009;**123**(5):1090-1097.
49. Brown SM, Koarai A, Sturton RG, Nicholson AG, Barnes PJ, Donnelly LE. A role for M₂ and M₃ muscarinic receptors in the contraction of rat and human small airways. *Eur J Pharmacol*. 2013;**702**(1-3):109-115.
50. House JS, Nichols CE, Li H, et al. Vagal innervation is required for pulmonary function phenotype in *Htr4*^{-/-} mice. *Am J Physiol Lung Cell Mol Physiol*. 2017;**312**(4):L520-L530.
51. Buels KS, Jacoby DB, Fryer AD. Non-bronchodilating mechanisms of tiotropium prevent airway hyperreactivity in a guinea-pig model of allergic asthma. *Br J Pharmacol*. 2012;**165**(5):1501-1514.
52. Kang JY, Rhee CK, Kim JS, et al. Effect of tiotropium bromide on airway remodeling in a chronic asthma model. *Ann Allergy Asthma Immunol*. 2012;**109**(1):29-35.
53. Gosens R, Bos IS, Zaagsma J, Meurs H. Protective effects of tiotropium bromide in the progression of airway smooth muscle remodeling. *Am J Respir Crit Care Med*. 2005;**171**(10):1096-1102.
54. Huo Y, Guan L, Xu J, Zhou L, Chen R. Tiotropium inhibits methacholine-induced extracellular matrix production via β -catenin signaling in human airway smooth muscle cells. *Int J Chron Obstruct Pulmon Dis*. 2018;**13**:1469-1481.

55. Kumawat K, Koopmans T, Gosens R. β -catenin as a regulator and therapeutic target for asthmatic airway remodeling. *Expert Opin Ther Targets*. 2014;**18**(9):1023-1034.
56. Hsiao YH, Tseng CM, Su KC, et al. Glycopyrronium bromide inhibits lung inflammation and small airway remodeling induced by subchronic cigarette smoke exposure in mice. *Respir Physiol Neurobiol*. 2018;**249**:16-22.
57. Cazzola M, Calzetta L, Rogliani P, Puxeddu E, Facciolo F, Matera MG. Interaction between corticosteroids and muscarinic antagonists in human airways. *Pulm Pharmacol Ther*. 2016;**36**:1-9.
58. Milara J, Serrano A, Peiró T, et al. Acridinium inhibits cigarette smoke-induced lung fibroblast-to-myofibroblast transition. *Eur Respir J*. 2013;**41**(6):1264-1274.
59. Calzetta L, Rogliani P, Page C, Rinaldi B, Cazzola M, Matera MG. Pharmacological characterization of the interaction between tiotropium bromide and olodaterol on human bronchi and small airways. *Pulm Pharmacol Ther*. 2019;**56**:39-50.
60. Global Initiative for Asthma. 2020 GINA Report, Global Strategy for Asthma Management and Prevention. Available at: https://ginasthma.org/wp-content/uploads/2020/06/GINA-2020-report_20_06_04-1-wms.pdf.
61. Bos IS, Gosens R, Zuidhof AB, et al. Inhibition of allergen-induced airway remodelling by tiotropium and budesonide: a comparison. *Eur Respir J*. 2007;**30**(4):653-661.
62. Kistemaker LE, Bos IS, Menzen MH, Maarsingh H, Meurs H, Gosens R. Combination therapy of tiotropium and ciclesonide attenuates airway inflammation and remodeling in a guinea pig model of chronic asthma. *Respir Res*. 2016;**17**:13.
63. Rogliani P, Matera MG, Facciolo F, Page C, Cazzola M, Calzetta L. Beclomethasone dipropionate, formoterol fumarate and glycopyrronium bromide: Synergy of triple combination therapy on human airway smooth muscle ex vivo. *Br J Pharmacol*. 2020;**177**(5):1150-1163.
64. Ciprandi G, Cirillo I, Pasotti F, Ricciardolo FL. FEF₂₅₋₇₅: a marker for small airways and asthma control. *Ann Allergy Asthma Immunol*. 2013;**111**(3):233.
65. Kerstjens HAM, Maspero J, Chapman KR, van Zyl-Smit RN, Hosoe M, Tanase AM, et al. Once-daily, single-inhaler mometasone-indacaterol-glycopyrronium versus mometasone-indacaterol or twice-daily fluticasone-salmeterol in patients with

