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"Development of new therapeutic strategies in the control of asthma"

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1.Introduction

1.1 Asthma

Asthma is one of the most common airway diseases and there is a large geographical variation in asthma prevalence, severity, and mortality (Kudo M., 2013).

Asthma affects more than 300 million people worldwide, and it is the most common disease of childhood (Stern J. P. J., 2020). In 50% of adults and 80% of children afflicted with asthma prevails the allergic form of this disease, IgE-mediated (Del Giacco S. R., 2016). In the last few years, the incidence of asthma is increasing quickly: the incidence is doubled in Europe in the last decade, while in the United States the number of asthmatic patients is increased by 60% and the total number of deaths caused by asthma is doubled (Nunes C., 2017).

In recent decades, the prevalence of asthma is increased in low- and middle-income countries, and the risk of asthma is higher in urban compared to rural-areas (Rodriguez A., 2019). Thus, the variation in the asthma prevalence seems to be related to the urbanization process but it is not known how asthma and urbanization are associated (Rodriguez A., 2019). Multiple hypotheses have been proposed to explain the increased incidence of asthma, this one is combined with an increase in other allergies such as allergic rhinitis (Dharmage SC., 2019). In 1980, has been suggested that increased exposure to allergens, such as house dust mites, and the increased use of plush furniture may have contributed to increasing asthma prevalence (Baxi S. N., 2010). In 1989, the "hygiene hypothesis", proposed by Strachan, suggested that decreased exposure to unhygienic environments during childhood could lead to the increased prevalence of asthma incidence (Dharmage S. C., 2019).

It is known that asthma begins in childhood, but it can occur at any time during life. In childhood, asthma shows a higher incidence and severity in boys, but this trend reverses during puberty (Trivedi M., 2019).

1.1.1 Asthma risk factors

Both individual and environmental factors are involved in the development of asthma (Mukherjee A. B., 2011). The identified genes responsible are more than 100, while the environmental factor involved are allergens, air pollution, tobacco smoke, and respiratory infection (S.M., 2010).

Allergens, such as dust mites and pollen, can induce the sensitization process that is a major risk factor in the development of asthma (Baxi S. N., 2010).

Environmental factors

- *Air Pollution*

  Air pollution is associated with an increase in bronchial hyper-responsiveness (BHR) and asthma exacerbation. The main pollutants in the atmosphere of urban areas are ozone, nitrogen dioxide, and particulate matter (PM) (Guarnieri M., 2014).

  Ozone

  Ozone is generated by photochemical reactions involving the ultraviolet radiations. About 50% of inhaled ozone is absorbed in the nasal airways and the other 50% can affect the lower airways (D'Amato G., 2005). Inhaled ozone induces alterations in lung function and increases epithelial cell permeability, and this facilitates the transition of allergens and toxins in the airways (Mumby S., 2019). This process leads to an increase in pro-inflammatory mediators' level (IL-1, IL-6, IL-8, TNF, etc.) (D'Amato G., 2005).

Nitrogen dioxide (NO_2)

NO_2 is a toxic compound with a strong smell, and it is a precursor of ozone and is responsible for photochemical smog. NO_2 induces irritation of the airway system and alterations of lung functions (D'Amato G., 2005) (Dharmage S. C., 2019) .

Particulate Matter

PM is the main component of urban air pollution, and it is a mixture of solid and liquid components of different sizes and compositions. PM are categorized according to size: PM10 (less than 10µm of diameter) and PM 2.5 (less than 2.5 µm). PM has long been suspected to trigger asthma development and acute exacerbation. PM2.5 can reach human lung parenchyma while PM10 reaches proximal airways and can be eliminated by mucociliary clearance (Colarusso C., 2019).

- *Viral Respiratory Infection*

  Viral infections associated with wheezing episodes include respiratory syncytial virus (RSV), human rhinovirus (HRV), coronavirus, and many others. The association between asthma and viral respiratory infection is present both in children and adults. These viral infections have many similarities with asthma, for instance wheezing, rapid breathing, and airway inflammation. Many respiratory viruses are associated with the development and exacerbation of asthma (Busse W. W., 2010).

- *Tobacco*

  Tobacco smoke is an important risk factor for airway hyperreactivity (Silverman E.K., 1996). Morbidity and mortality are both increased in people who are cigarette smokers compared to non-smokers. Tobacco from cigars, cigarettes, and pipe induces airway inflammation and hyperreactivity (Tamimi A., 2012). Many studies show a correlation between parental smoking and the development of asthma in children (Carlsen K.H., 2005) (Pattenden S., 2006). Moreover, it was observed that maternal smoking during pregnancy increases the risk of asthma in childhood (Carlsen K.H., 2005). Like exposure in utero, post-natal tobacco

exposure causes pulmonary inflammation with an increase in the T helper type 2 (Th2) pathway (Stern J., 2020).

Individual Factors

- *Genetic predisposition*

  Many genes have been associated with the development of asthma. Some of these genes are *ADAM 33, DPP10, PHF11, NPSR1, HLA-G, CYFIP2, IRAK3, and OPN3* (Stern J., 2020).

  ADAM33 is a member of the "A Disintegrin and Metalloprotease" (ADAM) family proteins, this gene has been localized on chromosome 20p13 and it is associated with bronchial hyperreactivity and accelerated decline in lung function (Sun F.J., 2017). Some studies show that *ADAM33* polymorphisms influence lung function and epithelial-mesenchymal dysfunction (Li HF., 2019) (Farjadian S, 2018). This gene contains over 55 single nucleotide polymorphisms (SNPs), some of these are associated with a decline in forced expiratory volume in the first second (FEV1) (Sun F.J., 2017). Another gene associated with an increase in asthma development is *Interleukin-4 (IL-4)* (Kousha A., 2020), this gene is located on chromosome 5 at the position q31 and it is responsible for *IL-4* production, a cytokine secreted by Th2 cells. Polymorphisms of this gene are associated with alteration in IgE levels and hyperreactivity (Imani D., 2020).

- *Obesity*

  Recent studies have shown the link between asthma and obesity (Peters U., 2018). This link could be explained by the hypothesis that obese patient shows a reduction in respiratory function because of an alteration of elastic properties of the thoracic wall (Dixon A.E., 2018). Moreover, both conditions are characterized by systemic inflammation. Indeed, the main hormones involved in the regulation of systemic inflammation in obese patients, adiponectin, and leptin play an important role also in airway inflammation (Zheng H, 2018). For instance, leptin shows a pro-inflammatory effect both in obese and asthmatic patients, leading to

an increase in airway reactivity and in the expression of cytokines T helper type 2 (Zheng H., 2018).

- *Gender*

  The incidence of asthma is influenced by sex, especially by hormonal changes (Yung J.A., 2018). In childhood, boys show an increase in asthma incidence compared to girls while after puberty, in adult age, females show a higher severity and prevalence of asthma compared to men. This sex-switch in asthma prevalence suggests that sex hormones may play a role in the development of asthma (Yung J.A., 2018).

1.1.2 Pathophysiology of asthma

The National Asthma and Education and Prevention Program Expert Panel Report 3 (NAEPPR3) defines asthma as "a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role: mast cells, eosinophils, neutrophils (especially in sudden onset, fatal exacerbations, occupational asthma, and patients who smoke), T lymphocytes, macrophages, and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of coughing (particularly at night or early in the morning) wheezing, breathlessness, and chest tightness. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment" (Busse W.W., 2007).

Asthma is divided into two types:

- Allergic Asthma (AA)
- Non- allergic Asthma (NA)

The first one is characterized by BHR, eosinophilic inflammation, increased IgE levels, increased mucus production, and airway remodelling. NA is not an allergic condition and it is characterized by lower levels of inflammatory cells (Fang L., 2020).

An important molecular mechanism of asthma is the type 2 inflammation, which occurs in many but not all patients (Bacerra-Diaz M., 2017).The inhaled allergens, such as house dust mite, animal dander, fungi, and pollen, stimulate the T-helper type 2 cell proliferation, Th2 cytokines, IL-4, IL-5, and IL-13 production and release (Lambrecht

B.N., The Cytokines of asthma, 2019). Anyway, many studies have shown that asthma is not a simple Th2 disease, because of the involvement of many other pro-inflammatory mediators as Th17 and Th9 cells. These inflammatory cells contribute to airway inflammation and enhance airway contraction (Fahy J. V., 2015).

Airway Inflammation

Asthma is characterized by a Th2 immune response, with $CD4^+$ T helper type 2 differentiation cells and the production of type 2 cytokines, such as IL-4 and IL-13 (Lloyd C.M., 2010). The release of these cytokines causes airway inflammation and mucus production (Lambrecht B.N., 2019). To induce the activation of the adaptative immune system, cells of the innate immune system need to acquire peptides from antigens and translate this signal from exposure to allergens into allergen-specific T cell response (Boonpiyathad T., 2019).

Airway epithelial cells induce the release of IL-33, this one activates dendritic cells (DCs) and Th2 pro-inflammatory mediators *(Fig.1.1)* (Lambrecht B.N., 2019). DCs play a role both in the initiation and maintenance of allergic airway inflammation and asthma, indeed these can capture inhaled allergens and present them to CD4 T-cells on MHCII (Morianos I., 2020). Moreover, epithelial cells induce the production of cytokine, which can increase DCs function and promote the Th2 cells production (Byoungjae K., 2018). Th2 cells produce cytokines IL-4, IL-5, and IL-13, which are responsible for airway inflammation and IgE production (Fahy J. V., 2015). Th2 cytokines also induce the expression of adhesion molecules (VCAM-1 and ICAM-1) for eosinophil extravasation (Johansson MW., 2017). IL-4 promotes the B-cell isotype switching, eotaxin production, and BHR. Also, IL-13 shows most of these actions and it has a direct effect on BHR, upregulating RhoA protein. Another cytokine that plays a key role in airway inflammation is IL-5, which is produced by Th2-lymphocytes, mast cells, and eosinophils and it is involved in eosinophil growth, maturation, and activation. IL-5 is also involved in the airway remodelling and BHR (Lambrecht B.N., 2019). Another important pro-inflammatory mediator that is involved in airway reactivity and inflammation is Sphingosine-1-phosphate (S1P). Indeed, high level of this lipid mediator was found in the sputum of asthma patients (Roviezzo F., 2014)

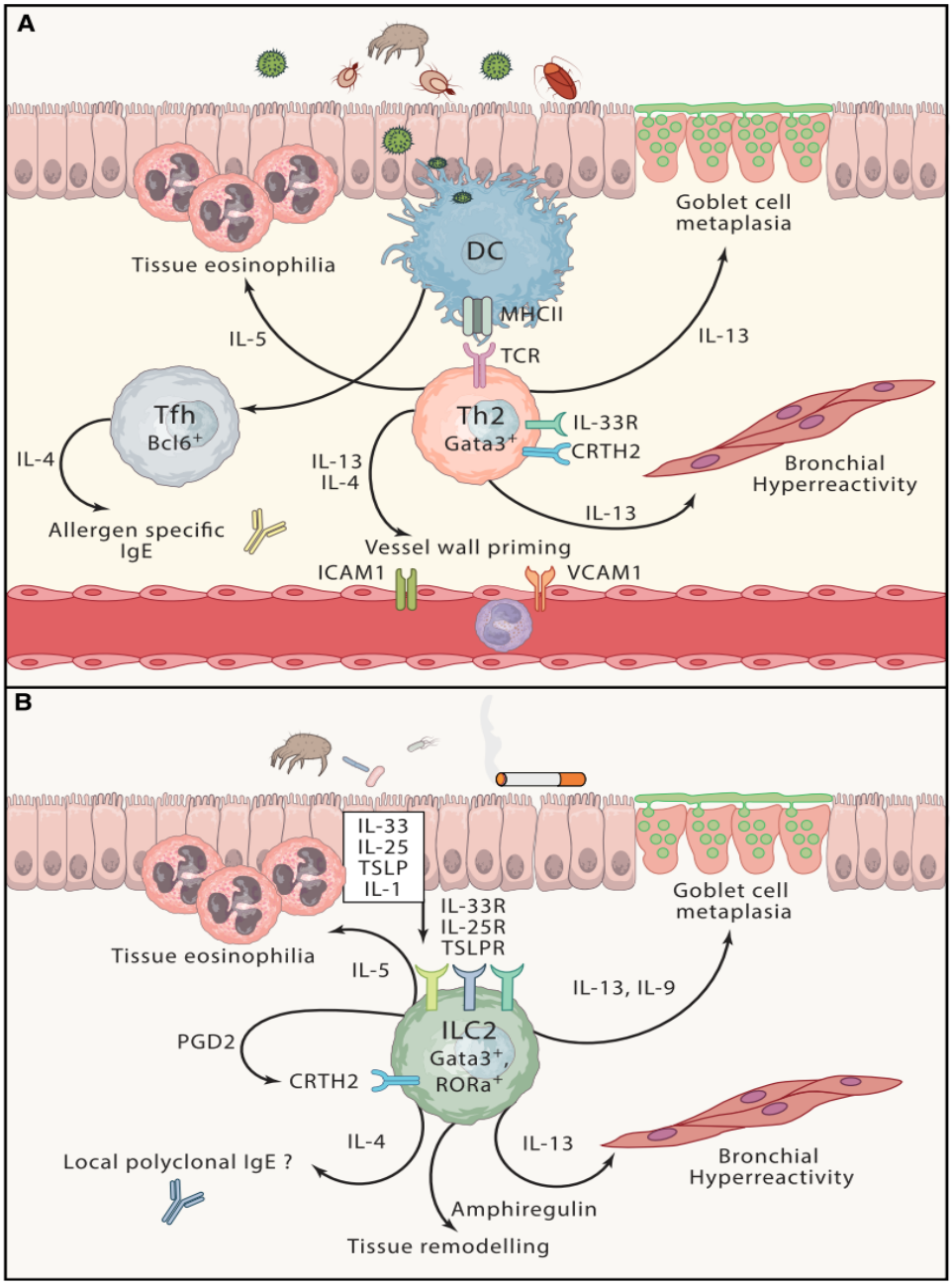


Figure 1. 1 Th2 proinflammatory pathway

A. Allergen, such as house dust mite, is processed by dendritic cells. These cells can activate Th2 cells through the interaction with TCR. IL-4 and IL-13, cytokines secreted by Th2 cells, switch B cells to produce IgE, while IL-5 promotes the development and survival of eosinophils. Moreover, IL-13 is involved in bronchial hyperreactivity and Globet cell metaplasia. **B.** Other stimuli, such as cigarette smoke, induce the release of IL-33R and IL-25R by epithelial cells that activate Type 2 innate lymphoid cells (ILC2). These cells express the transcription

factors GATA3 and RORa. ILC2s are very similar to Th2 cells, the only difference is the hight secretion of PGD2 and IL-9, this one induces Globet cell metaplasia (Lambrecht B.N., 2019)

Mucus Production

Mucus hypersecretion is a hallmark of chronic airway disease. The composition of mucus includes lymphocytes, eosinophils, neutrophils, and mucin produced by goblet cells. The activation of mucociliary cells leads to bronchoconstrictor mediators release (histamine and prostaglandin D2) and this induces the activation of other pro-inflammatory mediators as leukotrienes (LTC4, LTD4, LTE4) (Kesimer M., 2017).

Bronchial hyper-responsiveness (BHR)

Airway inflammation and structural changes of the airway induce BHR. Airway smooth muscle (ASM) contraction is due to an intracellular increase of calcium ions (Ca^{2+}) (Sommer B., 2017). The main source of Ca^{2+} in ASM is the intracellular sarcoplasmic reticulum (SR). After the activation of the G_q-protein coupled receptor (GPCR), phospholipase C (PLC) leads to the formation of two second messenger, the inositol triphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 can spread to the cytosol where it binds its specific receptor on SR (IP_3 R), inducing an increase in intracellular Ca^{2+}levels from SR (Sun MY., 2017). This increase of intracellular Ca^{2+} levels, caused by IP_3, induces the interaction with calmodulin (CaM) and the complex Ca^{2+}- CaM leads to the activation of the enzymatic domain of myosin light chain kinase (MLCK), resulting in ASM contraction (Sun MY., 2017). Many pro-inflammatory mediators are involved in the increase of Ca^{2+}. For instance, interleukin 1β (IL-1 β), IL-13, tumor necrosis factor α (TNF- α), and other Th2-derived cytokines can increase Ca^{2+} responses induced by contractile agents (Kudo M, 2013).

Airway remodelling

Another typical feature of asthma is the airway remodelling. This process refers to structural changes, that include subepithelial fibrosis, increased smooth muscle mass, and

epithelial alterations (Fehrenbach H., 2017). All these processes lead to the thickening of the airway wall, BHR, and airway edema formation (Bergeron C, 2010). An important contribution in airway remodelling is due to an increased ASM mass, this could be due to airway infiltration of myofibroblast and pro-inflammatory mediators (Lloyd CM, 2017). Various growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β, have a stimulatory effect on ASM migration. Indeed both (TGF)-β and PDGF induce ASM migration by modifying matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) balance (Ito I, 2009). Also, lipid mediators, including leukotrienes, promote ASM cell migration. PGD_2 mediates chemotactic effects through the binding with DP2 receptor on ASM cells (Domingo C, 2018). The function of various leukocytes is regulated by chemokines, which are important chemotactic molecules, for instance, eotaxin that can induce ASM migration in a dose-dependent manner (Kudo M, 2013).

1.1.3 Inflammatory mediators

As described above, asthma is characterized by airway inflammation, mucus production, airway hyperreactivity and remodelling. Different immune cells are involved in all these processes, like dendritic cells, T cells, B cells, basophils, eosinophils, and mast cells. The presence of these inflammatory cells causes the release of cytokines which are involved in the alteration of repair response, inflammatory and remodelling process. Other mediators involved in airway inflammation and hyperreactivity are leukotrienes and prostanoids.

Cytokines

Cytokines play a key role in the inflammatory process of asthma. These mediators can be classified in three stages according to their biologic activity (Lin SC., 2019). The Group 1 involves the cytokines for the epithelial environment stage, the Group 2 includes the cytokines for the Th2 polarization while the Group 3 cytokines for the tissue damage stage (Lin SC., 2019). The cytokines of the Group 1 are activated during the sensitization process, when there is the first contact with the allergen. This causes the damage of the airway tissue and the release of cytokines and chemokines from the respiratory epithelial cells. One of these cytokines is the IL-6, which is important for the activation of DCs, which can trap the allergen inducing the activation of Th2 immune system. The

respiratory epithelial cells also release the IL-33, which stimulates the cytokines of Group 2, like IL-13 and 5. The DCs could be consider the link between the stage 1 and 2, indeed, DCs induce the differentiation of naïve T cells in Th2 cells inducing the release of IgE from B cells. Besides Th2, naïve T cells can also differentiate in Th9 or Th17 cells releasing different cytokines, like IL-4, IL-5, IL-9, or IL-17. All these cytokines are involved in the activation of eosinophils and mast cells. The last group of cytokines involved the TGF-β and IL-10 which leads the development of regulatory cells and the tissue repair (Lin SC., 2019).

Leukotrienes

Leukotrienes (LTs) are potent lipid mediators synthesized from AA through the 5-lipoxygenase pathway (5-LO) *(Fig1.2)* (Hedi H., 2004) (Jo-Watanabe A., 2019). In the first step of this pathway, AA is oxygenated by 5-LO, this one is first activated by FLAP (5-lipoxygenase-activating protein), to give 5-hydroperoxy eicosapentaenoic (5-HpETE). The second step of this pathway is the dehydration of 5-HpETE, and this leads to the formation of LTA_4. This leukotriene is unstable and can be converted to LTC_4, through the conjugation with glutathione, or it can be converted to LTB_4, through the conjugation with water. These reactions are mediated by LTC_4 synthase (LTC_4S) and LTA_4 hydrolase (LTA_4 H), respectively, and the synthesis is localized near the nuclear membrane. Then LTB_4 and LTC_4 are transported by carrier proteins out of the cell. In extracellular space, LTC_4 is cleaved to LTD_4, and LTE_4. LTC_4, LTD_4 and LTE_4 contain a cysteine residue and, for this reason, they are called cysteinyl-LTs (cys-LTs) (Hedi H., 2004). The leukotrienes synthetic pathway is regulated by the amount of free AA and by the intracellular localization of 5-LO. In resting leukocytes, 5-LO is in the cytoplasm or the nucleoplasm, then it moves to the outer or inner nuclear membrane after FLAP activation (Pace S., 2017). LTs actions are mediated by specific seven-transmembrane domain receptors coupled to the G protein (GPCRs). B leukotrienes receptor 1 (BLT_1) is located on leukocytes and is a high-affinity receptor, while B leukotrienes 2 (BLT_2) is a lower-affinity receptor, and it is present on mononuclear cells, granulocytes, and bone marrow-derived dendritic cells (Golden MP, 2007). Cys-LTs act on cys-LT_1 or cys-LT_2 receptors, the expression of the first one is influenced by Th2 cytokines and it mediates activities as bronchoconstriction, mucus production, and edema in airways, while cys-LT_2 receptors mediate inflammation, fibrosis, and vascular permeability (Golden MP, 2007).

After the activation, cys-LT_1 and cys-LT_2 receptors interact with G_q protein, increasing intracellular calcium, or with G_i protein, decreasing intracellular cyclic AMP (cAMP) (Golden MP, 2007). Both pathways induce downstream kinase cascade, and this leads to various biological responses, such as ASM contraction and amplification of inflammatory responses mediated by Th2 cells (Golden M.P., 2007).

