

# Tiotropium reduces lung inflammation in a model of chronic gastro-oesophageal reflux

YongYao CUI<sup>1#</sup>, Philippe DEVILLIER<sup>2\*#</sup>, XiaoDong KUANG<sup>1#</sup>, Hao WANG<sup>1</sup>,  
Liang ZHU<sup>1</sup>, ZuPeng XU<sup>1</sup>, Zheng XIA<sup>1</sup>, Leila ZEMOURA<sup>2,3</sup>,  
Charles ADVENIER<sup>2</sup>, HongZhuan CHEN<sup>1\*</sup>

<sup>1</sup> Department of Pharmacology, Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai, 200025, China.

<sup>2</sup> Université Versailles St-Quentin, Faculty of Medicine Paris Ile-de-France Ouest, Research Unit UPRES EA220, Hôpital Foch, 11 rue Guillaume Lenoir, 92150 Suresnes, France.

<sup>3</sup> Department of Pathology, Hôpital Foch, 40, rue Worth, 92150 Suresnes, France.

\*Corresponding author:

Tel : 33-1-46252791

Fax : 33-1-46252422

E-mail address: p.devillier@hopital-foch.org

# These authors contributed equally to this work

## **ABSTRACT**

Gastro-oesophageal reflux is frequent in chronic airway diseases and is considered as a trigger for symptoms. In animal models, bilateral vagotomy or muscarinic antagonists prevent the increase in airway resistance and the microvascular leakage induced by acute oesophageal acid instillation.

The present study investigates lung inflammation and remodelling in an animal model of chronic gastro-oesophageal reflux disease (GORD) and the effectiveness of pretreatments with tiotropium, atropine and dexamethasone.

Mice were exposed to twice daily intra-oesophageal HCl instillations for 21 days. Exposure to HCl causes marked infiltration by inflammatory cells of the airways and of peribronchial areas, an increase in epithelial thickness, histological features of interstitial pneumonitis, an increase in cell numbers and in the levels of IL-8 and sICAM in bronchoalveolar lavage fluids, as well as of *in vitro* tracheal contractility. The administration of nebulised tiotropium or intraperitoneal atropine prior to each instillation of HCl, considerably inhibits all these changes.

These results indicate a major role of acetylcholine in airway inflammation and remodelling in a model of GORD, and demonstrate that tiotropium and atropine can prevent lung inflammation with similar effectiveness as intraperitoneal dexamethasone, providing additional evidence that anticholinergics might contribute to the control of inflammatory processes in airway diseases.

**KEYWORDS:** airway inflammation, airway smooth muscle, anticholinergics, glucocorticosteroids, gastroesophageal reflux, mouse.

## Introduction

Patients with severe chronic obstructive pulmonary disease (COPD) or asthma have a high prevalence of gastro-oesophageal reflux disease (GORD) which is considered as a potential trigger for airway symptoms and exacerbations [1-4]. Medical and surgical treatments for reflux improve wheezing and coughing in about 66% of asthmatic patients, reduce the use of on-demand inhalers in more than half, and improve lung function in nearly 25% of them [1]. Recent studies however, have challenged the role of GORD as a cause of poorly controlled asthma at least in patients with asymptomatic GORD [3,4].

The parasympathetic nervous system provides the dominant autonomic innervations from the larynx down to the smallest airways and alveoli. The efferent cholinergic pathways that travel in the vagus nerve and synapse in airway parasympathetic ganglia cause broncho-constriction [5]. The blockade of muscarinic receptors (M<sub>1</sub> and M<sub>3</sub>) with atropine or inhaled anticholinergics improves airway obstruction and, indirect evidence suggest that parasympathetic activity is increased in COPD and asthma [5].

In asthmatic patients, the reduction in forced expiratory flow at 50% of vital capacity induced by hydrochloric acid (HCl) instillation into the oesophagus was eliminated by atropine pretreatment [6]. In animal models, we and others have reported that bilateral vagotomy or atropine pre-treatment inhibit the increase in airway resistance and the microvascular leakage induced by acute oesophageal acid instillation [7,8]. We have also shown that airway microvascular leakage caused by single HCl intra-oesophageal instillation in guinea-pigs involved both M<sub>1</sub> and M<sub>3</sub> receptors [9]. These results indicate that the release of acetylcholine acting through the activation of muscarinic receptors contributes to the airway responses caused by oesophageal stimulation with HCl.

Acetylcholine is traditionally involved in airway smooth muscle contraction, microvascular leakage and mucus secretion. Recent evidence also indicates that acetylcholine may contribute to various aspects of lung inflammation and airway remodeling [5]. Through the activation of muscarinic receptors, acetylcholine can stimulate the proliferation of lung fibroblasts and smooth muscle cells, as well as the collagen synthesis in lung fibroblasts [5,10]. Acetylcholine can also promote the release of chemotactic activities from epithelial cells, macrophages and airway smooth muscle [5].