- inadequately controlled asthma (IRIDIUM): a randomised, double-blind, controlled phase 3 study. *Lancet Respir Med*. 2020;**8**(10):1000-1012.
66. Lavorini F, Pedersen S, Usmani OS. Dilemmas, confusion, and misconceptions related to small airways directed therapy. *Chest*. 2017;**151**(6):1345-1355.
67. Lipworth B, Manoharan A, Anderson W. Unlocking the quiet zone: the small airway asthma phenotype. *Lancet Respir Med*. 2014;**2**(6):497-506.
68. Jabbal S, Kuo CR, Lipworth B. Randomized controlled trial of triple versus dual inhaler therapy on small airways in smoking asthmatics. *Clin Exp Allergy*. 2020;**50**(10):1140-1147.
69. Usmani OS, Mignot B, Kendall I, De Maria R, Cocconi D, Georges G, et al. Predicting lung deposition of extrafine inhaled corticosteroid-containing fixed combinations in patients with chronic obstructive pulmonary disease using functional respiratory imaging: an *in silico* study. *J Aerosol Med Pulm Drug Deliv*. 2020;doi: 10.1089/jamp.2020.1601.
70. Rogliani P, Ora J, Girolami A, Rossi I, Cazzola M, Calzetta L. Beclomethasone, formoterol and glycopyrronium: ceiling effect in small airways of COPD patients (abstract). *Eur Respir J*. 2020;**56**(suppl 64):5236.
71. Virchow JC, Kuna P, Paggiaro P, Papi A, Singh D, Corre S, et al. Single inhaler extrafine triple therapy in uncontrolled asthma (TRIMARAN and TRIGGER): two double-blind, parallel-group, randomised, controlled phase 3 trials. *Lancet*. 2019;**394**(10210):1737-1749.
72. Usmani OS. Small airways dysfunction in asthma: evaluation and management to improve asthma control. *Allergy Asthma Immunol Res*. 2014;**6**(5):376-388.
73. Santus P, Radovanovic D, Pecchiari M, Ferrando M, Tursi F, Patella V, et al. The relevance of targeting treatment to small airways in asthma and COPD. *Respir Care*. 2020;**65**(9):1392-1412.
74. Calzetta L, Puxeddu E, Rogliani P. Gender-related responsiveness to the pharmacological treatment of COPD: a first step towards the personalized medicine. *EBioMedicine*. 2017;**19**:14-15.
75. Witt-Enderby PA, Yamamura HI, Halonen M, Lai J, Palmer JD, Bloom JW. Regulation of airway muscarinic cholinergic receptor subtypes by chronic anticholinergic treatment. *Mol Pharmacol*. 1995;**47**(3):485-490.