LTs play an important role in asthma pathogenesis, they serve to promote and to persist the airway inflammation (Watanabe AJ, 2019). Moreover, LTs are the most potent endogenous bronchoconstrictor known (Montuschi P. , 2010). Cys-LTs are increased in the airways of asthmatic patients and they are associated with changes in airway hyperresponsiveness (AHR) after allergen exposure (Matsuda M, 2018). Cys-LTs show many other functions on the airway: promote leukocyte migration, activate the eosinophils, basophils, mast cells, and the T lymphocytes (Dholia N, 2018). Asthmatic patients appear to be more sensitive to the inhalation challenge with LTE_4, and this leukotriene induces an increased number of the eosinophils in the lung (Laidlaw T. M., 2012). Many studies suggest that cys-LTs play an important role in prolonging eosinophils survival and enhancing the production of eosinophils from the bone marrow in association with granulocyte macrophage colony-stimulating factor (GM-CSF) (Thompson-Souza GA, 2017). Several studies have reported that LTD_4 can induce the cell proliferation in a dose-dependent manner, through the overexpression of the cys-LT_1 receptor (Dholia N, 2018). Also, LTB_4 has biological actions in airways, it has no direct bronchoconstriction effect, but it contributes to airway narrowing, edema formation, and increasing mucus production. Indeed, LTB_4 is a potent chemoattractant for the neutrophils and eosinophils, and it can enhance both the adhesion and migration of cells through the endothelium. Both cys-LTs and LTB_4 are enhanced in bronchoalveolar lavage (BAL) fluid of asthmatics after allergen challenge (Wenzel S.E., 2003).

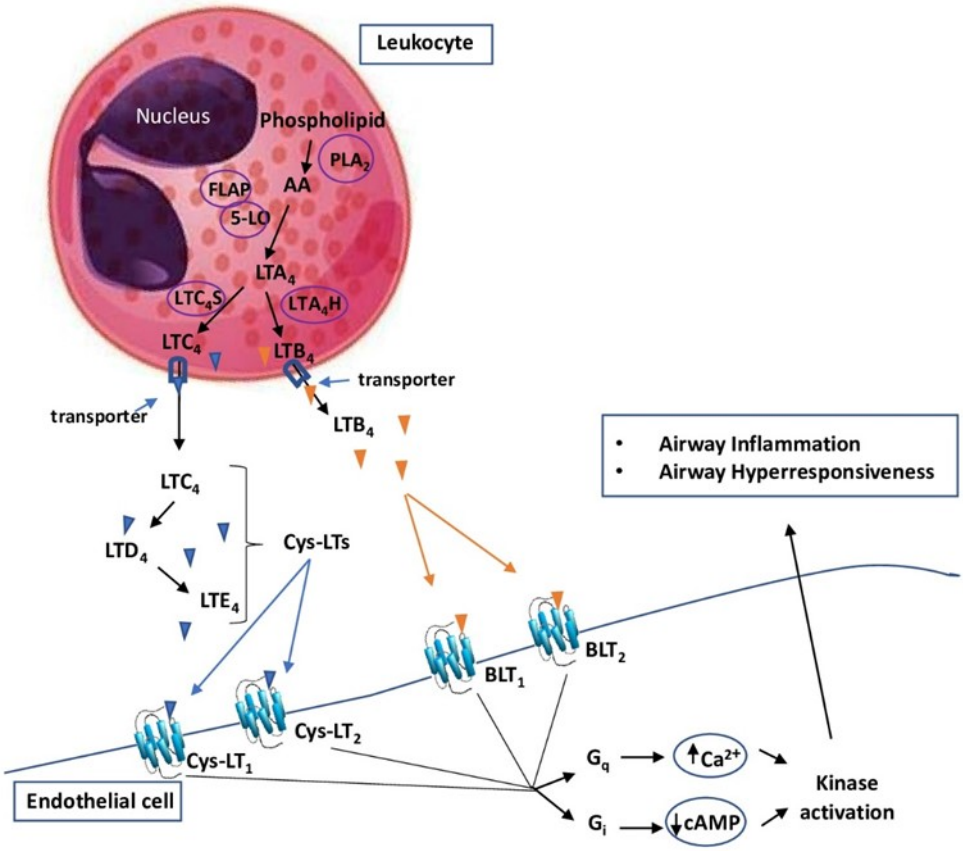


Figure 1. 2 Pathway of Leukotrienes

Prostaglandin

Prostaglandins are lipid mediators synthesized from Arachidonic acid (AA) through Cyclooxygenase (COX) enzyme (Kunio Yui1, 2015). There are two types of COX enzymes: COX-1 and COX-2. COX-1 is constitutively expressed in most tissues while COX-2 is activated by different inflammatory stimuli (Jane A. M, 2020).

Both enzymes catalyse a reaction that leads to the generation of prostaglandin PGG_2 starting from AA. Then this prostaglandin is converted into PGH_2, which is the precursors of many mediators, for instance, PGD_2, PGE_2, PGF_2, PGI_2, and thromboxane (TXA_2) (Peebles Jr. R.S, 2019). PGD_2 is one of the prostaglandins mainly involved in airway inflammation (Domingo C., 2018). This is released by mast-cells following allergic and non-allergic stimuli, but it can also be released from dendritic cells and Th2 cells. Once released, PGD_2 can exert different activity by binding with two types of receptors: the prostaglandin receptor D_2 1 (DP_1) and prostaglandin receptor D_2 2 (DP_2) (Ricciotti E.,

2011). DP_1 mediates non-inflammatory activity, as vasodilation and inhibition of cell apoptosis and migration, while DP_2 mediates many pro-inflammatory effects, for instance, the activation and the migration of different Th2-mediators (Domingo C., 2018).This suggests that DP_2 plays an important role in the pathogenesis of asthma-inducing an inflammatory process. The binding of PGD_2 with the receptor DP_2 located on Th2 cells induces the upregulation of different cytokines involved in airway inflammation, such as IL-13, IL-5, and IL-4 (Domingo C., 2018). Moreover, the secretion of these cytokines is also increased following the interaction of PGD_2 with DP_2 located on type 2 innate lymphoid cells (ILC2) and eosinophils surface. The DP_2 receptor could be a target in asthma treatment, indeed different DP_2 antagonists are under clinical investigation to reduce airway inflammation (Ricciotti E., 2011).

Sphingosine-1-phosphate

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid involved in many biological processes, such as the stimulation of cell proliferation and cell survival by acting both as an intracellular second messenger or extracellular mediators by the interaction with specific receptors (Yatomia Y., 2001). S1P is synthesized from Ceramide, which is the central core of sphingolipids. Ceramide is formed from the condensation of serine and palmitoyl-CoA by the action of palmitoyl-CoA transferase. Ceramide can be phosphorylated to Ceramide-1 Phosphate (CIP) by the action of ceramide kinase (CERK). Moreover, it can be converted to sphingosine and this one can be phosphorylated by sphingosine kinases (SphKs) to generate S1P (Yatomia Y., 2001). There are two types of SphKs, SphK1, and SphK2. The first one is expressed in many tissues, for instance, brain, heart, spleen, and lung while SphK2 is expressed in the kidney and liver. SphK1 activation is mediated by many effects, for instance, the stimulation by TNF-α, platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF), through the phosphorylation on serine 225 (Tsai H.C., 2016). This phosphorylation induces the translocation of the enzyme from the cytosol to the membrane. Extracellular actions of S1P are mediated by the interaction with GPCRs, $S1P_1$- $S1P_5$, while intracellular actions are independent of these receptors (Struba G. M., 2010). S1P shows many intracellular actions independently from its receptors, such as the activation of the transcriptional nuclear factor (NF-kB) involved in the prevention of apoptosis (Chalfant C.E., 2005). In literature is reported the involvement of S1P in asthma pathogenesis by the interaction

with $S1P_2$ and $S1P_3$ that are mainly expressed in the lung (Roviezzo F, 2007). Indeed, elevated levels of S1P are found in the airways of asthma patients. In a murine model of airway inflammation, it has been observed that S1P subcutaneous administration, at the dose of 1ng, induces an increase in acetylcholine response in vitro. S1P promotes the Th2 inflammation, increasing IL-4 and IL-13 levels in the lung (Roviezzo F., 2009). Moreover, subcutaneous administration of S1P induces an alteration in pulmonary parenchyma morphology, an increase in mucus production, and lung inflammation (Roviezzo F., 2009). The increase in ASM hyperreactivity reaches a maximum effect after 21 days by the first S1P administration. This hyperreactivity is flanked by mucus production and lung inflammation. Moreover, S1P administration induces a significant increase of CD23, which is an important regulatory receptor for the IgE production, and this demonstrates that the IgE/CD23 signalling is a key role in the S1P effect in the airways (Roviezzo F., 2014). Indeed, anti-CD23 treatment in mice significantly reduces the hyperreactivity and lung inflammation caused by S1P administration (Roviezzo F., 2014).

1.2 Bronchial hyperresponsiveness induced by non-allergic stimuli.

Bronchial hyperresponsiveness is a condition that develops from the presence of different factors, for instance, the reduction of the airway diameters, the increase of the airway-wall thickness, and the increase of smooth muscle mass and reactivity (Bujarsky S., 2015).

Bronchial hyperresponsiveness is a hallmark of asthma but it can also be present in other respiratory diseases, like a chronic obstructive pulmonary disease (COPD), and maybe induced also by non-allergic stimuli (Borak J., 2016).

COPD is a heterogeneous disease characterized by emphysema and chronic bronchitis (Bujarsky S., 2015). These conditions cause lung damages and increase mucus production. About 60% of patients with COPD show a prevalence of bronchial hyperresponsiveness, being a marker for a more severe form of the disease (Bujarsky S., 2015).

The main symptoms of COPD are cough, shortness of breath, and physical activity limitation. Smoking is the main risk factor of COPD, but other risk factors can also be the genetics and air pollution. Cigarette-smoke exposure induces an inflammatory process in

the lungs, with the increase of neutrophils, macrophages, and T cells (Bartal, M., 2005). The activation of macrophages by cigarette-smoke induces the release of inflammatory mediators, for instance, interleukin 8 and leukotriene B4, inducing emphysema and destruction of epithelial cells (Bartal, M., 2005). Thus, while asthma is mostly characterized by eosinophilia and Th2 inflammation, COPD inflammation is driven by neutrophils and CD8 lymphocytes.

Cigarette-smoke exposure affects airway epithelium and smooth muscle cells inducing airway inflammation and bronchial hyperresponsiveness (Chiba Y, 2005). Many proinflammatory cells and molecules were found in the smoker's lungs. Neutrophils are the first cells that appear in the site of inflammation following cigarette-smoke exposure releasing many pro-inflammatory mediators involved in the development of emphysema (Bagdonas E, 2015).

1.3 Asthma treatment

Hyperreactivity is the focus of asthmatic response. The goal of bronchospasm treatment is to reduce the calibre of the airway, restoring the resting state (Cazzola M, 2019). For this reason, the main pharmacological treatment used involved the administration of bronchodilator drugs. There are different types of bronchodilators: β_2-agonists, muscarinic receptor antagonist, and xanthine. Besides bronchodilators, another therapeutic strategy involves the use of glucocorticoids to reduce airway inflammation (Cazzola M, 2019).

To understand the activities of these bronchodilator drugs it is necessary to know the ASM physiology. Airway tone is controlled by the vagus nerve and parasympathetic nerve that produces a baseline tone of ASM and it is involved in airway bronchoconstriction induced by irritants such as histamine. Acetylcholine (ACh) is the main neurotransmitter of the parasympathetic system and it induces bronchoconstriction through muscarinic receptors (mAChRs). These receptors are divided into five subtypes ($M_1 – M_5$) and they are coupled to the G protein (GPCRs) (Pelaia G, 2008). M_1, M_3 and M_5 are coupled to G_q, while M_2 and M_4 are coupled to $G_{i/o}$. ASM contraction induced by ACh is mainly mediated by the M_3 receptor. Instead, the ASM relaxation is mediated by β receptors, that are divided into three type β_1, β_2, and β_3. The β_2-receptor is expressed in ASM, epithelium, vascular smooth muscle, and many pro-inflammatory mediators. The

stimulation of β_2-receptor, which is coupled to G_s, induces an increase in cyclic-AMP inducing bronchial relaxation (Webb R. C., 2003).

β_2 – adrenergic receptor

The β_2-adrenergic receptor (β_2-AR) is a member of G-protein coupled receptor (GPCRs). All GPCRs are plasma membrane proteins with seven hydrophobic transmembrane domains (TM1-7) connected by three extracellular and three intracellular loops (Amrani Y., 2017). These receptors are characterized by an intracellular carboxyl terminus and an extracellular amino terminus. The third intracellular loop, between TM5-6, with a carboxyl terminus domain, constitutes the binding site for G protein, that can bind the nucleotides guanosine triphosphate (GTP) and guanosine diphosphate (GDP). The G proteins that bind GPCRs are heterotrimeric, they have three different subunits (alpha, beta, and gamma). Alpha subunit can bind both GTP and GDP, it depends on G protein activity (Billington C.K., 2017). GPCRs can be divided into five families:1) Rhodopsin family; 2) the adhesion family; 3) the taste family; 4) the glutamate family and 5) the secretin family. Receptors of all these families share a common structure. The most variable structure among these families is intracellular carboxyl terminus, extracellular amino terminus, and the transmembrane domains TM5 and TM6 (Kobilka BK, 2007). These receptors are targets of the catecholamine neurotransmitters, such as epinephrine and norepinephrine, that stimulate the sympathetic nervous system. The β_2-AR activation is due to N-glycosylation on amino acids in 6^{th}, 15^{th}, and 187^{th} position, this event allows to train ligand-receptor complex. This complex leads to the activation of G_s protein, leading to the release of α subunit that stimulates AC and the release of $\beta\gamma$ subunits, which can transduce the signal (Kobilka BK, 2007). This event induces an increase of the intracellular levels of the second-messenger cAMP, and the activation of PKA, which phosphorylates myosin light chain kinase and reduces Ca^{2+} intracellular levels inducing a reduction in ASM contraction (Webb R. C., 2003). PKA also leads to the phosphorylation of the transcription factor cAMP response element (CRE) binding protein (CREB) at serine 133, inducing an alteration of the gene transcription. The β_2-agonists can also induce the ASM relaxation through the activation of K^+ channels. The intracellular level of cAMP is controlled by phosphodiesterase (PDE). Another cAMP effector is EPAC, that is involved in the different cellular process reducing Rho pathways. All these events induce the ASM relaxation (Billington C.K., 2017).

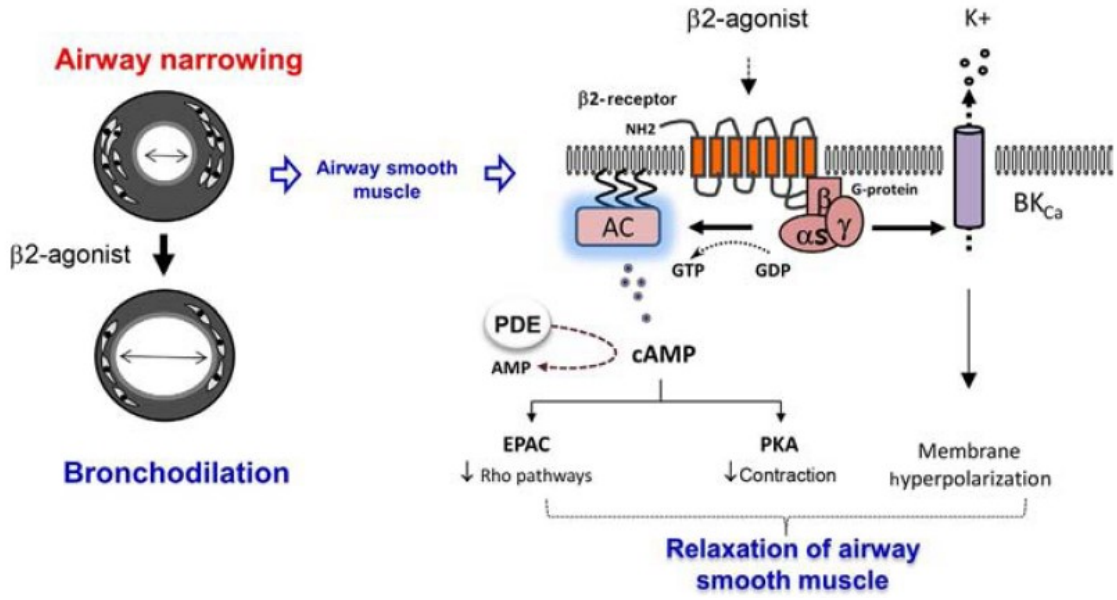


Figure 1. 3 β2 receptor activation

(Amrani Y., 2017)

The β_2-AR exists in an equilibrium between an inactive (R) and active (R^*) form, in resting condition this equilibrium hangs towards the inactive form. The active form in resting condition is due to allosteric changes in β_2-AR. The ligand-binding induces an alteration of equilibrium with a prevalence of the active form. The G_s protein is not the only protein which is coupled to β_2-AR. Indeed, there is another pathway of this receptor activated by the G_i protein which leads to the activation of p38 mitogen-activated protein kinase (MAPK) (Liapakis G, 2017).

Bronchodilators

The β_2-agonists are the main drugs used in the treatment of asthma because of their direct action on airway smooth muscle cells (Billington C.K., 2017). These drugs are characterized by a benzene ring with a side chain of two carbon atoms and an amine group. The first problem in the use of β-agonists was the non-selective actions of these drugs (Cazzola M., 2012). In the beginning, the β-agonists, such as epinephrine, show

many side effects due to the interaction with α_1 and β_1 receptors, inducing hypertension and tachycardia. Another β-agonist with non-selective action is isoproterenol, which induces tachycardia through the interaction with the β_1 receptor. Then Sir David Jack discovered β_2-agonist albuterol (called salbutamol in Europe), introducing selective β_2-agonists (Page C, 2012).

β-agonists are grouped into three subtypes: Short-acting β-agonists (SABAs), Long-acting β-agonists (LABAs), and Ultra-Long acting β-agonists (Ultra-LABAs). The first β-agonists show a short duration of the activity, between 4 or 6 hours, but then β-agonists with a long duration of the activity have been developed, about 12 hours (Billington C.K., 2017).

SABAs show bronchoprotective effects in minutes *(Tab.1)*. The first drugs of this group, like isoprenaline, have a very short action, about 2 hours, but other drugs, like albuterol, are active for 4 or 6 hours (Billington C.K., 2017). Albuterol shows a greater selectivity between β_1 and β_2 -receptors, inducing bronchial relaxation within 15 minutes of inhalation. SABAs are recommended only at the necessity, during a bronchoconstriction attack. They can be used in conjunction with LABAs, inhaled corticosteroids, or long-acting muscarinic agonists (Billington C.K., 2017). Anyway, the new guidelines GINA 2019 no longer recommend the use of SABA alone in adults and teenagers (Hogan AD., 2020)

LABAs, such as salmeterol and formoterol, show a bronchodilator effect about for 12 hours *(Tab.1)*. These drugs are used as a maintained therapy alone or in combination with glucocorticoids in adult patients. These drugs are generally added as a second-line treatment in asthma (Billington C.K., 2017). Currently, LABA are used in the treatment of patients with mild asthma at the necessity (Step 2) and also in treatment of patients with severe asthma (Step 4-5) in association with high doses of corticosteroids (Hogan AD., 2020).

Ultra-LABAs show a rapid action prolonged for about 24 hours. The ultra LABA indacaterol was approved by European Medicines Agency (EMA) in 2009. This drug is available by inhalation as a dry powder and it is indicated for maintenance therapy also in combination with long-acting muscarinic agonist (LABA) (Cazzola M., 2012).

All these β_2-agonists bind β_2-receptors that are coupled to G_s-protein. The interaction between ligand and the receptors induces the activation of Adenylyl Cyclase (AC) which

can mediate the hydrolysis of the adenosine triphosphate (ATP) in cAMP and the activation of Protein Kinase A (PKA) (Amrani Y, 2017). Many proteins are phosphorylated by PKA, like cAMP response-element binding-protein (CREB), a transcription factor involved in the regulation of gene transcription. The relaxant pathway includes the inhibition of the myosin light chain kinase activity that induces the alteration of intracellular calcium levels. Another pathway is involved in ASM relaxation, this is the inhibition of the Rho pathway through the activation of cAMP-mediated exchange protein (Epac). Anyway, the main pathway involved in ASM relaxation is a cAMP-dependent one (Amrani Y., 2017)

Even if the β_2-agonists are the first line-treatment of asthma, these drugs have a long story of side effects. A lack of sensitivity of the β_2-agonists induces side effects like tachycardia, binding β_1–receptor, while prolonged stimulation of β_2-receptors induces a reduction of its relaxation activity and this process is known as desensitization of β_2-receptor (Hsu E., 2020).

Glucocorticoids

Glucocorticoids are used in asthma treatment in step 2, 3 and 4 in combination with SABAs or LABAs to reduce airway inflammation (Papi A, 2020). The beneficial effects of these drugs are due to their anti-inflammatory activity, through the inhibition of pro-inflammatory cytokines (Ora J, 2020). For this reason, these drugs are currently the most efficacious drugs used in both in the control and treatment of asthma (Lin SC., 2019). Glucocorticoids move through the plasma membrane and bind their receptors (GR) in the cytoplasm. In resting conditions, these receptors are bind to chaperon molecules but after the binding with the glucocorticoids this complex ligand-receptor translocate to the nucleus and it can modify the pro-inflammatory gene expression by binding specific DNA sequences (Ora J, 2020). Inhaled glucocorticoids have anti-inflammatory effects in airways, reducing mast cells, macrophages, and other pro-inflammatory cells (Barnes P. J., 2010). Currently, low doses of corticosteroids at the necessity are used in the first treatment in patients with intermittent asthma (STEP 1). Low doses of corticosteroids in association with LABA are used in moderate asthma (STEP 2-3) while in severe asthma (STEP 4-5) the doses of corticosteroids are increased (Hogan AD., 2020). Despite the

introduction of biological drugs in the treatment of severe asthma (Step 5), the use of glucocorticoids as odd-on therapy has still an important role (Ora J., 2020).