The instillation of diesel particulates into the airways of anesthetized rats evokes neutrophilia in the lung which is abrogated by atropine pre-treatment or bilateral vagotomy [11]. In a guinea pig model of allergic asthma, a prominent role for acetylcholine has also been shown in the increase of airway smooth muscle thickening and contractility as well as mucous gland hypertrophy, goblet cell number and eosinophilia induced by repeated exposure to allergens [12,13]. Treatment with tiotropium bromide, a long-acting muscarinic receptor antagonist, prevented these different aspects of allergen-induced airway remodelling [12,13]. The anti-remodelling effects of tiotropium were comparable to those of budesonide [13] suggesting that the therapeutic effects of anticholinergics may contribute to the reduction in airway remodelling and lung inflammation in addition to their bronchodilatory effects.

In the present study, we established an experimental model of chronic GORD-induced lung inflammation by daily HCl intra-esophageal instillation in mice for 21 days and compared the effectiveness of atropine, tiotropium and dexamethasone in preventing various aspects of lung inflammation, including airway remodeling, cellular accumulation into the lung and airway smooth muscle contractility.

## **MATERIALS AND METHODS**

### *Animals*

Male BALB/c mice from Shanghai SLAC Laboratory Animal Co. Ltd, 8 weeks old, weighing 18–24 g were used. All experiments were conducted in accordance with the guidelines for the care and use of laboratory animals from Shanghai Jiao Tong University.

### *A novel mouse model of chronic airway inflammation induced by GORD*

Mice were exposed to the instillation into the distal part of the oesophagus of an acidic solution (0.3 ml; 0.1 N HCl and 0.5% pepsin) twice daily for 21 consecutive days using a calibrated feeding tube. Control animals were exposed to oesophageal instillations of saline. One hour prior to each instillation of the acidic solution, mice were treated either with dexamethasone (1 mg/kg) or atropine (1 mg/kg) administered intraperitoneally (i.p.) or with tiotropium (5 µg or 25 µg/kg) delivered by nebulisation for 20 min in a chamber using an Ultrasonic Nebulizer 4021, Shanghai Siling Medical Apparatus Factory (China). The doses of dexamethasone, atropine and tiotropium were chosen according to doses previously tested by us and others [10,12,14].

### *Evaluation of lung inflammation: bronchoalveolar lavage measurements and lung histology*

Animals were euthanised with urethane (100 mg/kg i.p.) 24 h after the last exposure to the acidic solution (day 22). The chest wall was then opened and the animals exsanguinated by cardiac puncture.

Immediately after cardiac puncture, bronchoalveolar lavage was performed by infusion and extraction of 1 ml of phosphate buffered saline (PBS) via a tracheal cannula. This was repeated twice, and the recovered bronchoalveolar lavage fluids (BALF) pooled. The BALF was centrifuged at 1500 g for 5 min at 4 °C, and the supernatant was stored at -70°C and used for interleukin-8 (IL-8) and soluble intercellular adhesion molecule-1 (ICAM-1) detection. IL-8 and ICAM-1 measurements were performed using ELISA kits (Rapidbio Lab, California, USA). The cell pellet was re-suspended in 1 ml PBS, and the total number of cells was counted with a hemocytometer. Differential counts of macrophages, lymphocytes, neutrophils and eosinophils were determined on cytopsin smears of BAL

samples from individual mice, stained with Wright-Giemsa after counting 400 cells. Results are expressed as cell number  $\times 10^6/L$  of BALF.

Immediately after BALF recovery, the esophagus and the right lung were removed, rinsed with PBS, instilled with 10% phosphate-buffered formalin and fixed with the same solution for 18-24 h. Parasagittal sections through the lung were then cut, embedded in paraffin and sectioned at 8  $\mu\text{m}$  in thickness. The slides were stained with hematoxylin and eosin (H&E) and examined by two histologists in a blinded fashion. The thickness of the bronchial epithelial and smooth muscle layers were measured in H&E-stained sections at 10 random non-overlapping locations using Image-Pro Plus 6.0 software (Olympus, Japan). The mean thickness was calculated on 7 medium-size bronchi (measuring 250–400  $\mu\text{m}$  in luminal diameter) for which the ratio of the maximum and minimum diameters was less than 2 to ensure that the airways were not obliquely cut. Segments of the esophagus were treated as described for lung tissues.