76. Newcomb R, Tashkin DP, Hui KK, Conolly ME, Lee E, Dauphinee B. Rebound hyperresponsiveness to muscarinic stimulation after chronic therapy with an inhaled muscarinic antagonist. *Am Rev Respir Dis*. 1985;**132**(1):12-15.
77. Ayala LE, Ahmed T. Is there loss of protective muscarinic receptor mechanism in asthma? *Chest*. 1989;**96**(6):1285-1291.
78. Ricci A, Mariotta S, Amenta F, Tayebati SK, Terzano C. Changes in muscarinic cholinergic receptor expression in human peripheral blood lymphocytes in allergic rhinitis patients. *Pulm Pharmacol Ther*. 2008;**21**(1):79-87.
79. Ricci A, Amenta F, Bronzetti E, Mannino F, Mariotta S, Tayebati SK. Expression of peripheral blood lymphocyte muscarinic cholinergic receptor subtypes in airway hyperresponsiveness. *J Neuroimmunol*. 2002;**129**(1-2):178-185.
80. Matera MG, Rinaldi B, Berardo C, Rinaldi M, Cazzola M. A review of the pharmacokinetics of M₃ muscarinic receptor antagonists used for the treatment of asthma. *Expert Opin Drug Metab Toxicol*. 2020;**16**(2):143-148.
81. Li X, Obeidat M, Zhou G, et al. Responsiveness to ipratropium bromide in male and female patients with mild to moderate chronic obstructive pulmonary disease. *EBioMedicine*. 2017;**19**:139-145.
82. Cohen J, Douma WR, Ten Hacken NH, Oudkerk M, Postma DS. Physiology of the small airways: A gender difference? *Respir Med*. 2008;**102**(9):1264-1271.
83. McDonald VM, Clark VL, Cordova-Rivera L, Wark PAB, Baines KJ, Gibson PG. Targeting treatable traits in severe asthma: a randomised controlled trial. *Eur Respir J*. 2020;**55**(3):1901509.
84. Sprio AE, Ciprandi G, Riccardi E, et al. The influence of smoking on asthma in the real-life. *Respir Med*. 2020;**170**:106066.
85. Price D, Bjermer L, Popov TA, Chisholm A. Integrating evidence for managing asthma in patients who smoke. *Allergy Asthma Immunol Res*. 2014;**6**(2):114-120.

Figure 1 – Synthesis of the cross-talk between LABA, LAMA and ICS on ASM. Several mechanisms have been suggested to explain the synergy between LABAs and LAMAs at the ASM level and presynaptically in parasympathetic nerve fibers. Bidirectional molecular interactions between corticosteroids and β_2 -AR agonists have also been suggested for the reciprocal potentiation of the pharmacological effects of ICS and LABAs. The pharmacological mechanisms of the interactions between mAChR antagonists and ICS are not yet clear. In asthma, M_2 mAChR/Gi-coupled expression increases, phosphorylation of Gs protein and inhibition of AC and reduced cAMP accumulation occur, and, consequently, this has a negative effect on ASM relaxation. ICSs reduce cholinergic hypersensitivity in the airways, possibly through regulation of the expression of M_2 and M_3 mAChRs.

AC: adenylyl cyclase; ACh, acetylcholine; β_2 -AR, β_2 -adrenoceptor; cAMP: cyclic adenosine monophosphate; ERK: extracellular signal-regulated kinase; GR, glucocorticoid receptor; IP3: inositol-3-phosphate; KCa^{++} , calcium-activated potassium channel; mAChR, muscarinic ACh receptor; MAPK: p38 mitogen-activated protein kinase; MLCP: myosin light chain phosphatase; PKA: protein kinase A; PKC: protein kinase C; PLC: phospholipase C. Solid line, activation; dotted line, inhibition.