Inhaled glucocorticoids show many local side effects, such as dysphonia and oropharyngeal candidiasis, but also systemic side effects, such as the inhibition of hormones as corticotropin, cortisol, and androgens, inducing the delay of puberty. For all these reasons, the prolonged use of glucocorticoids is not recommended, and the treatment with these drugs should last up to three months (Hogan AD., 2020) .

Anti-leukotrienes

Anti-leukotrienes (*a*nti-LTs) have been approved for asthma treatment (García-Menaya JM., 2019).There are two strategies to inhibit leukotriene's function. The first one is the inhibition of 5-LO activity (Zileuton), preventing LTs synthesis, while the second one is to bind LTs receptors (montelukast, zafirlukast, and pranlukast), inhibiting LTs activity. Both classes of drugs can effectively reduce the LTs activity. Montelukast is the most commonly anti-LTs prescribed in European countries (Sessa M., 2018). Cys-LTs receptor antagonists can improve the lung function reducing the pro-inflammatory mediators' activity (Tamada T., 2017). Anyway, glucocorticoids are more effective than anti-LTs in the reduction of allergen-induced airway hyperreactivity (Sessa M., 2018). Cys-LTs antagonist, like montelukast, are currently used in the treatment of asthma, and in literature is reported that these drugs can also modify the PGE_2 pathway (Trinh HKT., 2019). Pranlukast shows a lower potency in the inhibition of cys-LTs compared to montelukast, but the potency to suppress PGE_2 is the same (Trinh HKT., 2019). According to the GINA guidelines 2019, antileukotrienes are used as an alternative treatment to low dose of corticosteroids in patients with mild asthma (step 2). Moreover, the antileukotrienes can also be associated to corticosteroids (ICS) as an alternative therapy to low-dose ICS plus LABA in patient with moderate asthma (step 3) (Trinh HKT., 2019).

Sessa et al. demonstrated that there is a relationship between gender and Montelukast (Sessa M., 2018). It was already known that testosterone could modulate the leukotriene biosynthesis affecting the subcellular localization of 5-LOX (Pergola C, 2008), and this explain the increased effectiveness of montelukast in female (Sessa M., 2018).

Antileukotrienes show many side effects, the most common are headaches, abdominal pain, cough, and dyspepsia. In children can also be observed diarrhea, nausea, and otitis (Erdem SB., 2015).

Long-acting muscarinic antagonist (LAMA)

Cholinergic parasympathetic nerves play an important role in bronchial smooth muscle contraction and mucus secretion through the release of acetylcholine (ACh) that bind M-receptors. The inhibition of M_1 and M_3 anticholinergic receptors induce bronchorelaxation, while the inhibition of M_2 has the opposite action. Indeed, the muscarinic antagonist Ipratropium show many side effects blocking all three receptors whereas tiotropium, that is selective on M_1 and M_3, has a more potent bronchodilator effect compared to Ipratropium. Thus, tiotropium is the only LAMA approved for the treatment of asthma (Dusser D., 2019). Tiotropium is approved as add-on therapy in 6 years old patients or older. Moreover, in patients with severe asthma (Step 5) tiotropium (5µg/day) is suggested as a controller therapy before biological drugs (GINA 2019).

Biologic drugs

Biologic therapy is considered for patients with uncontrolled severe asthma. These drugs target pro-inflammatory mediators which play an important role in the pathogenesis of asthma, like IL-4, IL-13, and IL-5. The first monoclonal antibody (mAb) approved for the treatment of asthma is Omalizumab. This mAb binds the IgE high affinity-receptor (FcεRI) on mast cells and basophils, and down-regulates the expression of IgE receptor reducing the airway inflammation (McGregor MC., 2019) (Henriksen DP., 2020). Omalizumab is subcutaneously administrated every 2 or 4 weeks in patients with severe asthma which show a total serum IgE level between 30 and 1,300 IU/ml (McGregor MC., 2019). Other mAb approved in the treatment of severe asthma are mepolizumab and reslizumab, both inhibit IL-5 preventing the binding of IL-5 to its receptor. Currently, mepolizumab is approved for patients with 12 years or older while reslizumab is for patients aged 18 years or older (McGregor MC., 2019). Compared to mAb seen above, dupilumab can inhibit two pathway, IL-4, and IL-13, reducing mucus production and airway inflammation (Castro M., 2018).

Class of Drugs	Drug	Mechanism of action	Effects	Side effects
SABA	Salbutamol Fenoterol Terbutalin	bind on β2-adrenergic receptor	airway relaxation ↓ dyspnea	Tremor Tachycardia
LABA	Formoterol Salmeterol Indacaterol	bind on β2-adrenergic receptor	airway relaxation ↓ dyspnea	Tachycardia
SAMA	Ipratropium bromide Oxitropium bromide	blockage of Ach on cholinergic receptors	airway relaxation ↓ dyspnea	dry mouth prostatic disorders
LAMA	Tiotropium bromide Glycopyrronium Aclidinium	Interaction on M3 receptors	airway relaxation	vision blurred dry mouth constipation
Glucocorticoids	Budesonide Fluticasone	action on glucocorticoids receptors	↓ lung inflammation	dysphonia osteoporosis hyperlipidemia
Anti-LTs	Montelukast Zileuton Pranlukast	Cys-LTs receptor-antagonist 5-LOX inhibitor LTs receptor-antagonist	↓ lung inflammation airway relaxation	abdominal pain diarrhea headache
PDE-inhibitor	 Roflumilast 	increase the intracellular levels of cAMP	↓ lung inflammation	diarrhea weight loss
Biological drugs	Omalizumab Mepolizumab Reslizumab	bind with IgE block IL-5 block IL-5	↓ mucus production ↓ eosinophils ↓ eosinophils	nausea headache

Table 1 Drugs used in asthma treatment.

1.4 Limits of treatment with bronchodilators

It is well known that the use of bronchodilators is the most effective treatment in patients with airway hyperresponsiveness, both for asthma and BPCO or bronchial hyperresponsiveness induced by different stimuli (Page C., 2014). Unfortunately, a prolonged use of these drugs induces a decrease in receptor responsiveness, this process is known as desensitization (Shore S.A., 2003). Desensitization is the main limit of the asthma treatment. There are two types of desensitization, homologous and heterologous. The first one is due to a prolonged stimulation of β_2-agonists whereas the second type of desensitization is due to the interaction of many pro-inflammatory mediators with β_2-AR. The decrease of β_2-AR responsiveness is a process mediated by three pathways: 1)

receptor uncoupling from G-protein; 2) internalization; 3) down-regulation (Shore S.A., 2003).

Homologous desensitization is due to the combination of PKA and G-protein receptor kinase (GRK) activation. There are seven members of the GRK family but only GRK-2 and GRK-3 are involved in β_2-AR desensitization. PKA can directly phosphorylate β_2-AR and the bind with the agonist induces the translocation of GRKs from the cytosol to the membrane to phosphorylate β_2-AR. The phosphorylated receptor is a target for β-arrestin, and this interaction uncouples the receptor from the G protein, stopping the β_2-AR signalling. There are four members of the arrestin family, but only β-arrestin-1 and β-arrestin-2 are involved in receptor desensitization. After binding to β-arrestin, clathrin and β_2-adaptin are both involved in the internalization of the β_2-AR into endosomes, then the receptor can be recycled or sent to the lysosome for degradation. The downregulation of β_2-AR is a long-term event, that is due to transcriptional and translational events. This downregulation induces a decrease in the number of β_2-AR on the membrane surface, resulting in a reduction of the receptor response (Shore S.A., 2003).

Many pro-inflammatory mediators can induce the heterologous desensitization (Guo M, 2005). Increased levels of cytokines, such as IL-5, IL-13, and TNF-α, can reduce the cAMP production by ASM, resulting in a decrease in relaxation. IL-1 β and IL-17A are pro-inflammatory mediators that, through the activation of ERK and p38, can increase the COX-2 level, inducing an enhancement of PGE_2 release. PGE_2, through the EP2 binding, leads to the activation of PKA, that phosphorylates β_2-AR and induces the receptor internalization (Figure 4B) (Rumzhum N. N., 2016). Another mediator that induces the heterologous desensitization through the increase of COX-2 and PGE_2 is Sphingosine-1-phosphate (S1P) (Rumzhum N.N., 2016).

Also, Th2 cytokines IL-13 can induce the heterologous desensitization by interacting with its receptor IL-13Rα1 (Rovati G.E., 2006). This interaction induces the activation of Janus kinases (Jak's) and, through the Ras pathways, IL-13 leads to the ERK activation and β_2-AR desensitization (Figure 4A). Cys-LTs, which play a key role in asthma pathogenesis, induce the heterologous desensitization through the activation of PKC (Rovati G.E., 2006).

Moreover, another important limit in the treatment with bronchodilators is the age-related modification in lung mechanisms. The increasing of age induces a reduction in β_2-AR affinity resulting in a lower sensitivity to bronchodilator effects (Cazzola M, 2019).

1.5 Alternative therapeutic strategies to bronchodilators

An alternative therapeutic strategy in the treatment of bronchial hyperreactivity should consider:

1. β_2-receptor desensitization process due to prolonged use of β_2- agonist

2. Sex differences in airway function and inflammation

Strategies to overcome β_2-receptor desensitization

Since many proinflammatory mediators are involved in the β_2-receptor desensitization, a therapeutic strategy that could overcome this limit could be the association of β_2 agonist with other drugs. As described above, cysteinyl-LTs are involved in β_2 -receptor desensitization through the activation of Protein Kinase C (PKC) (Rovati G.E., 2006). *Rovati et al.* demonstrated that the exogenous LTD_4 reduces the isoproterenol-induced relaxation through the decrease in cAMP levels, but this effect is prevented by cys-LT_1R antagonist montelukast. Moreover, montelukast can also revert the reduced response to salbutamol caused by allergen exposure (Rovati G.E., 2006). Thus, an association of β_2-agonists with an anti-LTs drug could be an alternative therapeutic strategy to overcome β_2-receptor desensitization.

Sex differences in airway diseases

As for many allergic diseases, the incidence and prevalence of airway diseases differ between men and women, and during life (Raghavan D., 2016). Both the Incidence and severity of asthma are higher in males than in females during adolescence (Fuseini H., 2017). Anyway, puberty induces a switch, indeed adult female shows an increase in incidence and severity of this disease. This sex-switch in the incidence of asthma suggests the involvement of sex hormones. Male hormones, such as androgens, that are produced

in higher concentration in the post-pubertal phase, could have a protective role and could suppress the immune cell reactivity, this may explain the less incidence in man after puberty. Androgens, especially testosterone, can reduce the symptoms of asthma through genomic and non-genomic actions (Fuseini H., 2017). After binding to the androgen receptor (AR), androgens can modify pro-inflammatory mediators' expression and activity. Testosterone and its derivates, such as dehydroepiandrosterone (DHEA), decrease Th2 inflammation and IL-17 protein expression, showing a protective role through AR signalling (Laffont S, 2017). Many other actions of these hormones are independent of AR. Some studies have shown that androgens can block L-type voltage-dependent Ca^{2+} channels (L-VDCC), IP^3 receptors, and promote the prostaglandin E_2 (PDE2) synthesis in airway smooth muscle (Montaño L.M., 2020), inducing relaxation.

In contrast to male hormones, the female ones show pro-inflammatory effects in airway diseases (Keselman A, 2015). Many studies have shown that the lung eosinophils infiltrate and Th2 cells are enhanced in the airways of females compared to males, and this causes an increase in BHR (Keselman A, 2015). Anyway, the effects of estrogens seem to be complex and contradictory because of the expression of the estrogen receptors (ER_α- ER_β) in different tissues (Laffont S., 2017). Asthma symptoms in women change during life, during different phases of life such as pregnancy and menopause (Keselman A, 2015). Some studies have shown that women with a severe asthma during pregnancy show an increase in exacerbations compared to moderate asthma patients, while patients with mild asthma have an alleviation of symptoms during pregnancy but symptoms seem to return to normal after 3 months post-partum (Keselman A, 2015). Menopause could have a protective role in asthmatic women because of the decrease of estrogens level. However, some studies report that this phase is associated with increased respiratory symptoms, but others report a protective role of this phase. The European Community Respiratory Health Survey I (ECHRS I) found an increased risk of asthma during menopause in women who takes hormone replacement therapy (HRT), but women who do not take HRT show any differences in asthma risk between pre-menopause and post-menopause phase (Real FG, 2006). This suggests that female hormones have a great impact on asthma incidence and severities.

Based on that reported above, males and females show important differences in asthma features suggesting that a targeted therapy should consider both age and sex and in particular hormonal fluctuations.

Chapter 2

Chapter 2

2.Aim of the study

This study aims to investigate the mechanisms involved in the development of asthma features such as bronchial hyperreactivity and lung inflammation.

In particular the study focused on three main points:

1. the role of sphingolipids, such as sphingosine-1-phosphate, in airway.
2. the contribute of sex hormones that are widely involved both in the incidence and in the pathogenesis of airway disease.
3. The analysis of the constrictor and dilator pathways in airways and pulmonary arteries in parallel.

Chapter 3

Chapter 3

3.Materials and Methods

3.1 Animal Studies

3.1.1 Mice (University of Naples Federico II)

Female and male BALB/c, CD1, mice and C57BL/6 (8-9 weeks old) were purchased from Charles River (Calco, Italy) and were housed in the animal care facility of the Department of Pharmacy of the University of Naples Federico II, Italy. The animals were housed in a controlled environment (temperature 21± 2°C and humidity 60±10%) and provided with controlled rodent chow and water. Mice were allowed to acclimate for about four days before the experiments and they were subjected to a cycle of 12h light and 12h dark. All the experiments were conducted during the light phase (Rossi A, 2019) and the experimental procedures were approved by the Italian Ministry and all studies are reported in compliance with the ARRIVE guidelines (Kilkenny C, 2010).

3.1.2 Mice (Imperial College London)

Animal experiments in this study were performed in strict accordance with the regulations of Animals (Scientific Procedures) Act of 1986 and the EU Directive 2010/63/EU under a designated UK Home Office Project License 70/8422. C57BL/6 mice were bred under contract by Charles River Laboratories and all procedures were performed at National Heart and Lung Institute, Imperial College London and were approved by the Imperial College Animal Welfare and Ethical Review Panel. Male and female mice with an age range of 8-10 weeks were housed in individually ventilated cages with a 12-hour day/night cycle. Unlimited standard chow and water were provided.

Tissue collection

Weight and other details (experiment date, cage ID, mouse line, sex, number, genotype, ear mark, study ID) were recorded in all experiments. Mice were initially euthanised by CO2 narcosis for 5 mins. Mouse blood from inferior vena cava was collected immediately post-mortem in hirudin which has a blood anticoagulant property. Tissues including aorta, carotid arteries, pulmonary artery, and bronchi were collected for myography experiments (see below).

3.1.3 Ovalbumin-sensitization protocol

Adult female and male BALB/c and C57/BL6 mice (8-9 weeks old, males 24-27g and female 18-22g, Charles River, Calco, Italy) were subcutaneously (s.c.) treated with 0.4mL (100µg) of Ovalbumin (OVA) complexed with alum (13 mg mL^{1}) for the OVA group, or saline for the vehicle group, at the day 0 and 7. This experimental protocol, without OVA-challenge, increases the plasma IgE levels and induces a significant increase in the bronchial hyperreactivity and airway inflammation (Roviezzo F. B. A., 2015). All mice were sacrificed at a selected time point by an overdose of enflurane. Lungs, bronchi, and blood were collected to perform molecular and functional studies (Rossi A, 2019) (Cerqua I, 2020).

3.1.4 Cigarette-smoke experimental protocol

C57BL/6J mice were exposed to room air (control group) and cigarette-smoke (CS; Marlboro Red, 12mg of tar and 0.9mg of nicotine, 3 cigarettes per day up to 11 months) in especially designed macrolon cages (Tecniplast, Buggugiate, Italy) (De Cunto G, 2019). These cages were equipped with a filter cover having a hole (10-15mm) to flow the air out of the cage. Mice were exposed to smoke produced by the burning of cigarettes and introduced in the chamber through a mechanical ventilator (7025 Rodent Ventilator, Ugo Basile, Biological Research Instrument, Comerio, Italy). The initial flow rate was 33ml/min and then it was increased to expose mice to three cigarettes within 90 minutes (Cavarra E., 2001). Thus, in this chronic study mice were exposed to three cigarettes per day, five days a week up to 11 months, then mice were sacrificed at a selected time points (9,10 and 11 months) (De Cunto G, 2019).

3.1.5 Anti-LTs drugs experimental protocol

Male and female BALB/c mice were intraperitoneally (i.p.) treated with Montelukast (MS, 1mg/kg), 30 minutes before each OVA administration and 3 times per week until day 21. Similarly, another group of mice was i.p. treated with Zileuton (ZIL, 35mg/kg) and MK886 (MK, 0.1mg/kg). The vehicle group was treated with 0.5 mL DMSO 2% 30 minutes before each OVA injection and at 14 days. All these mice were then anesthetized with enflurane and sacrificed at selected time points to collect lungs, bronchi, and blood. (Rossi A, 2019).

3.1.6 5α-dihydrotestosterone (5α-DHT) experimental protocol

Female BALB/c mice were i.p. treated with 5α-DHT at the dose of 0.5mg/kg, and ZIL (35mg/kg), or vehicle (0.5mL DMSO 2%) 30 minutes before each OVA administration. The selected dose of 5α-DHT 0.5mg/kg had already demonstrated the capacity to inhibit LTs production (Pace S., 2017). Mice were anesthetized with enflurane and sacrificed at 14 or 21 days and lungs, bronchi, and blood were collected (Cerqua I., 2020).

3.2 Analysis of bronchial reactivity

3.2.1 Isolated organ baths

Bronchial tissues were rapidly dissected and cleaned from fat and connective tissue. Rings of 1-2mm were cut and mounted in 2.5mL organ baths containing Krebs solution (Mm: 118NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25$NaHCO_3$, and 11 glucose), at 37 °C, oxygenated (95% O_2 and 5% CO_2), and connected to an isometric force transducer (type 7006, Ugo Basile, Comerio, Italy) associated to a Powerlab 800 (AD Instruments). Rings were initially stretched until a resting tension of 0.5 g and allowed to equilibrate for at least 30 min. In each experiment bronchial rings were previously challenged with carbachol (10^{-6} M) until the response was reproducible. Once a reproducible response was achieved, bronchial reactivity was assessed performing a cumulative concentration-response curve to carbachol (10^{-6} M – $3x10^{-9}$ M). Results were expressed in dyne per mg tissue. Then the carbachol was washed out and the tissue was allowed to equilibrate for

10 minutes. On the other hand, a cumulative concentration-response curve to salbutamol (10^{-5} M – $3x10^{-8}$ M) on a stable tone of carbachol (1 µM) was performed to evaluate bronchial relaxation (Cerqua I., 2020).

S1P pathway on bronchial reactivity: experimental protocol

Bronchi harvested from C57BL/6J were subjected to a cumulative concentration-response curve to carbachol (10nM-30µM) or S1P (10nM-30µM). In other tissues bronchial reactivity was evaluated in presence of TY52156 ($S1P_2$-antagonist; 10µM, 15 minutes; Tocris Bioscience, UK), JTE-013 ($S1P_3$-antagonist; 10µM, 15 minutes), or SK-I (Sph-K inhibitor; 10 µM, 30 minutes; Tocris, Bristol, UK) (De Cunto G, 2019)

Prolonged stimulation of β_2-receptor in vitro

Bronchi from male and female CD1 and BALB/c mice were exposed to prolonged stimulation of β_2-receptor by both short-acting and long-acting β_2-agonist, salbutamol (0.01 mM-0.03µM) and formoterol (10 pM-0.03 µM), respectively. Other bronchial tissues were pretreated with Montelukast (1µM, 10 minutes on basal condition before salbutamol curve; 0.1µM for 5 minutes on carbachol tone before formoterol curve).

L-NAME and db-cAMP on bronchial reactivity

Bronchi from male and female BALB/c mice were preincubated with L-NAME (100µM,15 minutes), db-cAMP (100µM, 5 minutes), or IBMX (0.1 µM, 10 minutes) on carbachol tone, and then the concentration-response curve of salbutamol was performed.

3.2.2 Wire myograph

Wire myograph is an *in vitro* technique to analyse functional responses of isolated tissues. The system used in this thesis was the Multi Myograph System- model 610M to measure tissue contraction.