#### ***Airway function*** $\square$ ***isometric tension measurements*** $\square$

Immediately after BALF recovery, the trachea was removed and prepared free of serosal connective tissue. Single ring preparations were mounted for isometric recordings in organ baths containing modified Krebs-Henseleit (KH) solution (composition in mM: NaCl, 118.0; KCl, 4.7; CaCl<sub>2</sub>, 2.5; NaHCO<sub>3</sub>, 25.0; MgSO<sub>4</sub>, 1.2; KH<sub>2</sub>PO<sub>4</sub>, 1.2; glucose, 11.0, EDTA·Na<sub>2</sub> 0.5) maintained at 37°C and continuously gassed with 95% of O<sub>2</sub> and 5% of CO<sub>2</sub>. Each ring preparation was connected vertically to a force-displacement transducer under a resting tension of 0.5 g. Preparations were allowed to equilibrate for at least one hour during which the buffer solution was renewed each 15 mins. Resting tension was re-adjusted to 0.5 g and tracheal rings were precontracted twice with 40 mM KCl. Isometric contractions were recorded using a PowerLab 8sp life analysis system (Ad Instruments Co.). Following a thorough washing of the tissues over a period of at least 30 mins, cumulative concentration-response curves were constructed to acetylcholine (1nM-3mM) using 0.5 log increments. The changes in airway isometric tension were expressed as force generated (g) and pEC<sub>50</sub>, i.e.,  $-\log_{10}$  of the concentration of acetylcholine causing 50% of the maximal force generated (EC<sub>50</sub>). The EC<sub>50</sub> was calculated from the logarithmic regression of each concentration-response curve.

### ***Drugs***

Tiotropium was purchased from Phoesher chemical Co. Ltd (Jinan, China); dexamethasone from Tongyong pharmaceuticals company (Shanghai, China) and atropine and acetylcholine from Sigma Chemical (St Louis, Mo, U.S.A.). All other chemicals were of analytical grade.

### ***Data analysis***

All data are presented as mean±SEM. Differences between groups were analyzed using ANOVA, followed by a Dunnett t test for selected pairs if appropriate. Differences between means were considered to be significant at a p-value of < 0.05. All statistical analyses were performed using Prism version 5.0 (Graph-Pad Software, San Diego, CA, U.S.A.).

## **RESULTS**

### ***Repeated oesophageal instillation of HCl caused oesophagitis and lung inflammation***

Mice exposed to HCl exhibited consistent erosion of the esophageal stratified squamous epithelium associated with an impressive infiltration of inflammatory cells in the muscularis mucosa. The thickness of the muscularis mucosa was not significantly altered (data not shown).

Lung histopathology revealed that HCl exposure resulted in marked infiltrations by polynuclear phagocytes, lymphoplasmocytes and macrophages of the airway walls and of peribronchial and peribronchiolar areas. The formation of lymphoid follicles containing germinal centers was observed in certain areas (Fig.1, A & B vs C & D). The accumulation of inflammatory mucous exudates containing polynuclear phagocytes, lymphoplasmocytes and macrophages was also observed in the lumens of airways. In addition, histological features of interstitial pneumonitis were noted with a thickening of alveolar walls by infiltration by polynuclear phagocytes and lymphoplasmocytes associated with congested capillaries. The morphometric analysis of airway wall remodeling shows a significant

epithelial thickening associated with a weak but significant smooth muscle thickening (Fig. 2). The analysis of cells retrieved from the airways by bronchoalveolar lavages revealed a significant increase (1.8-fold) in the total cell number. Differential cell counts also revealed significantly increased numbers of macrophages, lymphocytes and neutrophils that were increased 1.7-, 2.0- and 3.1-fold respectively compared with saline control (Fig. 3). There was no increase in BALF eosinophil numbers. Furthermore, the concentrations of IL-8 and ICAM-1 were significantly elevated in BALF (Fig. 4).

### ***Reduced lung inflammation in mice treated with tiotropium and atropine***

Mice treated either with nebulised tiotropium (25 µg/kg) or intraperitoneal atropine (1 mg/kg) throughout exposure to HCl exhibited marked reductions in inflammatory cell infiltration and in the presence of congested capillaries in the alveolar walls (Fig. 1, E & F and G & H). A non-significant decrease of the epithelium thickening was observed in the group of mice treated with 5 µg/kg of tiotropium. However, no significant reduction of the smooth muscle thickening was observed in the three groups of mice treated with anticholinergics, but the smooth muscle thickness was however not significantly different from those in control mice not exposed to HCl (Fig. 2).

The number of cells in BALF was also decreased in mice treated either with nebulised tiotropium or intraperitoneal atropine (Fig. 3). In mice treated with tiotropium (5 µg/kg and 25 µg/kg), the reduction in the HCl-induced increase in BALF cell numbers reached respectively 76% and 89%(total cells), 79 % and 91% (macrophages), 83% and 89% (lymphocytes) and, 70% and 77%, (neutrophils). In mice treated with atropine, the reduction in the HCl-induced increase in BALF cell numbers reached 63% (total cells), 71% (macrophages), 65% (lymphocytes) and, 54% (neutrophils). There were no significant differences in the BALF cell numbers between the mice treated with tiotropium and atropine. IL-8 and ICAM-1 concentrations in BALF were reduced by atropine and the highest dose of tiotropium (Fig. 4).