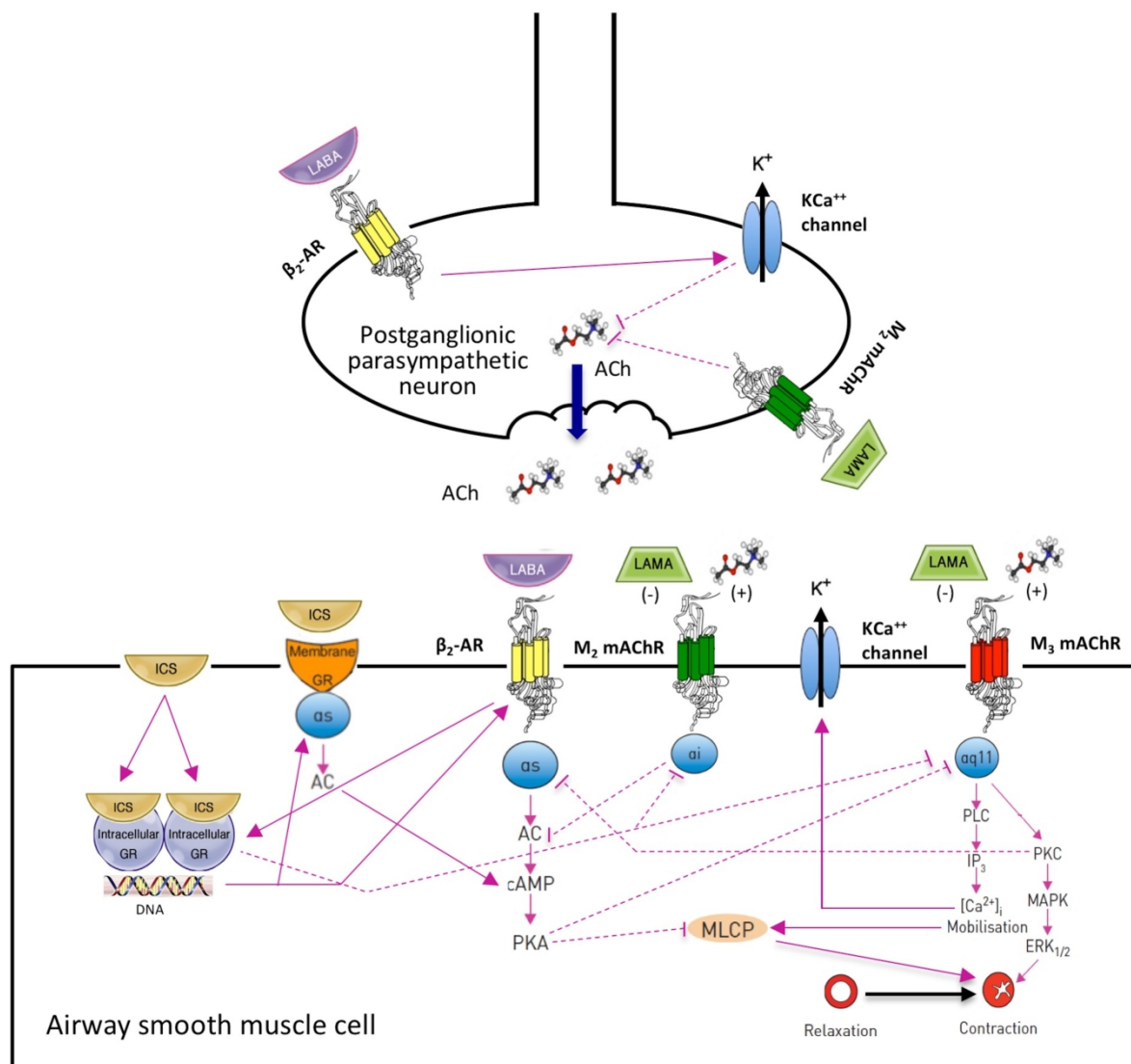


Figure 2 – Regional quantification of mAChRs and β -ARs in human airways. The total density of each receptor and their subtype ratio varied according to airway size and region. Data assumed from Ikeda et al. [20], modified.



Structure	Branch generation	Distribution of mAChR subtypes and β -AR subtypes (abundance, fmol·mg ⁻¹ of protein)			
		M ₂ mAChR	M ₃ mAChR	β_1 -AR	β_2 -AR
Trachea	0				
Primary bronchi	1				
Secondary bronchi	2				
Tertiary bronchi	3	47	48	0	50
Small bronchi	4				
Bronchioles	5-7				
Terminal bronchioles	8-16	11	28	0	85
Respiratory bronchioles	17-19	27	0	98	205
Alveolar ducts and sacs	20-23				

Figure 3 – Potential effects of ACh on asthmatic small airways. ACh, acetylcholine; ChAT, choline acetyltransferase; ECM, extracellular matrix.