Mouse bronchioles, pulmonary artery, carotid and aorta were dissected immediately post-mortem into phosphate-buffered saline (PBS), cleaned of fat then cut into equal size

pieces (2mm) before preparing for the myograph. Bronchioles were isolated from the right lung while the pulmonary artery from the left lung. Bronchi and vessels were mounted on myographs (DMT610M, Mulvany-Halpern, Danish Myotechnology, Denmark) in warm physiological salt solution (PSS) (120mM NaCl, 4.7mM KCl, 1.2mM MgSO4, 1.2mM KH2PO4, 25mM NaHCO3, 0.03mM EDTA, 5.5mM D-glucose; Sigma, UK) bubbled with oxygen (95%) and carbon dioxide (5%) and allowed to equilibrate for 30 minutes. ~8mN resting tension was applied to each vessel, then the vessels were allowed to stabilise for 30 mins. To ensure there had been no vessel damage before applying any chemicals, two times high potassium solution in the PSS (KPSS) responses were measured, with 3 mins of KPSS treatment and 3 mins of washing in between. KPSS responses for all vessels were over 1mN indicating no vessel damage, thus the bronchi and vessels were included in further experiments. Tissues were then washed with warmed gassed PSS. When the tissue reached the baseline, both the bronchial and vascular reactivity were analysed by the administration of concentration-response curves of Serotonin (5-HT) (1µM to 0.01M) (Sigma, UK), Carbachol (1µM to 0.01M) (Sigma, UK) and U46619 (10 µM to 1Mm) (Sigma, UK) dissolved in PSS. Each concentration of curve was given every 2 minutes. At the end of each concentration-response curve, tissues were washed with PSS until a new stable baseline was achieved and before the next concentration-response curve was added. For some groups, PSS with 1µM of Diclofenac (COX inhibitor), 1µM of Montelukast (cysteinil-leukotrienes receptor antagonist) or 1mM of L-NAME (NO synthase inhibitor) for 15 minutes was applied before the agonist were added.

3.3 Isolated perfused mouse lung preparation

To analyse the lung function, lungs were perfused in a non-recirculating fashion through the pulmonary artery at a constant flow of 1ml/min inducing a pulmonary artery pressure of 2-3 cmH_2O using an isolated and perfused mouse lung model. The medium used was RPMI-1640 lacking phenol red (37°C). Ventilation of the lungs was induced by a negative pressure (-3 and -9 cmH_2O) with 90 breaths per minute and hyperinflation (-20 cmH_2O) was performed every 5 minutes. A different pressure transduces (Validyne DP 45-24, Instrumentation Devices, Como, Italy) was used to measure the artificial thorax chamber pressure and airflow velocity was evaluated with a pneumotachograph tube connected to

a differential pressure transducer (Validyne DP 45-15, Instrumentation Devices, Como, Italy). The arterial pressure was evaluated with the cannula inserted in the pulmonary artery and connected to a pressure transducer (Isotec Health dyne, Irvine, USA). All data were analysed with Pulmodyn software (Hugo Sachs Elektronik, March Hugstetten, Germany) using the following formula: $P= V \cdot C^{-1} + R_L \cdot dV \cdot dt^{-1}$, (P=chamber pressure; C=pulmonary compliance; V= tidal volume; R_L= airway resistance). Then, the airway resistance was corrected for the resistance of the pneumotachometer. To evaluate the baseline state, lungs were perfused and ventilated for 45 min without any treatment, and then lungs were challenged with carbachol or S1P administered as a bolus. In another set of experiments, lungs were perfused with S1P inhibitors before the carbachol challenge (De Cunto G, 2019).

3.4 Plasma IgE levels

Blood was collected by intracardiac puncture using citrate (3.8%) as an anticoagulant. Then the plasma was obtained by centrifugation at 12.000 rpm at 4°C for 10 minutes and immediately frozen at –80°C. Total IgE levels were measured with an enzyme-linked immunosorbent assay (ELISA) using matched antibody pairs (BD Biosciences Pharmingen San Jose, CA) (Rossi A, 2019).

3.5 Cytokines, LTs, and PGD_2 levels

Lungs, harvested from male and female BALB/c mice, were isolated and homogenized in PBS (Sigma Aldrich, Milan, Italy). The homogenate was centrifuged (4° C, 6000 × *g*, 10 min). The levels of LTC_4 (Cayman Chemical, BertinPharma, Montigny Le Bretonneux, France) and cytokines (IL-4 and IL-13) (Affymetrix eBioscience San Diego, CA) were measured with commercially available ELISA kits according to the manufacturer's instructions. The levels of LT and IL-4 are expressed as pg mg^{-1} of tissue (Rossi A, 2019)

For PGD_2 levels analysis, lung homogenates were diluted with PBS (20µL of homogenates up to 1mL of PBS). Successively, PGB_1 (200ng) was added as an internal standard, and samples were frozen at -2°C for 2h. Samples were centrifuged at 800 x g, 15 minutes at 4°C, then the supernatants were diluted with PBS and HCL (1N), and PGs

were extracted by solid-phase extraction with liquid chromatography-tandem mass spectrometry (Rossi A, 2019).

3.6 S1P lung measurement

Lungs harvested from control and mice exposed to cigarette-smoke up to 11 months were mechanically homogenised with buffer (20-mM Tris-HCl, pH 7.4; 20% glycerol; 1mM β-mercaptoethanol; 1-mM EDTA; 1-Mm Na-orthovanadate; 15-mM NaF; 1-mM PMSF; protease inhibitor cocktail; 0.5-mM deoxypyridoxine; 40-mM β-glycerophosphate). S1P levels in lung homogenate were measured using a commercially available ELISA kit (Echelon, Tebu-bio, Magenta, Italy). Data were reported ad picomoles of S1P per micrograms of total protein (De Cunto G, 2019).

3.7 Lung tissue histology

Left lung lobes harvested from male and female mice (control group, OVA-group, and OVA+DHT group) were fixed in paraffin and then 7-µm cryosections were cut to perform Hematoxylin and eosin (H&E) staining. Periodic acid-Schiff (PAS; Sigma-Aldrich, Milan, Italy) staining was performed according to the manufacturer's instruction to detect glycoprotein (Roviezzo F., 2014). PAS-staining cryosections were analysed for the presence of goblet cells (PAS+cells) by imaging software (Zeiss, Germany). PAS+ cryosection were indicated with the following scores: 0:<5%; 1: 5-25%; 2: 25-50%; 3: 50-75%; 4: <75% positive staining/total lung area (Rossi A, 2019).

3.8 Morphology and morphometry (lungs exposed to cigarette smoke)

Lungs from control and smoking mice (up to 9, 10, and 11 months) were processed for histology and lung slides were used for morphology and morphometry analysis. All the lungs were intratracheally fixed with buffered formalin (5%) at a constant pressure of 20 cmH_2O for 24 hours. Lung slides were stained with H&E and peribronchial fibrosis was evaluated in paraffin-embedded lung slices after Masson's trichrome staining (De Cunto G, 2019).

3.9 Immunohistochemistry analysis

Left lung lobes harvested from mice (control group, OVA group, and OVA+DHT group) were fixed in paraffin and 7-µm cryosections were cut. Anti-mast cell tryptase (1:250, AbCam, UK) or isotype control anti-rabbit IgG were used. The diamino-benzidinic acid system was used to detect the complexes which were visualized through a Zeiss microscope (Germany) (Rossi A, 2019) (Cerqua I., 2020).

The lung tissue sections were harvested from mice (control group and smoking mice up to 9,10 and 11 months) were stained for α-smooth muscle action (α-SMA), sphingosine 1-phosphate receptor 2 ($S1P_2$), sphingosine 1-phosphate receptor 3 ($S1P_3$), sphingosine kinase 1 (Sph-K_1) and sphingosine kinase 2 (Sph-K_2). To recover the antigen, the sections were heated in a microwave for 20 minutes in 0.001M pH 6.0 citrate buffer and then allowed to cool at room temperature. Successively, the sections were incubated with 3% BSA for 30 minutes at room temperature to block the non-specific antibody binding. The sections were incubated overnight at 4°C with rabbit Ab to mouse Sph-K_1 (1:200 diluted; Bioss Cat# bs-2652R, RRID: AB_10856265), rabbit Ab to mouse Sph-K_2 (1:70 diluted; Abcam Cat# ab37977, RRID: AB_778046), rabbit Ab to mouse $S1P_3$ (1:100 diluted; Bioss Antibody, Woburn, US), and rabbit Ab to mouse $S1P_2$ (1:150 diluted; Biorbyt Cat# orb5558, RRID: AB_10938778). Then the slides were washed with TBST and incubated with the sheep anti-rabbit IgG (1:200) for 30 min. at room temperature followed by the incubation with peroxidase-antiperoxidase complex. Colour development was performed with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Burlingame, CA). The α-SMA protein detection was evaluated on the section by using mouse monoclonal α-SMA Ab (1:400 dilution; Sigma, St. Louis, USA) and MOM immunodetection kit (Vector Laboratories, Burlingame, USA) and DAB as a substrate. The MOM kit is designed to localize the mouse primary monoclonal and polyclonal antibodies on mouse tissues through a novel blocking agent. As negative controls, all primary antibodies were replaced by non-immunized-specific serum (De Cunto G, 2019).

3.10 Western Blot Analysis

Bronchial tissues harvested from male and female BALB/c mice were homogenized in RIPA buffer containing protease inhibitor cocktail and sodium orthovanadate and subjected to mechanical lysis with FAST-PREP instrument at 6 m/s of speed. The tissue was then centrifugated at 12.000 rpm, 4°C for 15 minutes. The protein concentration was

determined by using the Bradford assay (Bio-Rad Laboratories, Milano, Italy) and 42 µg of total proteins were separated by electrophoresis (10% SDS-PAGE). Then proteins were transferred to a PVDF membrane (Schleicher&Schuell, Munich, Germany). The nitrocellulose membrane was incubated in non-fat milk 3% in phosphate buffer saline (PBS) plus 0.1% (v/v) of tween 20 to block the non-specific site. Then, one membrane was incubated with Anti-ADBR2 rabbit polyclonal antibody (1:500 dilution, TA323758 ORIGENE) while the other one with PDE4a (1:1000 dilution; Cat no: 16226-1-AP Proteintech). After overnight incubation, all the membranes were washed out with non-fat milk 3% in PBS Tween, and then the anti-rabbit antibody was incubated for 2 hours (1:3000 dilution) at room temperature. After removing the secondary antibody, the membrane was washed out first with non-fat milk 3% in PBS Tween and then with PBS Tween only. Then, to confirm the equal amount of protein loading, membranes were incubated overnight with GAPDH monoclonal antibody (1:5000 dilution; Sigma Aldrich) followed by anti-rabbit antibody incubation for 2 hours at room temperature. Signal detection was obtained with ECL using ChemiDoc Imaging System (Bio-Rad, Italy). Densitometry analysis was performed with Image Lab Software (Bio-Rad, Milan, Italy) (Caiazzo E., 2020).

3.11 Flow cytometry analysis

Lungs were harvested from mice (control group, OVA-group, and OVA+DHT group) and digested with 1U · mL^{-1} collagenase (Sigma Aldrich, Milan, Italy). Cell suspensions were passed through 70 µm cell strainers, and red blood cells were lysed (Roviezzo F., 2014) to perform flow cytometry analysis 10^6 cells were incubated with antibodies: CD11c-FITC, cKit-PeCy5.5, IgE-APC, CD3PeCy5.5, CD4-FITC, IL-4-APC (eBioscience, San Diego, CA, USA). Isotype control was used (Cerqua I, 2020).

3.12 Cell culture

RBL-2H3 cells, purchased from ATCC (American Type Culture Collection, passage 5, population doubling time 38-40 h Manassas, VA, USA) were cultured in Minimum Essential Medium (MEM-sigma-Aldrich) supplemented with 10% of fetal bovine serum (FBS- Sigma-Aldrich) and 1% streptomycin/penicillin (Sigma-Aldrich) at 37°C in a humidified 5% CO_2 atmosphere (Cerqua I, 2020).

3.13 β-hexosaminidase release assay

Reached 80% of confluence, RBL-2H3 cells were seeded into a 96-well plate at 37°C at the density of 72 x 10^3 cells for well and incubated for 24h at 37°C in a humidified 5% CO_2 atmosphere to induce cell attachment. Cells were incubated overnight with anti-dinotrophenylated-human serum albumin (DNP)-IgE treatment (0.50 µg/mL). Successively, the Minimum Essential Medium was replaced with phenol-free DMEM plus 1mg/mL BSA. Vehicle (DMSO 0.1%), cromolyn (1mM) and DHT (0.01nM and 0.1nM) were incubated for 5 minutes at 37°C. Then, cells were treated with DNP (10ng/mL) to induce degranulation and with Triton-X-100 (0.1%) to induce β-hexosaminidase release. MEM was replaced with phenol-free DMEM supplemented with 1 mg/mL BSA and then, cells were treated with vehicle (DMSO 0.1%), cromolyn (1mM) and DHT (0.01nM and 0.1nM) for 5 minutes at 37°C. Successively, cells were treated with Ca^{+2} ionophore (A23187, 1µM) and Triton-X-100 (0.1%). After 1 hour of degranulation stimuli, 50µL of supernatants from each well were collected and added to 50µL of p-nitrophenyl-N-acetyl- β-D- glucosaminide 1.4mM in citrate buffer 0.2 M, pH 4.2. The enzymatic reaction was detected after 1h by adding 100µL /well of Trizma solution 0.3M, pH 9.4. The β-hexosaminidase release was measured at 405 nM in a multiplate reader (EnSpire, PerkinElmer, Milan, Italy) (Cerqua I, 2020).

3.14 Statistical analysis

The results are expressed as mean ± S.E.M. of the mean of n observations, n= number of experiments performed on different days, or the number of animals. Statistical analysis used was a two-tailed Student *t*-test for single comparison or by one-way or two-way ANOVA using GraphPad InStat (Graphpad Software Inc., San Diego, CA) followed by a Bonferroni post-hoc test for multiple comparisons, respectively. *Post-hoc* tests were run only when F achieved $P < 0.05$ and there was no significant variance in the homogeneity. A P value < 0.05 was used to define statistically significant differences between mean values (Rossi A, 2019).

3.15 Materials

The compound used were provided by the following suppliers: OVA by Sigma Aldrich (Milan, Italy), MS, and Zil by Sequoia Research Products (United Kingdom), MK by Cayman Chemical (BertinPharma, Montignery Le Bretonneux, France), S1P by Enzo Life Science (Rome, Italy), TY52156, JTE-013 and SK-I by Tocris Bioscience (United Kingdom), DHT by Sigma Aldrich (Milan, Italy).

Chapter 4

Chapter 4

4.Role of S1P in bronchial hyperreactivity induced by cigarette-smoke

4.1 Rationale

As we have already seen in Chapter 1, S1P is a lipid mediator widely involved in the airway function and pulmonary inflammation. *Roviezzo et al.* have demonstrated that S1P in vivo administration can cause the alteration in pulmonary parenchyma, increasing mucus production, and lung inflammation (Roviezzo F. S. R., 2015). Therefore, this demonstrates that the pathway of this lipid mediator is widely involved in airway diseases.

Cigarette-smoke is one of the main risk factors in the development of airway diseases and previous papers have found an alteration of sphingomyelinase levels in smokers (Petrache I., 2005).

4.2 Aim

These experiments aim to evaluate the role of S1P in a murine model of airway hyperreactivity induced by cigarette smoke.

4.3 Material and Methods

Material and methods related to these experiments are reported in Chapter 3.

4.4 Statistical Analysis

Statistical analysis related to these experiments is reported in Chapter 3. Details of each analysis and statistic parameters are explained in the description of the graphs.

4.5 Results

Cigarette smoke induces a progressive development of pulmonary emphysema.

Cigarette smoke is one of the main risk factors for the development of airway inflammation. Herein, pulmonary changes in cigarette-smoke exposed mice were evaluated *(Figure 4.1)*. C57BL/6J mice were exposed to cigarette smoke for 11 months, while the vehicle group was exposed to room air. After 9 months of exposure, smoke induced a mild increase in collagen deposition in the peri-bronchial and peribronchiolar region *(Figure 4.1b)* than the vehicle group *(Figure 4.1a)*. The collagen deposition became much more evident after 10 and 11 months of treatment *(Figure 4.1 c, d)*. In the insert in *Figure 4.1c,* it is also possible to observe how the infiltrate spread in the bronchial area.

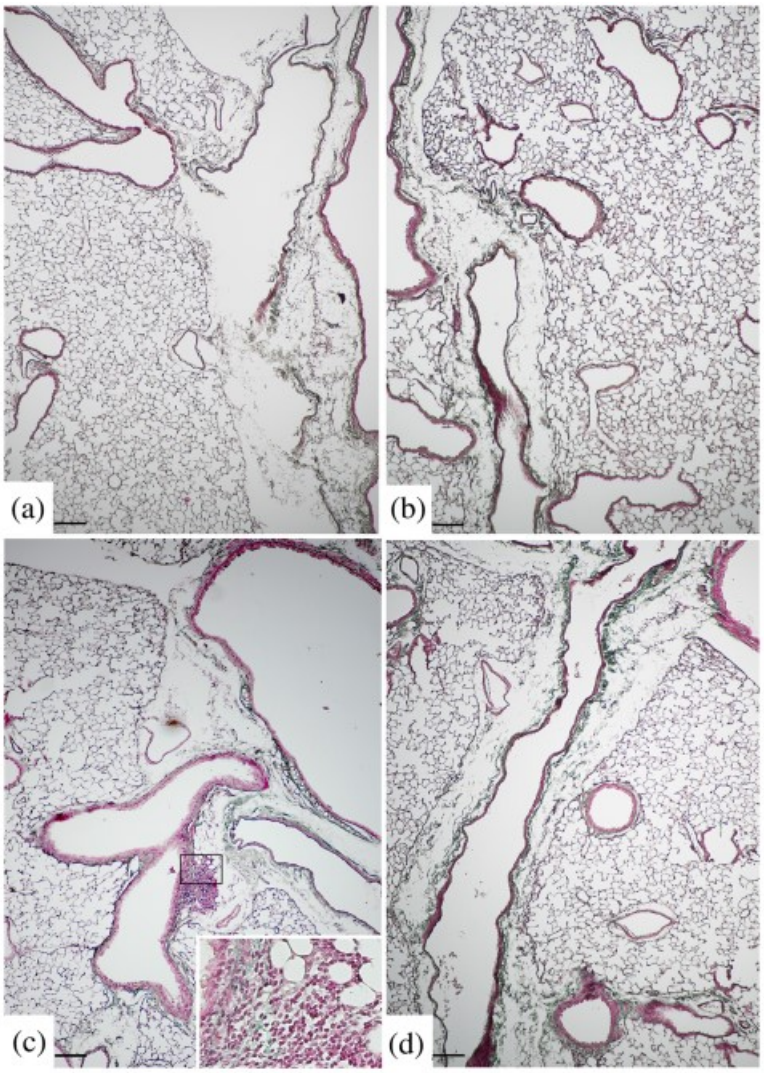


Figure 4. 1 Masson's trichrome staining

Lung slice of mice exposed to **A**. room air and cigarette-smoke for **B**. 9, **C**. 10, and **D**. 11 months. Inset in **C**: scale bars = 150 μM; 6 mice for each experimental group (De Cunto G, 2019).

Cigarette smoke gradually increases the response to cholinergic stimulation.

Bronchial responsiveness and lung resistance (R_L) were tested to evaluate both the upper and lower airway, respectively, following the cigarette-smoke exposure. Bronchial reactivity was analysed through the use of isolated organ bath while R_L through whole-body plethysmography. Cigarette-smoke induced a time-related increase in bronchial reactivity to carbachol *(Figure 4.2)*. This reactivity started to increase following 10 months of treatment reaching the plateau at 11 months *(Figure 4.2a)*. A similar effect was evaluated in bronchi challenged in vitro with S1P *(Figure 4.2b),* bronchial reactivity to S1P started to increase following 10 months of cigarette smoke exposure. Compared to carbachol reactivity, S1P induced bronchial contraction in control mice only at high concentrations *(Figure 4.2b),* while bronchi harvested from mice exposed to cigarette smoke for 10 and 11 months were more reactive to S1P already at a lower concentration.

Compared to the upper airway, the lower airway showed an early increased response to carbachol already after 9 months of cigarette smoke exposure, reaching the plateau at 11 months *(Figure 4.2c)*. R_L was significantly increased also following S1P in vitro administration *(Figure 4.2d),* also in this case mice exposed to cigarette smoke for 10 and 11 months were more reactive already at lower concentrations compared to control group.

To find out if this hyperresponsiveness induces by cigarette-smoke is coupled to the airway remodelling process, immunohistochemical analysis on the pulmonary section was performed to evaluate α-SMA expression in the peribronchial *(Figure 4.3 a, b)* and peribronchiolar region *(Figure 4.3 c, d)*. Mice exposed to cigarette smoke for 11 months *(Figure 4.3 b, d)* showed a thick layer of α-SMA positive staining compared to the control group *(Figure 4.3 a, c)* both in peribronchial and peribronchiolar regions, demonstrating that the hyperreactivity induced by cigarette smoke is sustained by airway remodelling.