### ***Reduced lung inflammation in mice treated with dexamethasone***

Mice treated with dexamethasone (1mg/kg, i.p.) throughout exposure to HCl exhibited a reduction in lung inflammation (Fig. 1, I & J) with a significant decrease of the epithelium



thickening and no alteration of the smooth muscle thickening (Fig. 2). Dexamethasone also markedly inhibited the recruitment of bronchoalveolar inflammatory cells (Fig. 3). The reductions in the increase in BALF cell numbers reached 93% (total cells), 98% (macrophages), 95% (lymphocytes) and 81% (neutrophils). IL-8 and ICAM-1 concentrations in BALF were both reduced by dexamethasone (Fig. 4).

***Inhibition by tiotropium, atropine and dexamethasone of the enhanced acetylcholine-induced contraction of mouse trachea.***

Three weeks of twice daily instillations of HCl into the distal part of the esophagus enhanced contraction of mouse tracheal rings in response to the cumulative addition of acetylcholine (Fig. 5). The maximal contractile response to acetylcholine increased from  $0.35 \pm 0.02$  g in saline-exposed mice to  $0.55 \pm 0.06$  g in HCl-exposed mice ( $p < 0.001$ ). Sensitivity to acetylcholine, however, was not affected ( $pEC_{50} = 5.7 \pm 0.3$  and  $6.0 \pm 0.7$  for saline- and HCl-exposed mice, respectively). Treatments with tiotropium (25  $\mu$ g/kg) and atropine completely prevented the increase in maximal contraction induced by HCl-exposure (tiotropium 25  $\mu$ g/kg :  $0.32 \pm 0.04$  g and atropine :  $0.41 \pm 0.02$  g (Fig. 5)). The sensitivity to acetylcholine was also not altered (tiotropium 25  $\mu$ g/kg :  $pEC_{50} = 5.4 \pm 0.4$ ; atropine :  $pEC_{50} = 5.4 \pm 0.2$ ). Similarly, pretreatment with dexamethasone completely prevented the increase in contractility caused by HCl-exposure (maximal response:  $0.37 \pm 0.23$ ) with no alteration of the sensitivity to acetylcholine ( $pEC_{50} = 5.5 \pm 0.3$ ).

## **DISCUSSION**

The main findings of the present study are that airway remodelling, inflammatory cellular infiltration of the lung and the increase in airway reactivity to acetylcholine caused by a twice daily instillation of HCl into the oesophagus of mice for 21 days, can be partially or even considerably prevented by muscarinic receptor antagonists including tiotropium, a long-lasting anticholinergic widely prescribed in COPD patients. We have previously shown in guinea pigs that the airway vascular leakage induced by acute intra-oesophageal HCl instillation involving acetylcholine release from vagus nerve terminals, was blocked by atropine and was mediated through the activation of  $M_1$  and  $M_3$  receptors. In this

previous study, the upper portion of the oesophagus was ligated to prevent stimulation of the larynx by HCl and to ensure an oesophageal origin of the cholinergic reflex-induced airway leakage [9]. In these previous experimental conditions, however, we used a concentration of HCl (1 N) far above the physiological range to trigger a cholinergic reflex. In the present study, it was not possible to ligate the upper part of the oesophagus since HCl was instilled twice daily for 21 days, but we used a concentration of HCl (0.1 N) in the physiological range [15]. Oesophago-laryngeal reflux as well as microaspirations of HCl likely occurred in this chronic model of GORD as well as microaspiration of gastric content, as suggested by the observation of microscopic food fragments in airways of some HCl-exposed untreated mice (data not shown). Pulmonary microaspiration of gastric content has also been demonstrated in children with chronic respiratory symptoms [16,17]. Stimulation of the larynx or the trachea with HCl causes a much greater bronchoconstriction than oesophageal stimulation [18-20] suggesting that oesophago-laryngeal reflux is a more likely mechanism for the bronchoconstriction and airway inflammation associated with GORD than an oesophageal-bronchial reflex. Results of studies in animals indicate that bronchoconstriction caused by activation of tracheal irritant receptors involved a vagally-mediated reflex [21]. Apart from microaspiration, acetylcholine appears therefore to be involved in the pulmonary changes associated with GORD through cholinergic reflex originating either from the oesophagus or from the upper airways. In addition, nonneuronal acetylcholine release from inflammatory and epithelial cells [5] might also contribute to the development of airway inflammation and hyperresponsiveness in the present model of chronic GORD.

The present results show that the efficacy of either atropine administered intraperitoneally or tiotropium administered by nebulisation in preventing airway remodeling and inflammatory cellular infiltration of the lung is very similar to that of dexamethasone administered intraperitoneally. In our model of GORD, the increase in the epithelial and smooth muscle thickness of the airways was rather weak in comparison to the increases previously reported after chronic exposure of mice to allergen [22]. The high dose of tiotropium, atropine, and dexamethasone fully prevented this moderate increase in epithelial thickening. Acetylcholine stimulates the proliferation of rat airway epithelial cells through the activation of M1 muscarinic receptors and nicotinic receptors [4], an

observation that may contribute to explain the inhibitory effects of atropine and tiotropium on the epithelium thickening. A non significant increase in the smooth muscle thickness was observed in mice treated with anticholinergics or dexamethasone but the weak increase in untreated mice precludes any firm conclusion on the preventive effect of these drugs. However, in sensitized guinea pigs exposed to allergen, tiotropium markedly prevented the increase in airway smooth muscle thickening but was less effective than budesonide [13]. In the present study, the marked increase in the contractility of isolated trachea from untreated mice exposed to HCl was considerably inhibited by tiotropium, atropine and dexamethasone. The inhibitory effect of tiotropium is unlikely to be due to a residual blockade of the muscarinic receptors caused by the persistence of this long acting muscarinic antagonist in the trachea (more than 24 h after the last administration), since no residual effect on methacholine-induced guinea pig tracheal contraction has been previously shown at 24h after in vivo exposure [12] and since a similar inhibitory effect was observed with atropine, a short-acting anticholinergic [23]. We did not study the smooth muscle thickness of the trachea, but the weak increase in thickness in the peripheral airways did not support a relationship between the increase in contractility and the increase in smooth muscle mass. In the previous study from Bos et al. [13], the tracheal contractility to methacholine was increased without concomitant changes in airway smooth muscle content in the large airways in contrast with the increase in peripheral airways, suggesting that the central airways have acquired a hypercontractile phenotype after repeated allergen challenge. As observed in the present study, tiotropium completely prevented the increase in contractility in this model of allergen-driven lung inflammation.

Treatment of mice with tiotropium, atropine and dexamethasone markedly prevented the infiltration by polynuclear phagocytes, lymphoplasmocytes and macrophages of the airway walls and of peribronchial and peribronchiolar areas caused by repeated oesophageal instillation of HCl. This cellular infiltration of the lung tissues was associated with an enhanced number of macrophages, lymphocytes and neutrophils in BALF and an increase in BALF levels of IL-8 and ICAM-1. Activated neutrophils are known to play a key role in pulmonary inflammation and oxidative damage. IL-8 - a major chemoattractant for neutrophils, is produced by various pulmonary cells including macrophages and epithelial cells and sICAM-1 which is mainly produced by alveolar epithelial cells in the

alveolar lining fluid - can activate leukocytes [24,25]. In children with GORD and asthma-like symptoms, neutrophilia positively correlated with IL-8 levels in BALF [26] and both neutrophilia and IL-8 levels correlated with the number of proximal reflux events [16]. In vitro studies have revealed that muscarinic receptor stimulation, particularly the M<sub>3</sub> receptor subtype, triggers the release of pro-inflammatory mediators involved in neutrophil recruitment, such as IL-8 and leukotriene B<sub>4</sub>, from airway smooth muscle, epithelial cells and alveolar macrophages [27-29]. IL-8 and leukotriene B<sub>4</sub> are also involved in monocyte chemoattraction [24,30]. In the three most recent studies, tiotropium suppressed the release of IL-8 and LTB<sub>4</sub> explaining at least in part the preventive efficacy of blocking muscarinic receptors with tiotropium or atropine on the increased number of neutrophils and macrophages in the airways in our model.

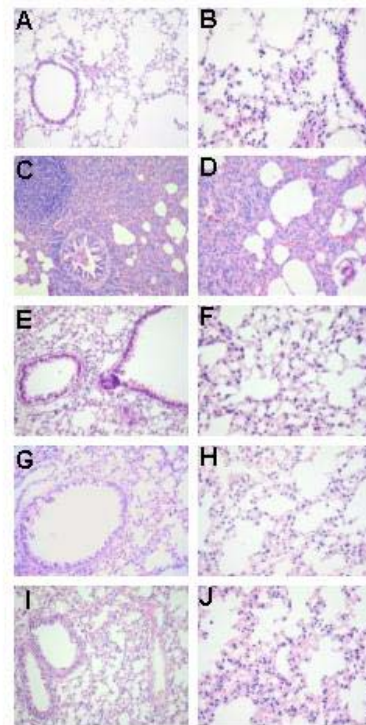
In conclusion, the results of the present study demonstrate that muscarinic receptor blockade by atropine or tiotropium prevents airway inflammation and remodeling in an animal model of chronic GORD. The preventive efficacy of the two muscarinic antagonists is similar to those of dexamethasone. The present study extends the beneficial effects of tiotropium in allergen-induced airway inflammation and remodelling to a different model of pulmonary inflammation, providing further evidence that the beneficial effects of anticholinergics might exceed those of bronchodilation both in asthma and in COPD, particularly in patients suffering from GORD