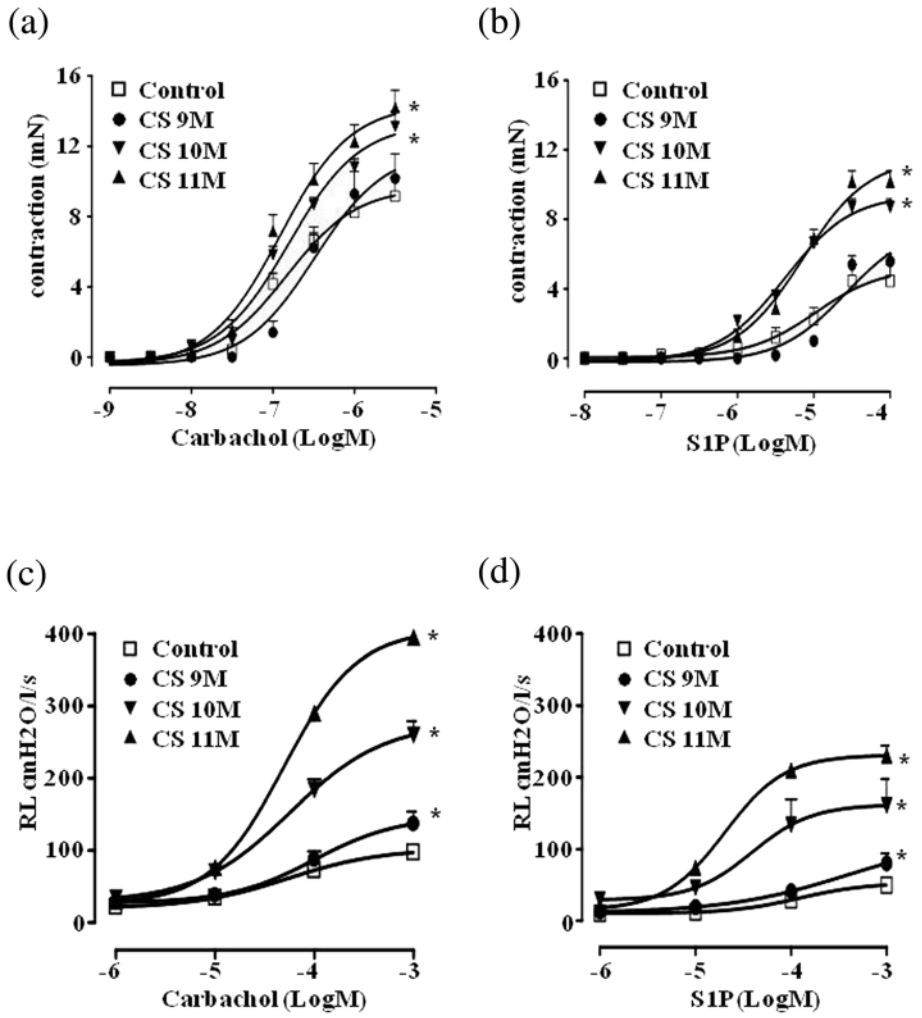


Figure 4. 2 Analysis of bronchial reactivity and lung resistance

Mice were exposed to room air (Control) or cigarette-smoke for 9, 10 and 11 months. Bronchial reactivity to **a**. carbachol **b**. S1P was analysed in vitro. Lung resistances to **c**. carbachol **d**. S1P were measured in a plethysmography. Data represent mean ± S.E.M. 5 mice used for each group. Statistical analysis used: two-way ANOVA followed by Bonferroni test. *P<0.005, **P<0.01 and ***P<0.001 (versus control) (De Cunto G, 2019).

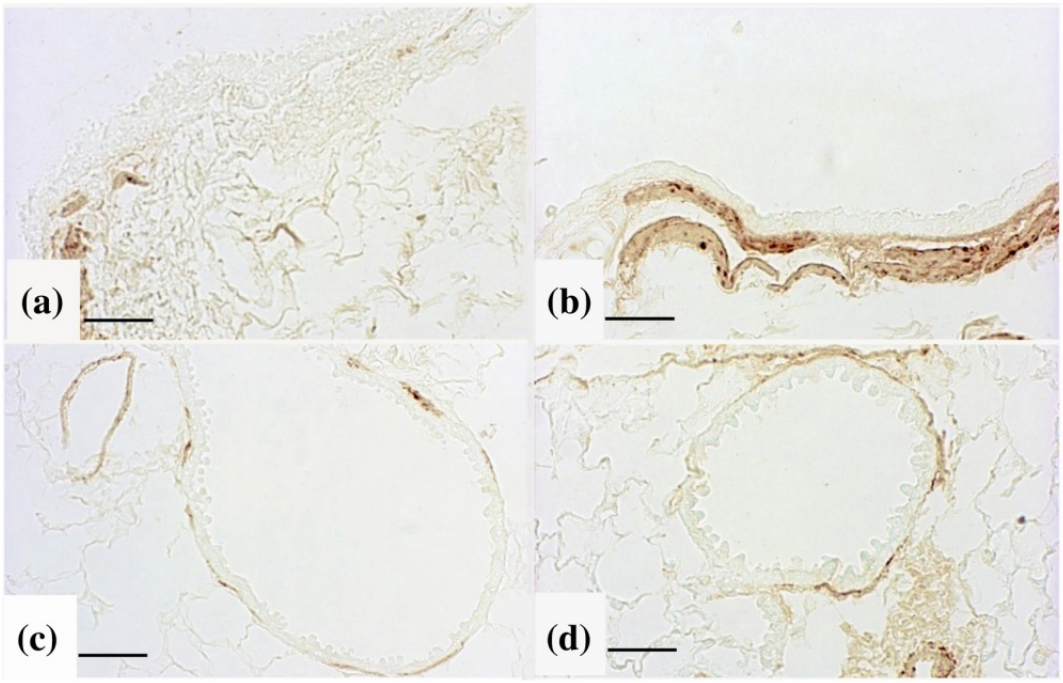


Figure 4. 3 α-SMA expression

α-SMA expression (a) in peribronchial region of control group and (b) mice exposed to cigarette smoke for 11 months. α-SMA expression in peribronchiolar region of control group (c) and mice exposed to cigarette smoke for 11 months (d). Scale bar = 40 μm. 6 mice for each group (De Cunto G, 2019).

S1P pathway affects carbachol-induced contraction in bronchi from smoking mice.

To further analyse the role of S1P in airway function, the activity of sphingosine kinase inhibitor, SK-I, was tested on carbachol-induced contraction in bronchi harvested from control and smoking mice *(Figure 4.4)*. Pretreatment of bronchi with SK-I did not affect carbachol reactivity in smoking mice up to 9 months *(Figure 4.4a)*. SK-I significantly decreased the carbachol reactivity in mice exposed to cigarette smoke for 10 months *(Figure 4.4b)* and the effect became even more evident after 11 months of treatment *(Figure 4.4c)*. Additionally, R_L was evaluated using the whole-body plethysmography through bolus administration in the pulmonary artery, before the carbachol challenge. This SK-I administration significantly decreased the R_L of mice exposed to cigarette smoke for 11 months *(Figure 4.4d)*.

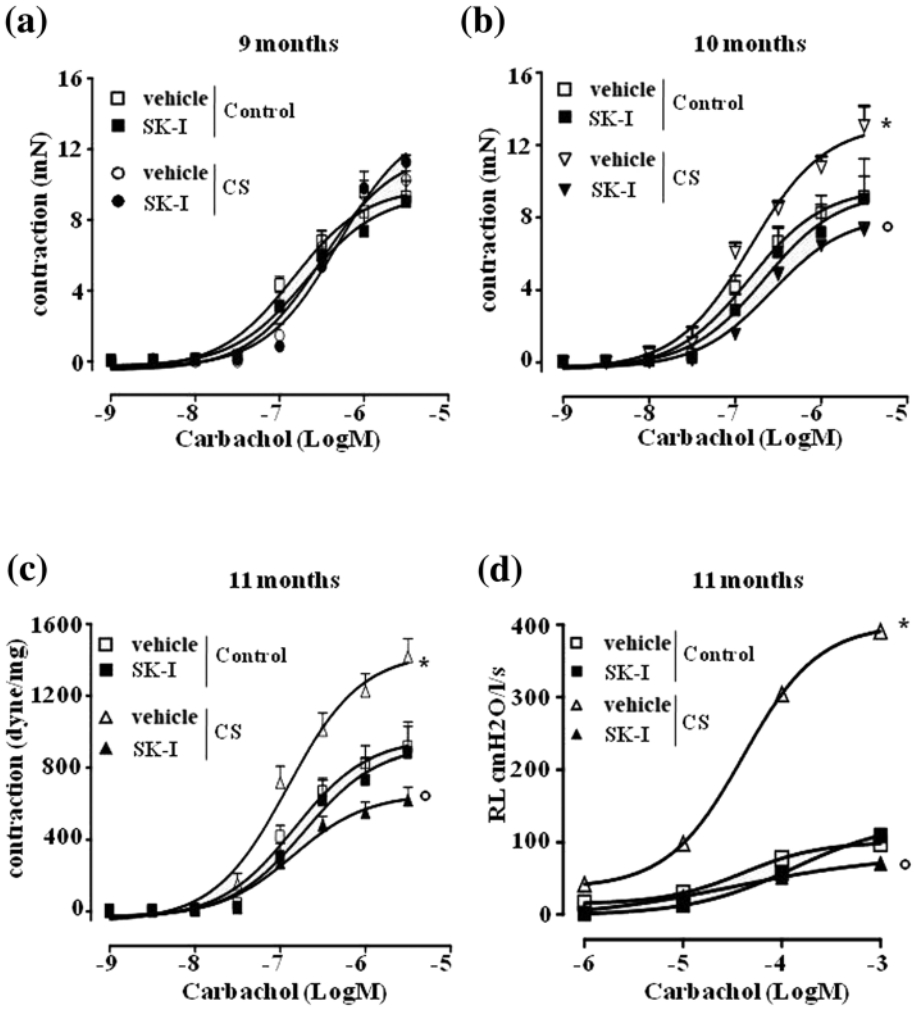


Figure 4. 4 Analysis of SK-I on bronchial reactivity

Analysis of SK-I on carbachol induced contraction in **(a,b,c)** bronchi from control and smooking mice up to **a**. 9, **b**. 10 and **c**. 11 months. Tissues were pre-incubated with SK-I (30 min. 10μM) and exposed to concentration-response curve of carbachol. **d**. Analysis of SK-I on R_L in control and smooking mice up to 11 months. SK-I was introduced in pulmonary artery and carbachol reactivity was measured. Data represent mean ± S.E.M. Panel (a), number of mice: 7 mice control, 5 mice control+SK-I, 8 mice cigarette-smoke exposed (CS) up to 9 months, 5 mice 9months+ SK-I. Panel (b), number of mice: 7 mice control, 5 mice control+SK-I, 8 mice CS up to 9 months, 5 mice 9months+ SK-I. Panel (c), number of mice: 8 mice control, 5 mice control+SK-I, 8 mice CS up to 9 months, 5 mice 9months+ SK-I. Panel (d), number of mice: 5 mice for each group. Statistical analysis used: two-way ANOVA followed by Bonferroni test. *P<0.05, versus control vehicle; °P<0.05 versus CS vehicle (De Cunto G, 2019).

S1P receptors responsible for S1P activity on airways

S1P activity is mediated by different receptors. $S1P_2$ and $S1P_3$ are widely expressed on bronchial tissue. For this reason, bronchi were pretreated with antagonists of these receptors, JTE-013 ($S1P_2$), and TY52156 ($S1P_3$) and then exposed to S1P *(Figure 4.5)*. Both antagonists abrogated the S1P-induced contraction in bronchi harvested from mice exposed to CS for 11 months *(Figure 4.5a, b)*. To confirm this result, JTE-013 and TY52156 were administrated in the pulmonary artery to measure R_L. Both drugs completely abrogated the increased R_L induced by S1P in mice exposed to CS for 11 months *(Figure 4.5c, d)*.

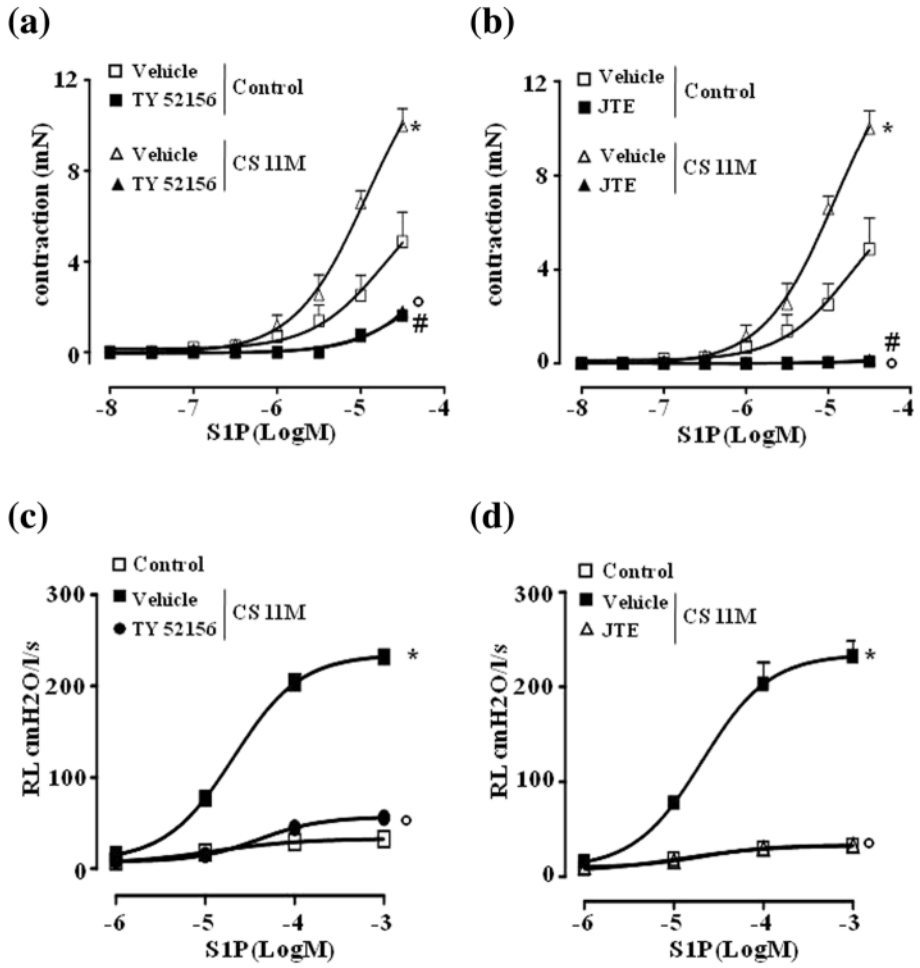


Figure 4. 5 Analysis of S1P receptors-antagonist on bronchial reactivity

S1P receptors-antagonists, JTE-013 ($S1P_2$- antagonist) and TY52156 ($S1P_3$-antagonist), **(a, b)** on bronchial reactivity and **(c, d)** R_L. Bronchi harvested from control and mice exposed to cigarette-smoke for 11 months were pre-incubated with **a**. TY52156 and **b.** JTE-013 and then exposed to S1P administration in vitro. **c, d.** R_L were measured following **c.** TY52156 and **d.** JTE-013 administration in pulmonary artery and thed exposed to S1P activity. Panel (a,b,c and d), number of mice: 5 mice for each group. Statistical analysis used: two-way ANOVA followed by Bonferroni test. *P<0.05, versus control vehicle; °P<0.05 versus CS vehicle; #P<0.05 versus **a.** control + TY52156 or **b.** control + JTE-013 (De Cunto G, 2019).

S1P pathway is up-regulated by cigarette-smoke.

Cigarette-smoke exposure up to 11 months induced an increased expression of $S1P_3$ in lung tissue *(Figure 4.6 d, e, f)* compared to control mice exposed to room air *(Figure 4.6 a, b, c).* Control mice showed mild positive staining of *$S1P_3$* in epithelial cells of main bronchi *(Figure 4.6 a)*, of the small airway *(Figure 4.6 b)*, of bronchioles *(Figure 4.6 c),* and macrophages *(Figure 4.6 c*). Cigarette-smoke increased the expression of $S1P_3$ in airway epithelium *(Figure 4.6 d*), bronchial epithelial cells *(Figure 4.6 e*), and macrophages *(Figure 4.6 f*).

Besides, the $S1P_2$ regulation was detected *(Figure 4.7*) in control mice *(Figure 4.7 a*), mice exposed to cigarette smoke up to 11 months *(Figure 4.7 b, c*), and up to 9 months *(Figure 4.7 d*). Cigarette-smoke exposure up to 11 months induced upregulation of $S1P_2$ on epithelial cells *(Figure 4.7 b*), and smooth muscle cells *(Figure 4.7 c*), while exposure up to 9 months increased the expression of $S1P_2$ in smooth muscle cells *(Figure 4.7 d*).

Since Sphingosine-kinases affect bronchial hyperreactivity, Sph-K isoforms were detected by immunohistochemistry analysis with selective antibody *(Figure 4.8)*. The Sph-K_1 expression was not modified by cigarette smoke exposure (these data are not shown), while Sph-K_2 expression was increased in the airway epithelium of main bronchi *(Figure 4.8 b)* and the bronchiolar epithelium and macrophages *(Figure 4.8 d)* in mice exposed to cigarette-smoke up to 11 months.

S1P levels were evaluated in control and smoking mice of 11 months *(Figure 4.8 e).* Mice exposed to cigarette smoke for 11 months showed a significant increase in S1P levels in lung tissue compared to control mice. These data demonstrate that cigarette-smoke up-regulates the S1P pathway in lung tissue.

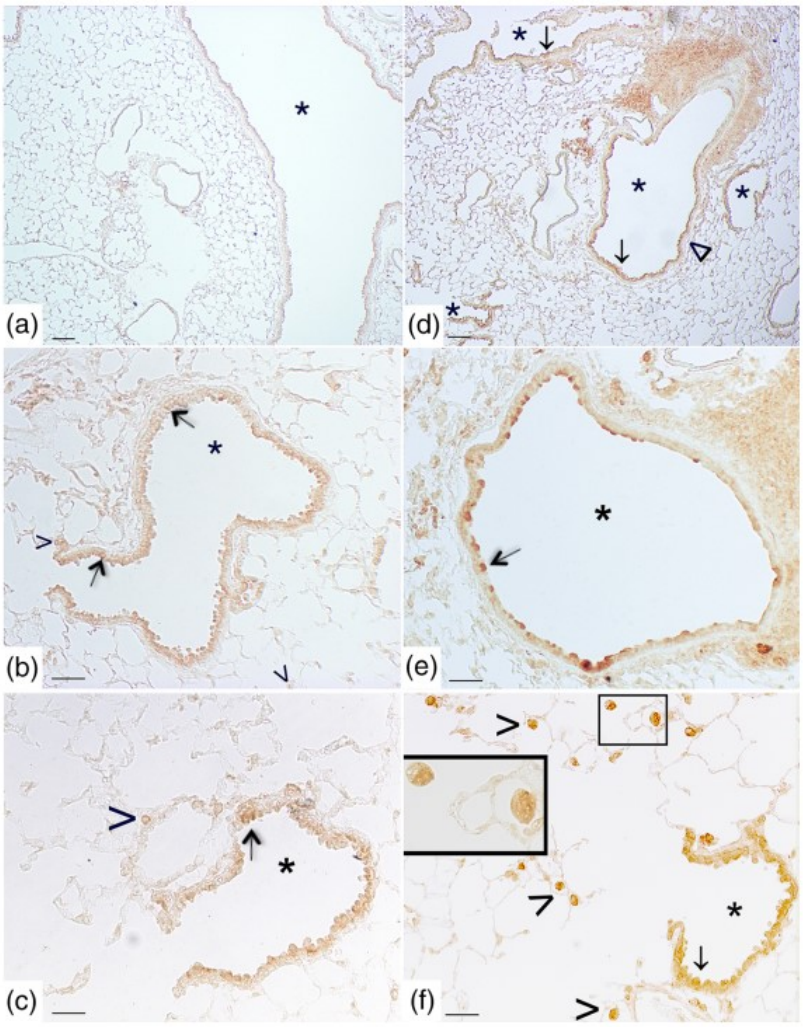


Figure 4. 6 Immunohistochemical analysis for the S1P3 detection

$S1P_3$ analysis in lung tissue of mice exposed to **(a,b,c)** room air and **(d,e,f)** cigarette-smoke for 11 months. **a.** Ephitelial cells of main bronchi and **b.** ephitelial cells of small airway (arrow) showed a mild $S1P_3$ positivity staining in control mice. **b.** macrophages (arrowhead) showed a faint staining for $S1P_3$. A mild $S1P_3$ staining is detected also on **c.** epithelial cells of bronchiole (arrow) and macrophages (arrowhead) in control mice. $S1P_3$ staining increased in mice exposed to cigarette-smoke for 11 months **(d,e,f)**, particularly on **d.** airway epithelium (arrow) and smooth muscle cell layer (wedge), **e.** bronchial epithelial cells and **f.** epithelial cells from small airways and macrophages. Scale bars: **a,d.** 100µM, **b,e**. 60 µM and **c,f.** 30 µM. Number of mice used: 6 mice for each group (De Cunto G, 2019).