### **Acknowledgements**

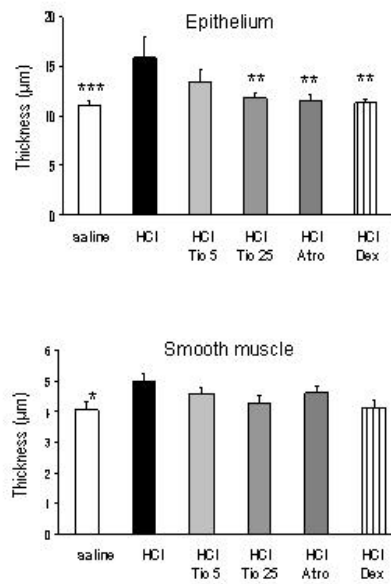
This study was supported by the National Natural Science Foundation of China (No. 30600269, 30873109), the Key Project of Science and Technology Commission of Shanghai Municipality (No. 09JC1408800) and the National Basic research Program of China (No. 2010CB529806).

### **Legends:**

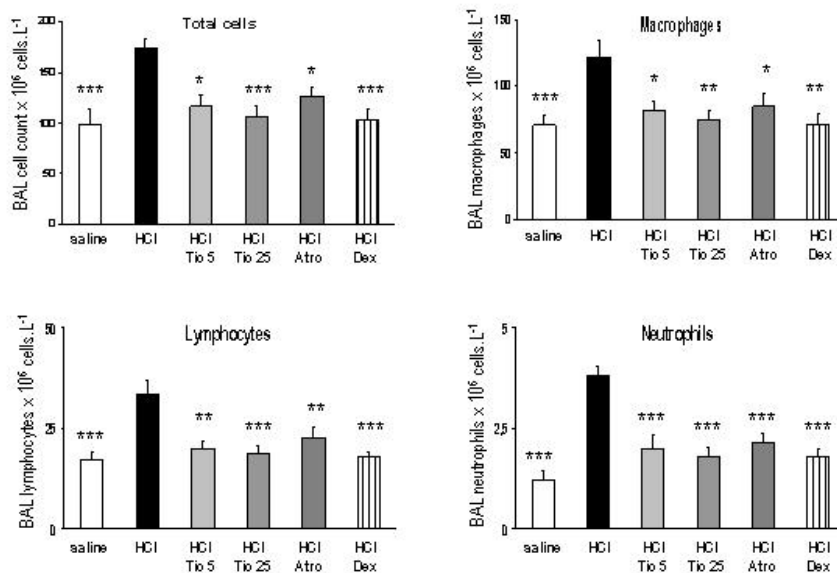
**Figure 1:** Representative photomicrographs of lung sections stained with hematoxylin and eosin. Photographs were taken at 100X (left) and at 200X (right): saline-exposed animals (A, B), untreated HCl-exposed animals (C, D), HCl-exposed animals treated with nebulised tiotropium (25 µg) (E, F) or treated with intraperitoneal injection of atropine (1 mg/kg) (G, H) or dexamethasone (1 mg/kg) (I, J).



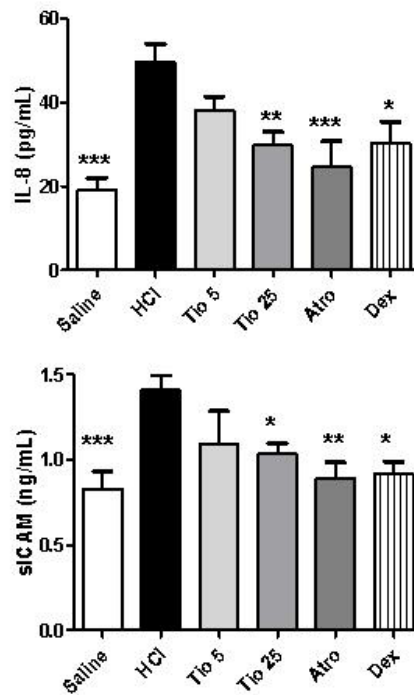
**Figure 2:** Effects of twice daily exposure to HCl for 21 days and treatment by nebulisation of tiotropium 5 µg (Tio 5) or 25 µg (Tio 25) or by intraperitoneal injection of atropine 1 mg/kg (Atro) or dexamethasone 1 mg/kg (Dex) on epithelial and smooth muscle thickness in medium size bronchi. Data are means +/- SEM (7 animals per group) \*P<0.05; \*\*P<0.01; \*\*\*P<0.001 when compared to untreated HCl-exposed animals.



**Figure 3:** Effects of twice daily exposure to HCl for 21 days and treatment by nebulisation of tiotropium 5 µg (Tio 5, n=9) or 25 µg (Tio 25, n=12) or by intraperitoneal injection of atropine 1 mg/kg (Atro, n=12) or dexamethasone 1 mg/kg (Dex, n=11) on total and differential cell counts in BALF. Data are means +/- SEM. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001 when compared to untreated HCl-exposed animals (n=17). Control animals exposed to saline for 21 days (n=16).

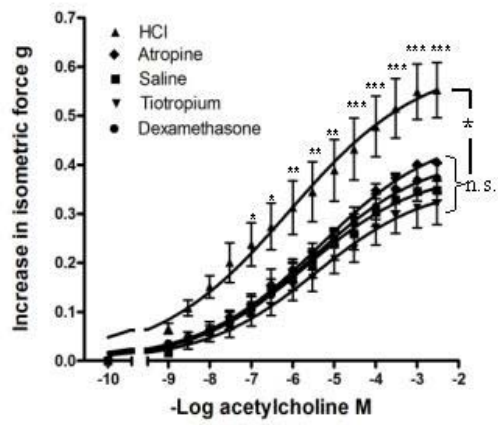


**Figure 4:** Effects of twice daily exposure to HCl for 21 days and treatment by nebulisation of tiotropium 5  $\mu\text{g}$  (Tio 5, n=7) or 25  $\mu\text{g}$  (Tio 25, n=11) or by intraperitoneal injection of atropine 1 mg/kg (Atro, n=7) or dexamethasone 1 mg/kg (Dex, n=7) on BALF levels of IL-8 and ICAM-1. Data are means  $\pm$  SEM. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001 when compared to untreated HCl-exposed animals (HCl, n=11). Control animals exposed to saline for 21 days (n=11).



**Figure 5:** Effects of twice daily exposure to HCl for 21 days and treatment by nebulisation of tiotropium (25  $\mu$ g) or by intraperitoneal injection of atropine (1 mg/kg) or dexamethasone (1 mg/kg) on acetylcholine-induced isometric contraction of tracheal preparations. Data are means  $\pm$  SEM (6 animals per group). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  when compared to untreated HCl-exposed animals (HCl, ANOVA for repeated measures); n.s. = not significant when compared to saline exposed animals. \* $P < 0.05$  when compared to HCl.





## References:

1. Fox M, Forgacs. Gastro-oesophageal reflux disease. *BMJ* 2006;332:88-93.
2. Terada K, Muro S, Sato S, Ohara T, Haruna A, Marumo S, Kinose D, Ogawa E, Hoshino Y, Niimi A, Terada T, Mishima M. Impact of gastro-oesophageal reflux disease symptoms on COPD exacerbation. *Thorax* 2008; 63: 951-955.
3. American Lung Association Asthma Clinical Research Centers, Mastronarde JG, Anthonisen NR, Castro M, Holbrook JT, Leone FT, Teague WG, Wise RA. Efficacy of esomeprazole for treatment of poorly controlled asthma. *N Engl J Med.* 2009; 360: 1487-99.
4. DiMango E, Holbrook JT, Simpson E, Reibman J, Richter J, Narula S, Prusakowski N, Mastronade JG, Wisa RA for the American Lung Association Asthma Clinical Research Centers. *Am J Respir Crit Care Med* 2009; 180: 809-816.
5. Gwilt CR, Donnelly LE, Rogers DF. The non-neuronal cholinergic system in the airways: an unappreciated regulatory role in pulmonary inflammation? *Pharmacol Ther* 2007; 115: 208-222.
6. Herve P, Denjean A, Jian R, Simonneau G, Duroux P. Intraesophageal perfusion of acid increases the bronchomotor response to methacholine and to isocapnic hyperventilation in asthmatic subjects. *Am Rev Respir Dis* 1986; 134: 986-989.
7. Hamamoto J, Kohrogi H, Kawano O, Iwagoe H, Fujii K, Hirata N, Ando M. Esophageal stimulation by hydrochloric acid causes neurogenic inflammation in the airways in guinea pigs. *J Appl Physiol* 1997; 82: 738-745.
8. Daoui S, D'Agostino B, Gallelli L, Alt XE, Rossi F, Advenier C. Tachykinins and airway microvascular leakage induced by HCl intra-oesophageal instillation. *Eur Respir J* 2002; 20: 268-273.
9. Cui YY, Zhu L, Wang H, Advenier C, Chen HZ, Devillier P. Muscarinic receptors involved in airway vascular leakage induced by experimental gastro-oesophageal reflux. *Life Sci* 2008; 82: 949-955.
10. Haag S, Matthiesen S, Juergens UR, Racké K. Muscarinic receptors mediate stimulation of collagen synthesis in human lung fibroblasts. *Eur Respir J* 2008; 32: 555-562.
11. McQueen DS, Donaldson K, Bond SM, McNeilly JD, Newman S, Barton NJ, Duffin R. Bilateral vagotomy or atropine pre-treatment reduces experimental diesel-soot induced lung inflammation. *Toxicol Appl Pharmacol* 2007; 219 :62-71.