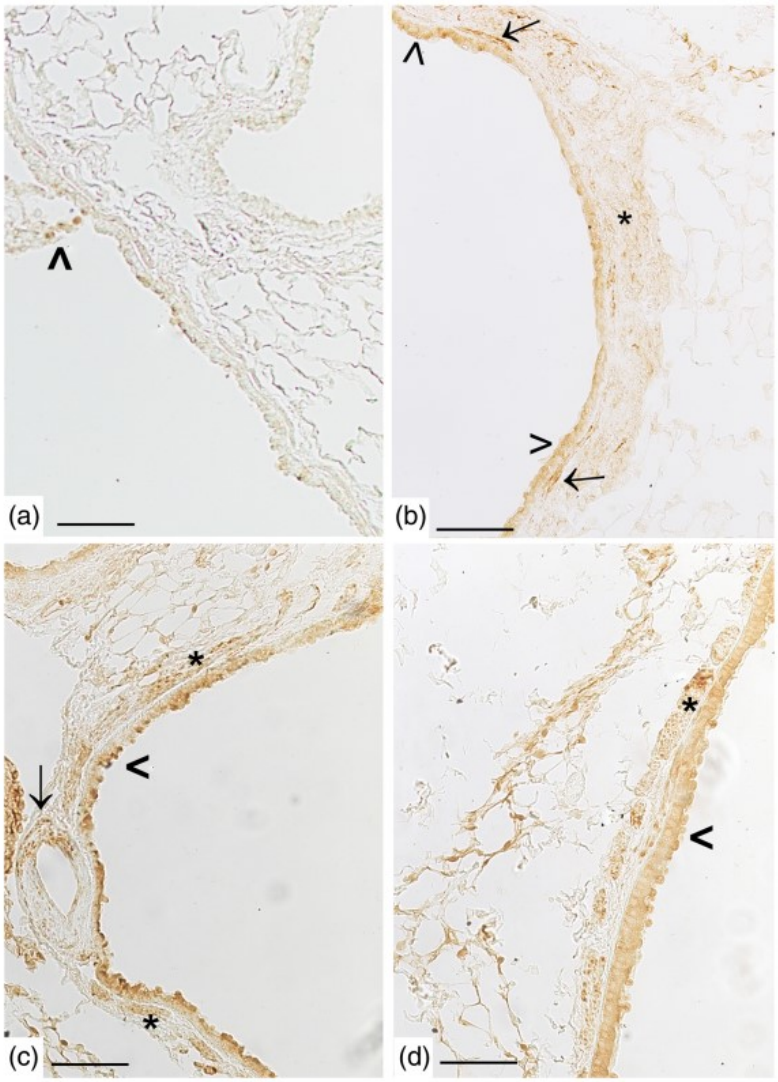


Figure 4. 7 Analysis of S1P2 in the lung

Immunohistochemical analysis for the detection of $S1P_2$ in lung tissue of mice exposed to **a.** room air and **b,c.** cigarette-smoke for 11 months and **d.** for 9 months a. Airway epithelium from control mice showed a mild $S1P_2$ positive staining. **b.** Mice exposed to cigarette-smoke for 11 months showed high $S1P_2$ positive staining on epithelial cells (arrowhead), on spindle-shaped cells (arrow) and smooth muscle cell layer (asterisk), and **c.** on smooth muscle cells (asterisk), on epithelial cells od bronchus (arrowhead) and blood vessels (arrow). **d.** after 9 months of cigarette-smoke exposure $S1P_2$ positive staining is detected in smooth muscle cells. Scale bar: 100 μM. Number of mice used: 6 mice for each group (De Cunto G, 2019).

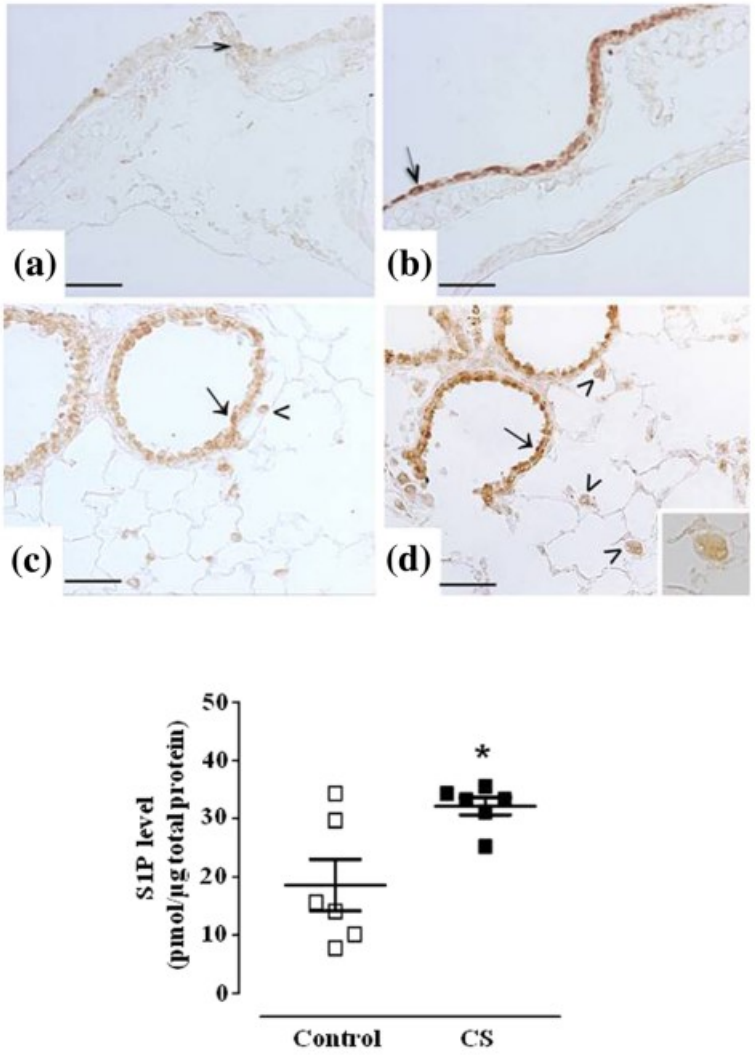


Figure 4. 8 Analysis of Sph-k2 and S1P levels in airways

Immunostaining for Sph-K_2 in **(a, b)** airway epithelium of main bronchi from **a.** control and **b.** mice exposed to CS for 11 months, and in **(c, d)** bronchiolar epithelium (arrow) and macrophages (arrowhead) in **c.** control and **d.** mice exposed to cigarette-smoke for 11 months. Scale bar: 50 µM. Number of mice used: 6 mice for each group. **e.** S1P levels have been detected in control and mice exposed to cigarette-smoke for 11 months. Data represent mean ± S.E.M. Number of mice used: 7 mice for each group. *P<0.05 versus control (De Cunto G, 2019).

4.6 Discussion and Conclusion

These experiments showed that the exposure of C57BL/6J mice to cigarette smoke for 11 months result in major structural changes in the lung. Particularly, pulmonary emphysema

developed just after 9 months of treatment and this was sustained by an increase of collagen deposition both in peribronchial and peribronchiolar regions. After 11 months of treatment, this increase in collagen deposition became much more evident and collagen started to spread in the bronchial area from 10 months of cigarette-smoke exposure, causing important time-dependent changes both in peribronchial and bronchial areas.

All these structural changes were sustained by the alteration in bronchial reactivity to carbachol. After 9 months of treatment, these alterations were not evident, but they started to become more evident after 10 months reaching a maximal effect after 11 months of exposure. Thus, this demonstrates that also these alterations in carbachol reactivity are time related. In the same manner, also the bronchial reactivity to S1P was increased after 10 months of treatment. Compared to upper airways that were involved after 10 months of exposure, the lower airways, evaluated by R_L, showed an alteration in contractility both to carbachol and S1P after 9 months of cigarette-smoke exposure reaching a maximal alteration after 11 months. Moreover, this increased bronchial hyperreactivity was coupled to an airway remodelling process. Indeed, immunohistochemistry analysis showed that the expression of α-SMA protein was increased in peribronchial and peribronchiolar regions at 11 months of exposure. Therefore, cigarette-smoke sparks lung inflammation and bronchial hyperresponsiveness that first affects lower and then upper airways.

S1P pathway is involved in bronchial hyperreactivity induced by cigarette smoke. To analyse the role of S1P in airway function, we evaluated the activity of sphingosine kinase inhibitor (SK-I) on carbachol reactivity. SK-I abrogated the hyperreactivity induced by carbachol after 10 and 11 months of cigarette-smoke exposure while had not significant effect on carbachol reactivity after 9 months of exposure. The same effect was observed on R_L, SK-I reduced the increase in R_L to carbachol challenge at 11 months of exposure. The main SK-I isoform involved in airway dysfunction induced by cigarette-smoke is Sph-K_2. The expression of this enzyme was increased in the airway epithelium of main bronchi and bronchiolar epithelium and macrophages in mice exposed to cigarette smoke for 11 months.

S1P activity in airway dysfunction induced by cigarette-smoke exposure was mediated by two types of receptors: $S1P_2$ and $S1P_3$- receptors. JTE-013 ($S1P_2$-antagonist) and TY52156 ($S1P_3$- antagonist) abrogated the bronchial hyperreactivity to carbachol and S1P

administration induced by cigarette-smoke exposure for 11 months. The involvement of these receptors was confirmed by the analysis of R_L.

Moreover, smoking mice for 11 months showed an increased expression of both receptors, $S1P_2$ and $S1P_3$, in airway epithelium, bronchial epithelial cells, and macrophages. To further confirm the involvement of the S1P pathway in cigarette-smoke induced airway dysfunction, S1P levels were evaluated. This altered airway function in smoking mice of 11 months was coupled to higher S1P levels in the lung than control mice.

All these data confirm the involvement of the S1P pathway in airway dysfunction induced by cigarette smoke exposure. This allows us to assume that S1P could be a therapeutic target for the treatment of both airway inflammation and bronchial hyperreactivity in smokers, that are more exposed to airway diseases development than other patient categories.

Chapter 5

Chapter 5

5.1 Sex differences in LTs production during allergic airway sensitization

5.1.1 Rationale

The incidence and prevalence of airway diseases are related to sex and age. Indeed, in childhood asthma has a higher prevalence in boys but, during adolescence, there is a switch in incidence and prevalence of this disease. Adult females show an increase both in incidence and severity of asthma, suggesting that changes in sex hormones are related to this disease (Fuseini H., 2017).

Sex hormones influence the immune system, modifying the pro-inflammatory mediators' synthesis, such as LTs. The synthesis of these lipid mediators is influenced by the amount of free Arachidonic acid (AA) and by the localization of 5-LO. In resting neutrophils, which are the major source of LTs production in blood, 5-LO is in the cytoplasm and, after the activation by Ca^{2+}, the enzyme moves to the nuclear membrane where is located FLAP protein that induces the activation of 5-LO, inducing LTs synthesis (Pergola C, 2008). Pergola *et al.* have shown that this hypothesis is true in neutrophils harvested from female mice, but in resting neutrophils from males, 5-LO is located both in the cytoplasm and in the nuclear compartment, and after the activation by Ca^{2+} this localization is not significantly altered, resulting in a reduction in LTs production. The 5-LO subcellular localization is regulated by extracellular signal-regulated kinases (ERK), through the phosphorylation of the enzyme. Male hormones influence the subcellular localization of 5-LO and ERK activity, inducing sex-differences in LTs production (Pergola C., 2008).

5.1.2 Aim

Based on the above, these experiments aim to evaluate sex differences in LTs production in a murine model of airway hyperreactivity induced by OVA-administration.

5.1.3 Material and Methods

Material and methods related to these experiments are reported in Chapter 3.

5.1.4 Statistical Analysis

Statistical analysis related to these experiments is reported in Chapter 3. Details of each analysis and statistic parameters are explained in the description of the graphs.

5.1.5 Results

Analysis of sex-differences in airway function

The influence of sex was analysed in different strains of mice (BALB/c, CD1, and C57/Bl6). Bronchi harvested from these mice were placed in an isolated organ bath and subjected to a cumulative concentration-response curve of carbachol to analyse airway contraction.

In figure 5.1 is shown the airway reactivity to a cumulative concentration-response curve of carbachol in physiological conditions. Bronchial tissues harvested from female mice, regardless of strains, showed an increase in carbachol reactivity compared to male mice ($p<0.0001$), this effect was already evident at the concentration of 10^{-7} M in all strains of mice *(Fig.5.1 A, B and C).*

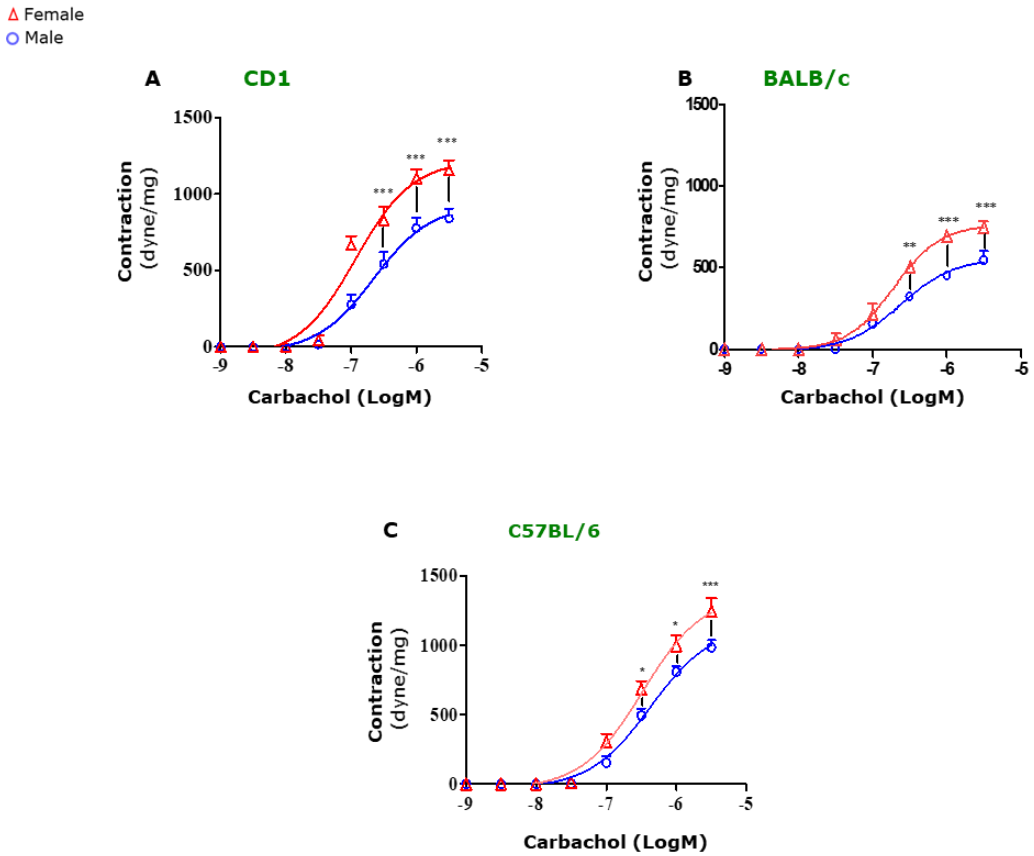


Figure 5. 1 Analysis of airway contraction in different strains of mouse

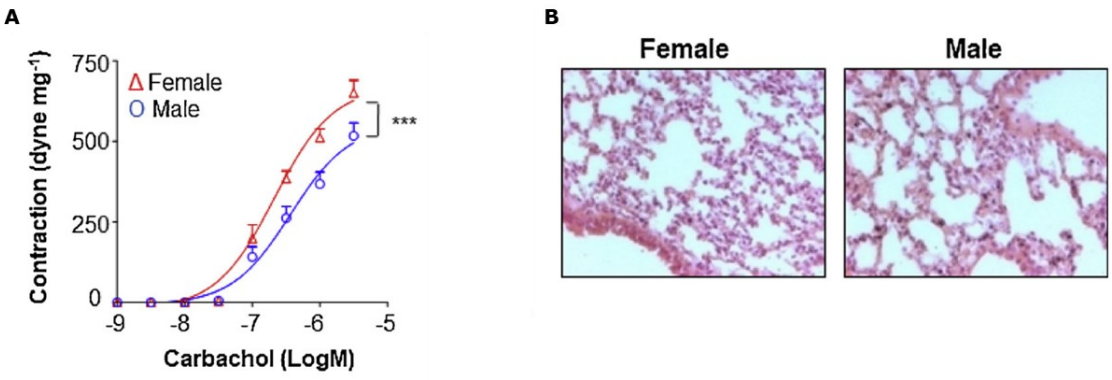
Bronchi harvested from male and female CD1 (A), BALB/c (B) and C57BL/6 (C) mice were placed in isolated organ bath to analyse airway contraction to carbachol. Data represent mean ± S.E.M. and significant is represented by *P<0.005, **P<0.01 and ***P<0.001. Statistical analysis used is Two-way ANOVA plus Bonferroni.

Sex Differences in lung function of non-treated BALB/c mice is not related to LTs pulmonary levels.

Since BALB/c mice show an increase in T-helper type 2 immune system, they are more likely to develop airway disease compared to other strains of mice. For this reason, we focused our attention on the analysis of lung function of non-treated BALB/c mice.

As reported above, female BALB/c mice showed an increase in airway reactivity following carbachol ex-vivo administration *(Fig.5.2 A)*. This increased airway contraction in female mice is associated with an increase in bronchial thickness *(Fig.5.2 B)*, demonstrating that female and male BALB/c mice show a different pulmonary structure during physiological conditions and this may explain the different airway reactivity.

Moreover, as shown in *Figure 5.2 C*, there is another difference between male and female BALB/c mice, indeed, female mice showed higher plasma levels of IgE in physiological conditions than male mice. Anyway, in physiological conditions there were no differences in Leukotrienes production between male and female BALB/c mice *(Fig.5.3 A, B)*, demonstrating that these sex differences in airway function are not related to LTs pulmonary levels.



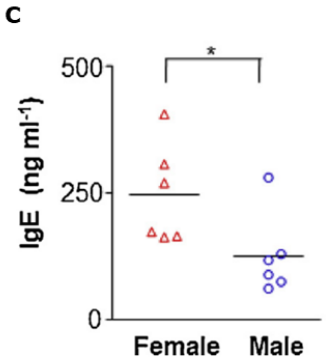


Figure 5. 2 Airways reactivity in BALB/c mice

A) Analysis of airway reactivity to carbachol in non-treated BALB/c mice. **B)** Immunohistochemical analysis of lung harvested from male and female BALB/c mice. **C)** analysis of IgE plasma level in BALB/c mice. Data represent mean ± S.E.M. (A) and mean with single data (C). Significant is represented by *P<0.005 and ***P<0.01. Statistical analysis used is Two-way ANOVA plus Bonferroni (A) and two-tailed Student t-test (C).

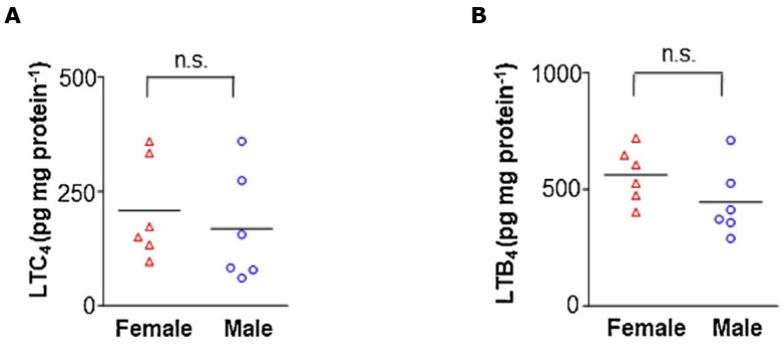


Figure 5. 3 Analysis of LTs levels in the lung

Analysis of pulmonary level of Leukotrienes LTC_4 **(A)** and LTB_4 **(B)** in male and female BALB/c mice. Data represent mean with single data and significant is represented by n.s., not significant (P>0.05). Statistical analysis used is two-tailed Student t-test.

Allergen sensitization induces sex differences in LTs levels in BALB/c mice.

LTs play an important role in airway diseases, so herein we analysed sex differences related to pulmonary LTs levels following allergen sensitization. Following OVA-sensitization at days 0 and 7, mice were sacrificed at different time points, 3, 8, 14, and 21 days *(Fig.5.4 A)*, to perform an ELISA kit. OVA sensitization induced a significant increase (°P<0.05) in LTC_4 *(Fig.5.4 B)* and LTB_4 levels *(Fig.5.4 C)* in female mice compared to vehicles. This increase in female mice was time-dependent, starting after 14 days of sensibilization and reaching a maximum peak after 21 days. Allergen-sensitization did not induce an increase in LTs levels in male mice.

To further analyse allergen sensitization, we performed ELISA-kit to evaluate the IgE plasma levels. IgE levels increased in both sexes without significant difference, demonstrating that OVA sensitization happened correctly in both sexes *(Fig.5.4 D)*. This proves that the difference in LTs levels in male and female mice was not due to a different OVA-sensitization. This sex difference in LTs levels could explain the sex dimorphism in bronchial reactivity in the murine asthma-like model.

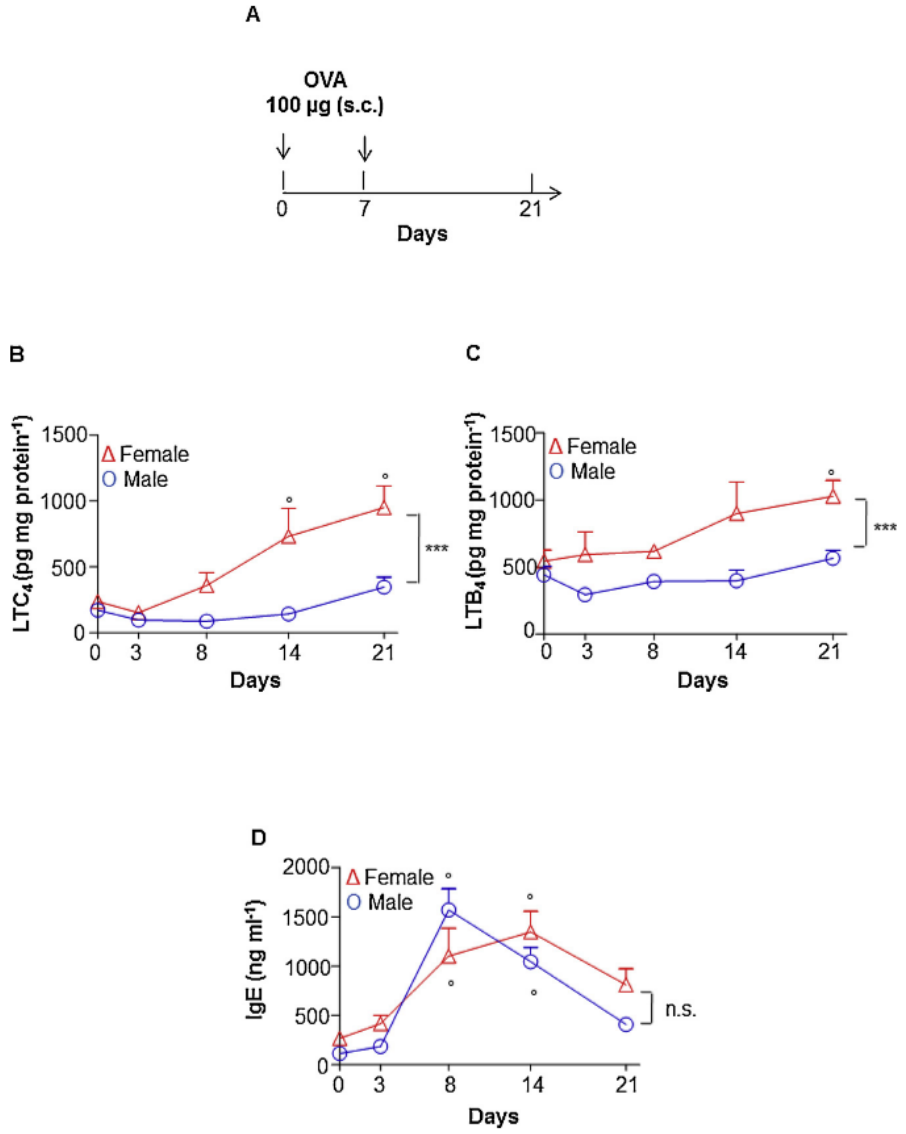


Figure 5. 4 Sex differences in LTs levels in sensitized-BALB/c mice

A. Time-course of OVA sensitization in male and female BALB/c mice **B.** LTC_4 **C.** LTB_4 levels in male and female BALB/c mice. **D**. plasma levels of IgE. N=6 mice for each group. Data represent mean ± S.E.M. Statistical significance: °P<0.05 vs. day 0 (one-way ANOVA plus Bonferroni) and ***P<0.001 (male vs. female) (Two-way ANOVA plus Bonferroni).

Sex differences in LTs production correlate with differences in airway hyperreactivity. To analyse the sex differences during the allergen sensitization, BALB/c mice were s.c. treated with OVA (see protocol above) and then sacrificed at different time points to analyse the airway function.

Sex differences induced by allergen sensitization started from 3 days after the first OVA administration in female vehicle mice compared to vehicle male (***p<0.001) and increased after 21 days *(Fig. 5.5 A, D)*. Bronchi harvested from female mice were more reactive to carbachol in-vitro administration already after 3 days of OVA-administration (°°°p<0.001) *(Fig. 5.5 A)*, while male mice have shown a significant increase in airway contraction to carbachol after 21 days of sensitization (°°p<0.01) *(Fig. 5.5 D)*.

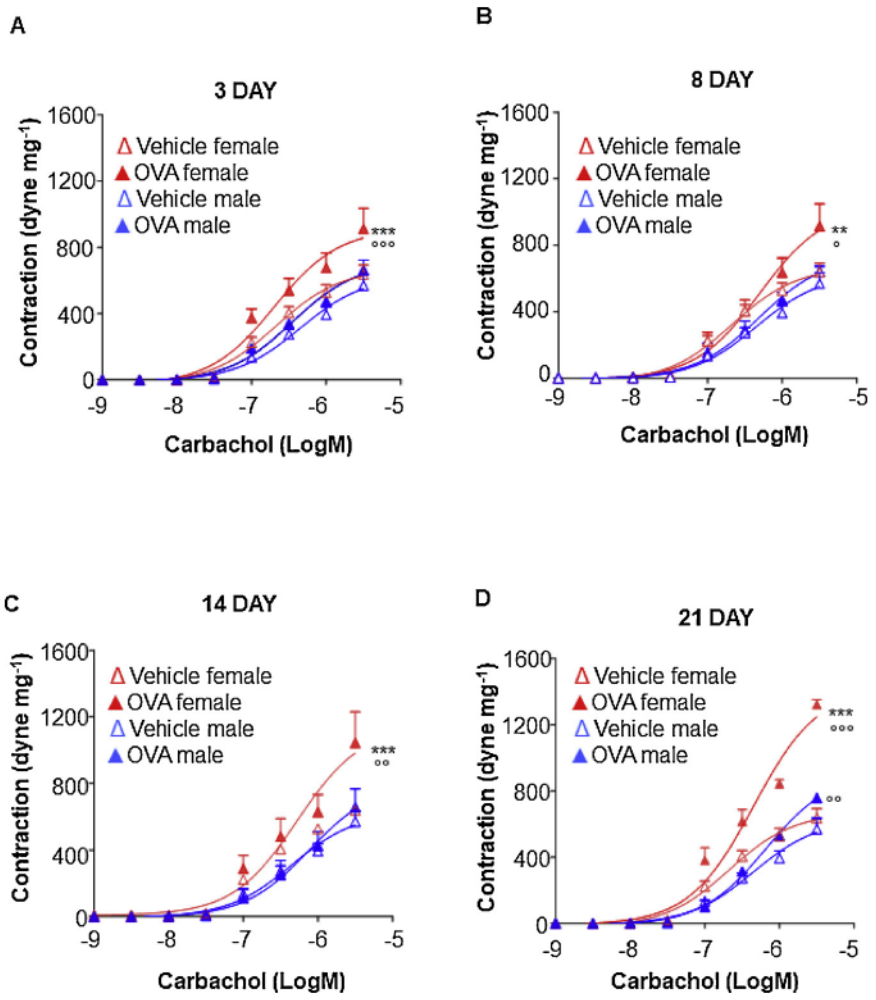


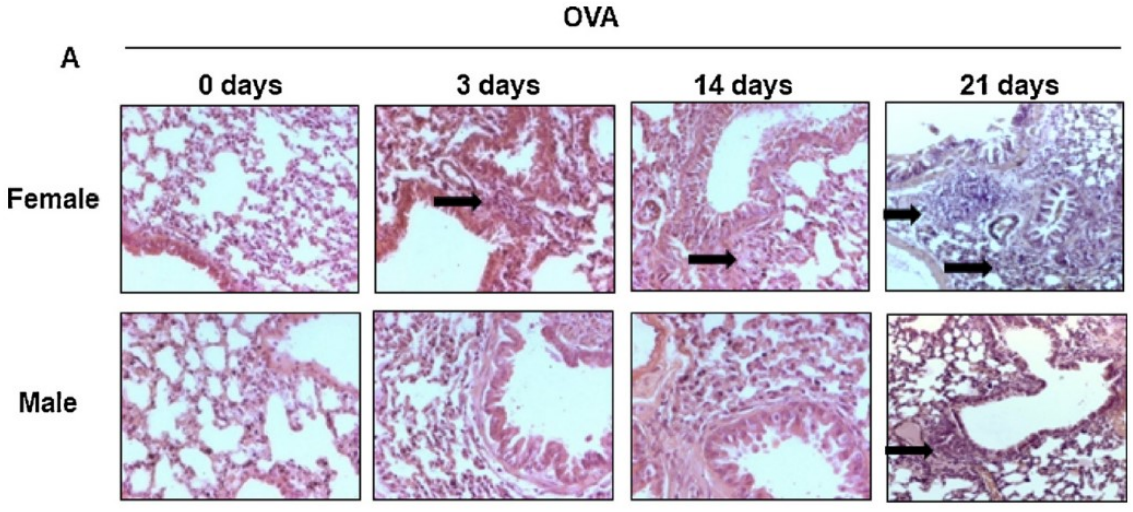
Figure 5. 5 Analysis of airway reactivity in BALB/c mice exposed to OVA

Male and female BALB/c mice were sacrificed at 3 days (A), 8 days (B), 14 days (C) and 21 days (D) after OVA s.c. administration to analyse in-vitro bronchial reactivity to carbachol. Data represent mean ± S.E.M. and significant is represented by **P<0.01 and ***P<0.001 female *vs* male mice; °°P<0.01 and °°°P<0.001 female *vs* vehicle; n=6 mice for each group. Statistical analysis used is Two-way ANOVA plus Bonferroni.

Sex differences in allergic airway inflammation

Another important feature of asthma is the airway inflammation, and LTs are widely involved in the process. Ova-sensitization induced airway hyperplasia associated with pulmonary cell infiltration *(Fig.5.6)*. In female mice, this increase in cell infiltration started after 3 days by the first OVA-administration and reached the maximal levels following 21 days. While cell infiltration increased in male mice directly after 21 days of OVA-sensitization *(Fig.5.6A)*. PAS^{+} staining analysis showed that cell infiltration was faster and higher in females compared to male mice *(Fig. 5.6B)*.

Moreover, OVA administration induced a marked increase in the lung resistance (R_L) to carbachol in female mice than male mice *(Fig 5.6 C)*.



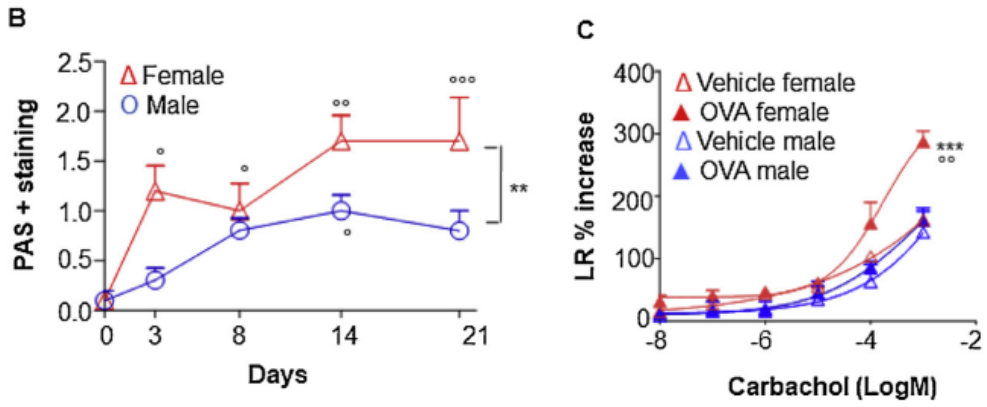


Figure 5. 6 Sex differences in pulmonary inflammation

Male and Female BALB/c mice received a subcutaneous administration of OVA and were sacrificed after 21 days. **A**. In female mice the cell infiltration starts from 3 days of sensitization while in male starts afters 21 days (as indicated with clack arrows). **B**. PAS+ Staining was carried out to analyse lung inflammation with scores: 0:<5%; 1: 5-25%; 3: 50-75%; 4<75%. **C**. Lung resistance (LR) was evaluated after 21 days by the fist OVA administration. Value represent means ± SEM; 5 animals for each group. Significance: °P < 0.05, °°P < 0.01 and °°°P < 0.001, statistical test used: one-way ANOVA (plus Bonferroni); **P< 0.01 male vs female, statistical test used: Two-way ANOVA (plus Bonferroni).

Sex differences in LTs production are Th2-related

To evaluate if the sex differences in the LTs production are related to the Th2 environment, C57BL/6 mice were investigated because of their lower Th2 response than BALB/c mice.

As observed in BALB/c mice, carbachol administration induced a higher bronchial reactivity in non-sensitized C57BL/6 female mice compared to male *(Fig.5.67A)*. Following OVA treatment, mice of both sexes showed an increase in bronchial hyperreactivity *(Fig.5.7 B)* and IgE production *(Fig. 5.7 C)*.

Compared to BALB/c mice, OVA-sensitization did not induce a significant increase in LTs production in male and female C57BL/6 mice *(Fig.5.7 D)*, suggesting the involvement of Th2 environment in LTs production.

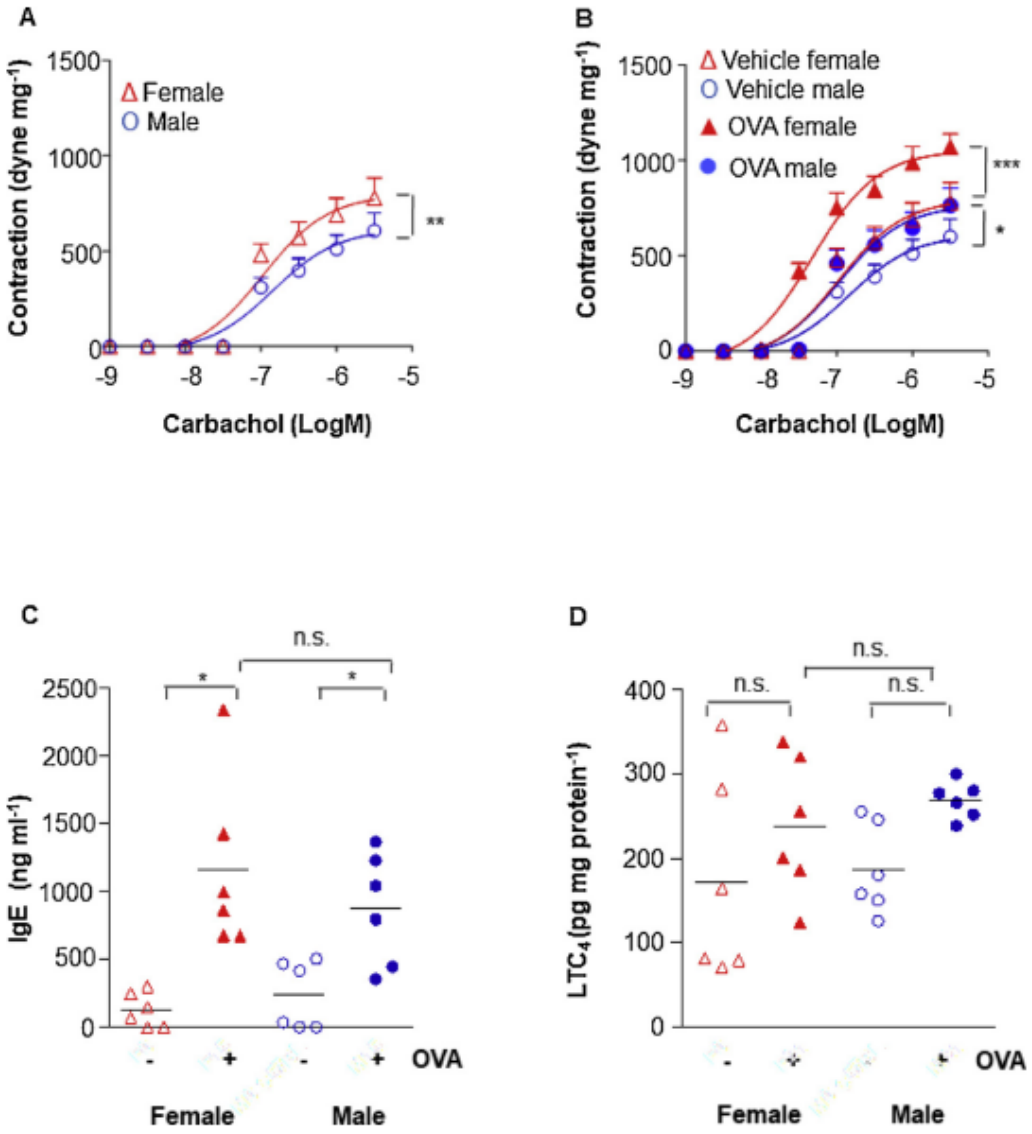


Figure 5. 7 Airway reactivity and LTs levels in C57BL/6 mice

Sex differences in airway hyperreactivity and LTs production in C57BL/6 male and female mice. Mice were s.c. treated with OVA complexed with alum or saline (vehicle) at the day 0 and 7. Lung, bronchi and blood were collected after 14 days of sensitization. **A**. Bronchial reactivity to carbachol in non-sensitized C57BL/6 mice. **B**. Bronchial reactivity to carbachol in OVA-sensitized C57BL/6 mice. **C**. IgE plasma levels **D**. LTC_4 pulmonary levels. Value represent means ± SEM; 6 animals for each group. Significance: *P < 0.05, **P < 0.01, ***P < 0.001 and n.s.=not significant (P>0.05). Statistical test used: Two-way ANOVA plus Bonferroni (A, B) and one-way ANOVA plus Bonferroni (C, D).

Sex differences in pulmonary mast-cell activity following OVA-sensitization

The main cells responsible for the release of leukotrienes are mast-cells. For this reason, the recruitment and the activation of mast-cells were both evaluated in the lung of mice treated with OVA. As demonstrated by tryptase positive staining analysis *(Fig. 5.8 A)*, there was a thickening in the basal membrane of lung harvested from female mice already after 3 days of sensitization. Male mice showed a positive staining just after 21 days of sensitization. Another important difference is the release of IL-13 *(Fig. 5.8 B)*. OVA-sensitized female mice show a significant increase in IL-13 release than OVA-sensitized male mice. Also, there was no increase in the release of IL-13 in OVA-sensitized male mice versus vehicle male *(Fig. 5.8 B)*. The PGD_2 levels were evaluated as an index of mast cell activation *(Fig. 5.8 C)*. The result was like that observed in IL-13 release, there was no difference in PGD_2 levels between OVA-sensitized male mice and vehicle male mice while OVA sensitization enhances the release of PGD_2 in females versus males *(Fig. 5.8 C)*.

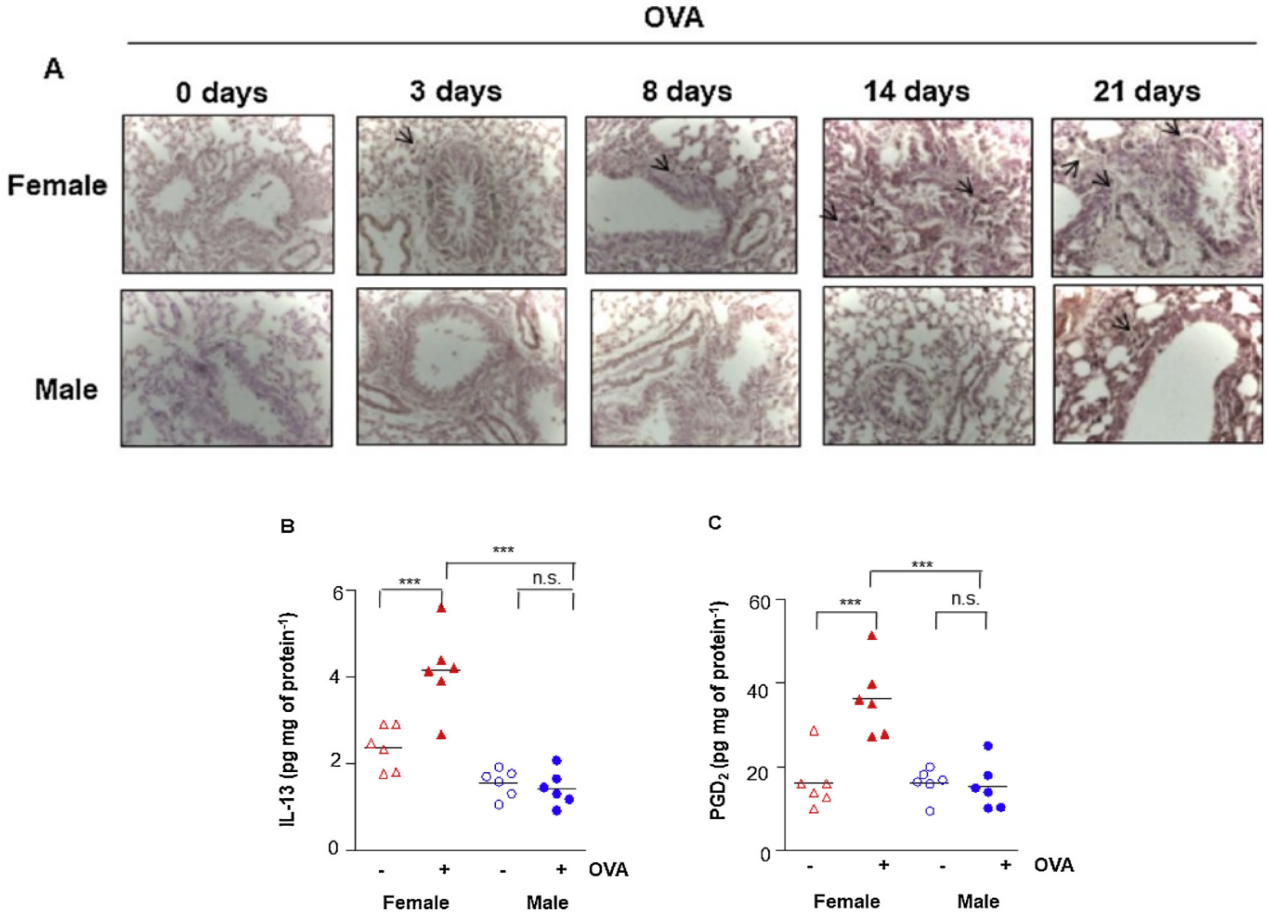


Figure 5. 8 Sex differences in mast-cell activity

Male and female BALB/c were treated with OVA on day 0 and 7. **A**. Tryptase lung immunohistochemistry analysis. **B**. IL-13 and **C**. PGD_2 levels analysis. Value represent means with single data; 6 animals for each group. Significance: ***P < 0.001 and n.s.=not significant (P>0.05). Statistical test used: one-way ANOVA plus Bonferroni.

Sex differences in LTs-modifying drugs activity

To confirm sex-difference in the LTs levels, male and female BALB/c mice were i.p. pre-treated with receptor-antagonist Montelukast (MS), 5-LOX inhibitor Zileuton (ZIL), and FLAP-inhibitor MK886 (MK). All these drugs significantly reduced (***P<0.001) the bronchoconstriction induced by OVA administration in female BALB/c mice *(Fig. 5.9 A)*, but not in males *(Fig. 5.9 B)*. Moreover, the efficacy of these drugs was also evident

in LTs pulmonary levels. ZIL and MK reduced LTs pulmonary levels in female mice but not in males *(Fig. 5.9 C)* suggesting that the decrease of bronchial hyperreactivity in the female was associated with LTs pulmonary levels.