12. 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: 1096-1102.
13. Bos IS, Gosens R, Zuidhof AB, Schaafsma D, Halayko AJ, Meurs H, Zaagsma J. Inhibition of allergen-induced airway remodelling by tiotropium and budesonide: a comparison. *Eur Respir J* 2007; 30: 653-661.
14. Kumar RK, Herbert C, Thomas PS, Wollin L, Beume R, Yang M, Webb DC, Foster PS. Inhibition of inflammation and remodeling by roflumilast and dexamethasone in murine chronic asthma. *J Pharmacol Exp Ther* 2003; 307 :349-55.
15. Lang IM, Haworth ST, Medda BK, Roerig DL, Forster HV, Shaker R. Airway responses to esophageal acidification. *Am J Physiol Regul Integr Comp Physiol* 2008; 294: R211-219.
16. Starosta V, Kitz R, Hartl D, Marcos V, Reinhardt D, Griese M. Bronchoalveolar pepsin, bile acids, oxidation, and inflammation in children with gastroesophageal reflux disease. *Chest* 2007; 132: 1557-1564.
17. Ravelli AM, Panarotto MB, Verdoni L, Consolati V, Bolognini S. Pulmonary aspiration shown by scintigraphy in gastroesophageal reflux-related respiratory disease. *Chest* 2006; 130:1520-1526.
18. Lopes FD, Alvarenga GS, Quiles R, Dorna MB, Vieira JE, Dolhnikoff M, MartinsMA. Pulmonary responses to tracheal or esophageal acidification in guinea pigs with airway inflammation. *J Appl Physiol* 2002; 93: 842-7.
19. Tuchman DN, Boyle JT, Pack AI, Schwartz J, Kokonos M, Spitzer AR, Cohen S. Comparison of airway responses following tracheal or esophageal acidification in the cat. *Gastroenterology* 87; 872-881, 1997.
20. Ishikawa T, Sekizawa SI, Sant'Ambrogio FB, Sant'Ambrogio G. Larynx vs. esophagus as reflexogenic sites for acid-induced bronchoconstriction in dogs. *J Appl Physiol* 1999; 86:1226-1230.
21. Coleridge HM, Coleridge JCG. Pulmonary reflexes: neural mechanisms of pulmonary defense. *Annu Rev Physiol* 56 ; 69-91, 1994.
22. Min MG, Song DJ, Miller M, Cho JY, McElwain S, Ferguson P, Broide DH. Coexposure to environmental tobacco smoke increases levels of allergen-induced airway remodeling in mice. *J Immunol* 2007; 178: 5321-5328.

23. Urso R, Segre G, Bianchi E, Bruni G, Dal Pra P, Fiaschi AI. Plasma kinetics of atropine and ipratropium in rats after different routes of administration evaluated by a radioreceptor assay. *Eur J Drug Metab Pharmacokinet* 1991; 3: 111-115.
24. Viola A, Luster AD. Chemokines and their receptors: drug targets in immunity and inflammation. *Annu Rev Pharmacol Toxicol* 2008; 48: 171-197.
25. Mendez MP, Morris SB, Wilcoxon S, Du M, Monroy YK, Remmer H, Murphy H, Christensen PJ, Paine R 3rd. Disparate mechanisms of sICAM-1 production in the peripheral lung: contrast between alveolar epithelial cells and pulmonary microvascular endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 2008; 294: L807-14.
26. Sacco O, Silvestri M, Sabatini F. IL-8 and airway neutrophilia in children with gastroesophageal reflux and asthma-like symptoms. *Respir Med* 2006; 100: 307-315.
27. Bühling F, Lieder N, Kühlmann UC, Waldburg N, Welte T. Tiotropium suppresses acetylcholine-induced release of chemotactic mediators in vitro. *Respir Med* 2007; 101: 2386-2394.
28. Profita M, Bonanno A, Siena L, Ferraro M, Montalbano AM, Pompeo F, Riccobono L, Pieper MP, Gjomarkaj M. Acetylcholine mediates the release of IL-8 in human bronchial epithelial cells by a NFkB/ERK-dependent mechanism. *Eur J Pharmacol* 2008; 582: 145-53.
29. Gosens R, Rieks D, Meurs H, Ninaber DK, Rabe KF, Nanninga J, Kolahian S, Halayko AJ, Hiemstra PS, Zuyderduyn S. Muscarinic M3 receptor stimulation increases cigarette smoke-induced IL-8 secretion by human airway smooth muscle cells. *Eur Respir J* 2009; in press.
30. Koyama S, Sato E, Nomura H, Kubo K, Nagai S, Izumi T. Type II pneumocytes release chemoattractant activity for monocytes constitutively. *Am J Physiol* 1997; 272: L830-7