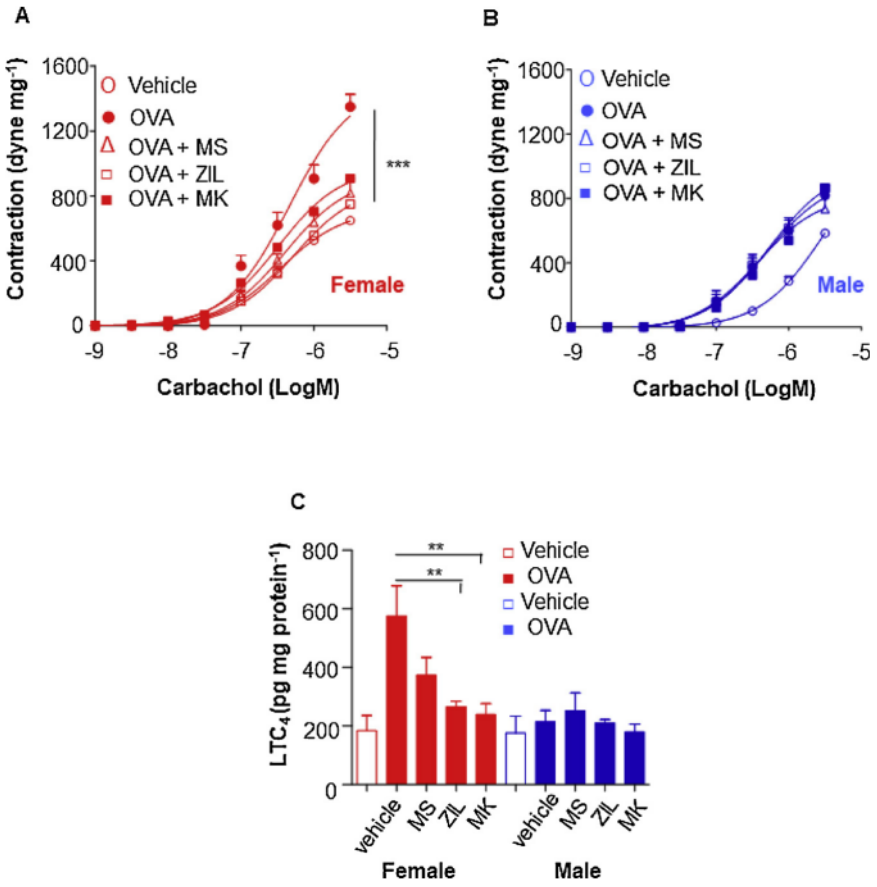


Figure 5. 9 Sex difference in LTs drugs activity

Sex difference in efficacy of LTs drugs on bronchial reactivity in female (A) and male (B) BALB/c mice and LTs production (C). Mice were s.c. treated with OVA (or saline for the control group) on day 0 and 7. Both male and female mice were pre-treated with MS (1mg/kg), ZIL (35mg/kg) and MK (0.1mg/kg) and then were sacrificed after 21 days to analyse bronchial reactivity and LTC_4 pulmonary levels. Data represent mean ± S.E.M. Statistical analysis used is two-way ANOVA (A and B) and one-way ANOVA (C) plus Bonferroni test; n=6 mice per group; **P<0.01 and ***P<0.001.

5.1.6 Discussion and Conclusion

The above data demonstrated that sex differences in airway function were already present in the physiological conditions in different mice strains. Anyway, in BALB/c mice, these sex differences in airway reactivity to carbachol were not related to sex differences in the LTs pulmonary levels. OVA-sensitization enhanced these sex differences increasing bronchial reactivity to carbachol in female mice than male. Compared to non-sensitized-mice, this increased hyperreactivity in OVA-sensitized female mice was sustained by increased LTs production in lung tissue. These sex differences were present not only in airway function but also in airway inflammation. Indeed OVA-sensitization induced pulmonary cell infiltration in female mice after 3 days of the administration, while this increase of the cellular infiltration in male mice started after 21 days of sensitization. Another important sex difference is in R_L, OVA-sensitization significantly increased the lung resistance in female mice than male. These sex differences in the LTs production were related to Th2-environment. Indeed, OVA-sensitization did not induce sex differences in LTs pulmonary levels in C57BL/6 mice. Since the release of LTs in the airway is mainly due to mast cells, the recruitment and the activation of these cells was evaluated. The recruitment of mast cells was evident in OVA-sensitized female mice just after 3 days of treatment increasing more up to 21 days. In line with this result, IL-13 and PDG_2 levels were higher in female mice after OVA-sensitization but not in male mice.

All these data were confirmed by LTs modifiers treatment. Indeed, all LTs modifiers used for these experiments, Montelukast, Zileuton, and MK886, reduced the bronchial hyperreactivity to carbachol in female mice that have a higher LTs level compared to male.

In conclusion, the data demonstrated that sex differences in bronchial reactivity were present both in physiological and pathological conditions. Anyway, this sex difference in airway reactivity was related to sex differences in the LTs production only in a pathological condition, particularly in the Th2 environment. Moreover, our data showed that LTs modifiers had greater benefits in females because of the increased LTs levels. Thus, this suggests that targeted therapy, which also considers sex, could be a good innovative therapy.

5.2 Role of 5α-dihydrotestosterone in airway function

5.2.2 Rationale

As we have already seen, sex hormones are involved in the incidence and prevalence of airway diseases. Indeed, women are prone to develop a more severe form of asthma. Before this difference was linked to the different lung structures between males and females, but now the role of sex hormones in this process is clear. Particularly, estrogens are involved in the inflammatory process through the bind with Erα-β receptors. During the menstrual cycle phases, when estrogen levels increase, a worsening of asthmatic symptoms has been reported (Keselman A., 2015). While male sex hormones seem to have a protective role that could be due to relaxing activity mediated via non-genomic pathway (Kouloumenta V, 2006).

5.2.2 Aim

Since male hormones seem to have a protective role in airway function and inflammation, herein we have investigated the effect of 5α-dihydrotestosterone administration in OVA-sensitized female mice.

5.2.3 Material and Methods

Material and methods related to these experiments are reported in Chapter 3.

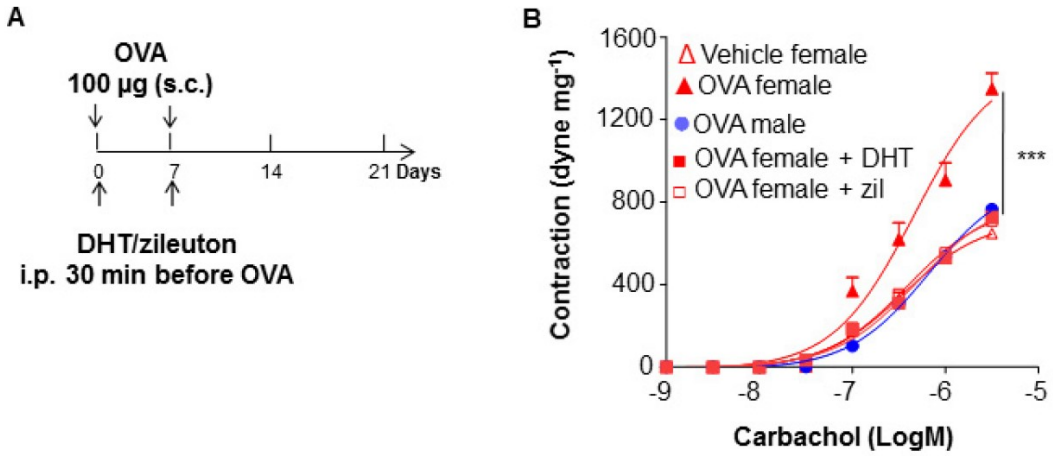
5.2.4 Statistical Analysis

Statistical analysis related to these experiments is reported in Chapter 3. Details of each analysis and statistic parameters are explained in the description of the graphs.

5.2.5 Results

5α-dihydrotestosterone (5α-DHT) affects bronchial hyperreactivity in female mice.

Female BALB/c mice were s.c. treated with OVA administration, demonstrating a significant increase in bronchial reactivity to carbachol than vehicle mice of both sexes. Pretreatment with 5α-DHT (0.5mg/kg, i.p. before each OVA administration) reverted OVA hyperreactivity in female mice bringing back the OVA-curve to vehicle male one (***P<0.001) *(Fig. 5.10 B)*. Moreover, DHT treatment showed the same effect of Zileuton (ZIL) administration both in airway reactivity *(Fig. 5.10 B)* and in the analysis of LTs pulmonary levels (*Fig. 5.10 C, D)*. Moreover, to further analyse the airway inflammation, leukotrienes (LTs) levels were evaluated. According to the previous data, OVA treatment increased both LTC_4 *(Fig. 5.10 C)* and LTB_4 *(Fig. 5.10 D)* pulmonary levels while pretreatment with DHT, as well as Zileuton pretreatment, reduced both LTC_4 and LTB_4.



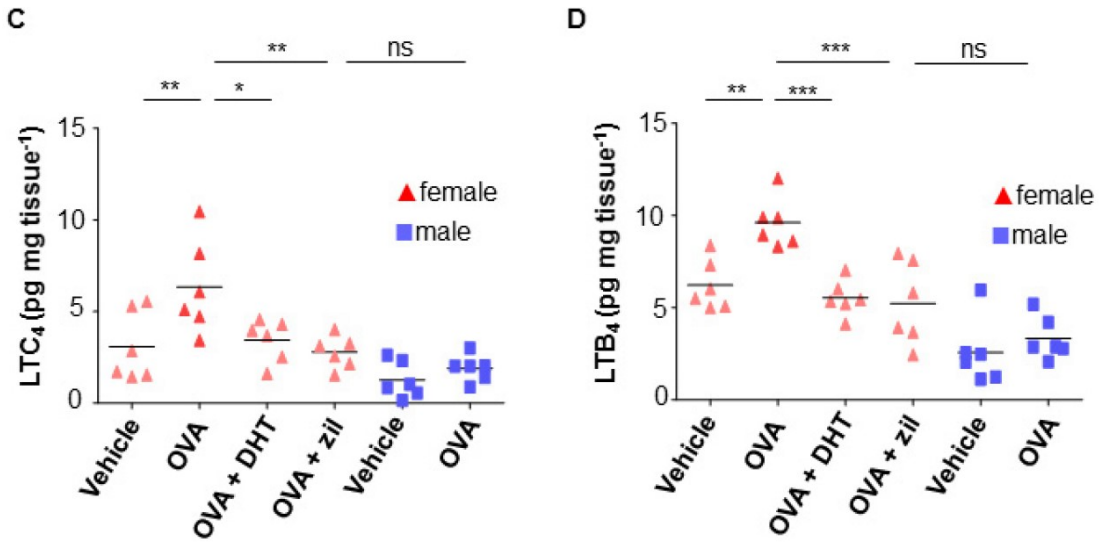


Figure 5. 10 DHT analysis in airway function

Role of DHT in airway reactivity and LTs pulmonary levels following allergen sensitization. **A.** Female BALB/c mice were s.c. treated with OVA (100µg) at the day 0 and 7 (OVA group). Pretreatment with 5α-DHT (0.5mg/kg) and ZIL (35mg/kg) were carried out 30 minutes before each OVA administration. Mice were sacrificed after 21 days to analyse bronchial reactivity (**B**) and LTs pulmonary levels (**C** and **D**). Data represent mean ± S.E.M. Statistical analysis used is two-way ANOVA (B) and one-way ANOVA (C and D) plus Bonferroni test; n=6 mice per group; statistical significance: ns: not significant; *P<0.05; **P<0.01; ***P<0.001.

5α-DHT blocks OVA-induced allergen inflammation in female mice.

Following DHT treatment, airway inflammation parameters, as airway hyperplasia, mucus production, and cell infiltration, were evaluated.

As shown in Figure 6.2, OVA sensitization increased the pulmonary cell infiltration *(Fig. 5.11 A, B)* and mucus production *(Fig. 5.11 C)*, causing airway hyperplasia. Anyway, PAS staining demonstrated that 5α-DHT pretreatment significantly reduced both cell infiltration and mucus production (**P<0.01) in female mice sensitized with OVA *(Fig. 5.11 B, C)*.

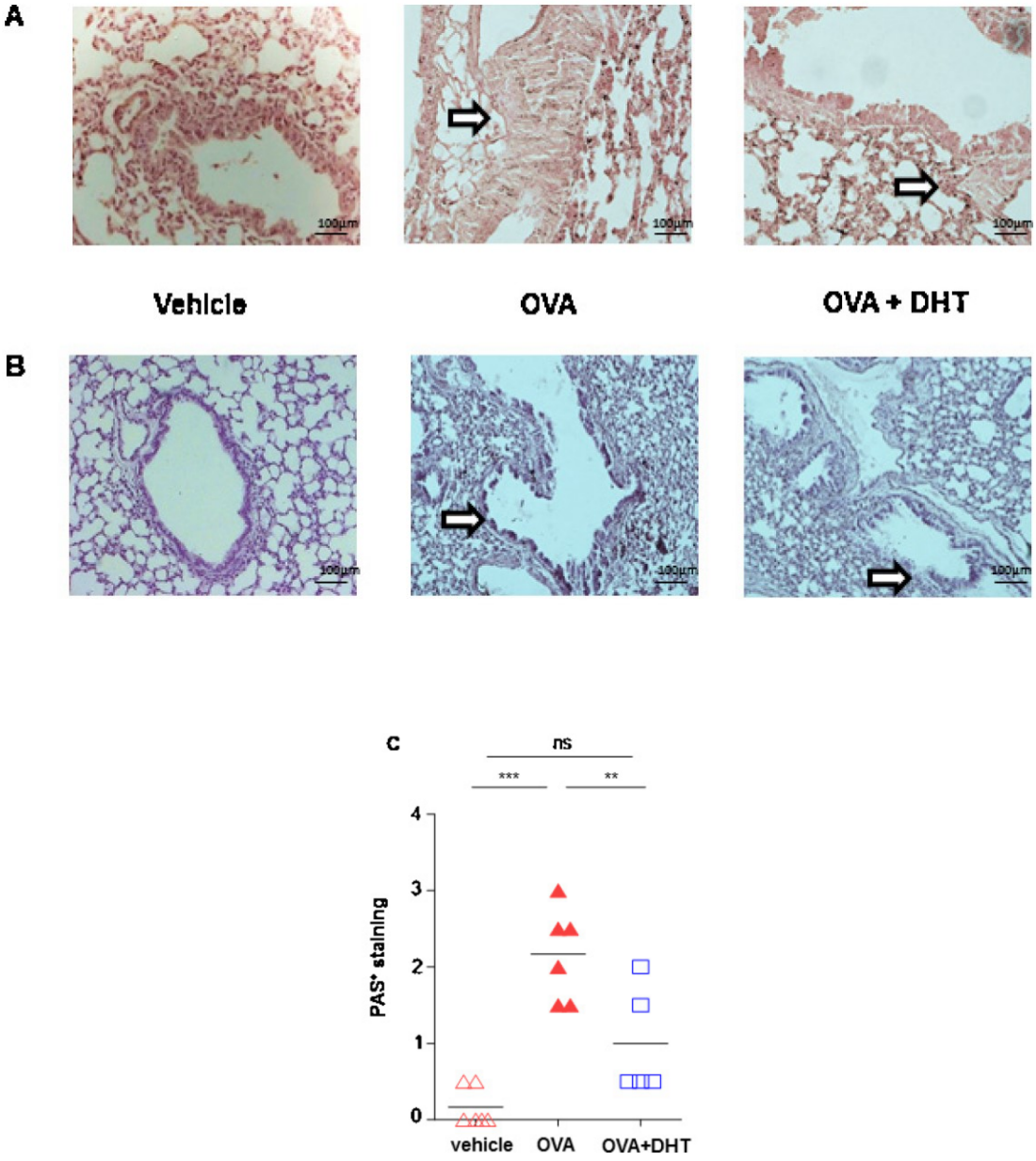


Figure 5. 11 Effect of 5α-DHT in pulmonary inflammation

Following OVA-sensitization mice were sacrificed at day 14 to evaluate H&E staining of lung tissue (**A**), lung cryosection for PAS (**B**), PAS^+ staining (**C**) with following scores: 0:<5%; 1: 5-25%; 2: 25-50%; 3: 50-75%; 4:<75%. Statistical analysis used: one-way ANOVA plus Bonferroni; **P<0.01; ***P<0.001.

Effect of 5α-DHT on OVA-induced sensitization

Female mice treated with OVA showed an increase in the percentage of $CD3^+CD4^+IL4^+$ cells *(Fig. 5.12 A)*, IgE^+ c-KIT^+ cells *(Fig. 5.12 B),* and IgE plasma level *(Fig. 5.12 C)* compared to the vehicle group. Since 5α-DHT was administrated during the sensitization phase, following 14 days by the first OVA administration, the effect of 5α-DHT on Th2 response was analysed through Flow cytometry analysis. Anyway, as reported in Figure 6.3, DHT did not affect Th2 response. Indeed OVA-sensitized mice pretreated with 5α-DHT did not report any reduction in the percentage of $CD3^+CD4^+IL4^+$ cells *(Fig. 5.12 A)*, IgE^+ c-KIT^+ cells *(Fig. 5.12 B),* and IgE plasma levels *(Fig. 5.12 C)* compared to OVA-group.

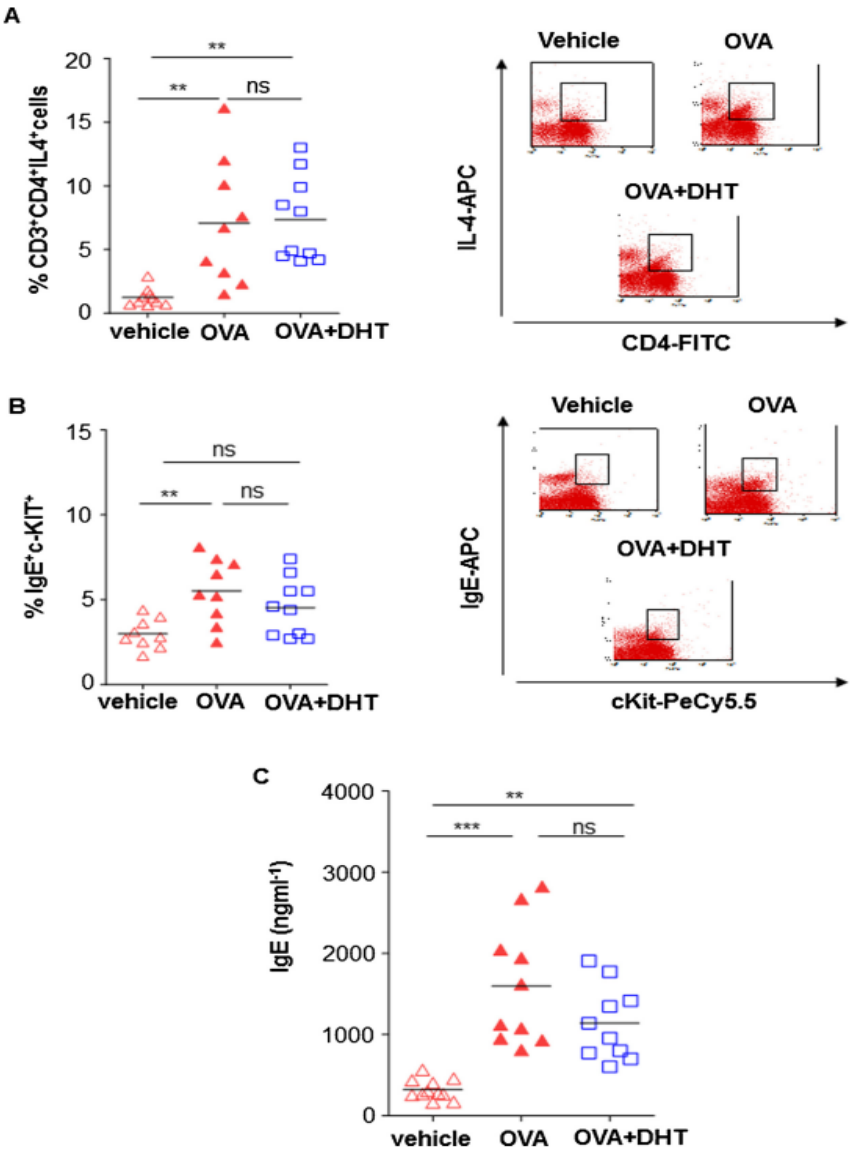


Figure 5. 12 Effect of DHT in BALB/c mice exposed to OVA

Mice were pretreated with 5α-DHT (0.5mg/kg) 30 minutes before OVA administration and then were sacrificed after 14 days to analyse pulmonary infiltration of $CD3^+$ $CD4^+$ IL4 (**A**) and IgE^+ c-KIT^+ cells (**B**), also IgE plasma level was evaluated (**C**). Statistical analysis used: one-way ANOVA plus Bonferroni; **P<0.01; ***P<0.001.

Effect of 5α-DHT on mast cell activation

Female BALB/c mice sensitized with OVA showed a marked thickening in the lung parenchyma as shown in tryptase positive staining *(Fig. 5.13 A)*, demonstrating an increase in mast cell activation and recruitment in lung tissue. Pretreatment with 5α-DHT (0.5mg/kg) reduced this thickening bringing back the aspect of parenchyma as vehicle group *(Fig. 5.13 A)*. The same effect was on the percentage of IgE^+tryptase$^+$ c-KIT^+ cells *(Fig 5.13 B)*, mice pretreated with DHT show the same percentage of IgE^+tryptase$^+$ c-KIT^+ cells of the vehicle group *(Fig 5.13 B).*

Moreover, OVA sensitization induced a significant increase in the IL-13 levels in lung tissue, but this effect was not reduced by the 5α-DHT pretreatment *(Fig. 5.13 C)*.

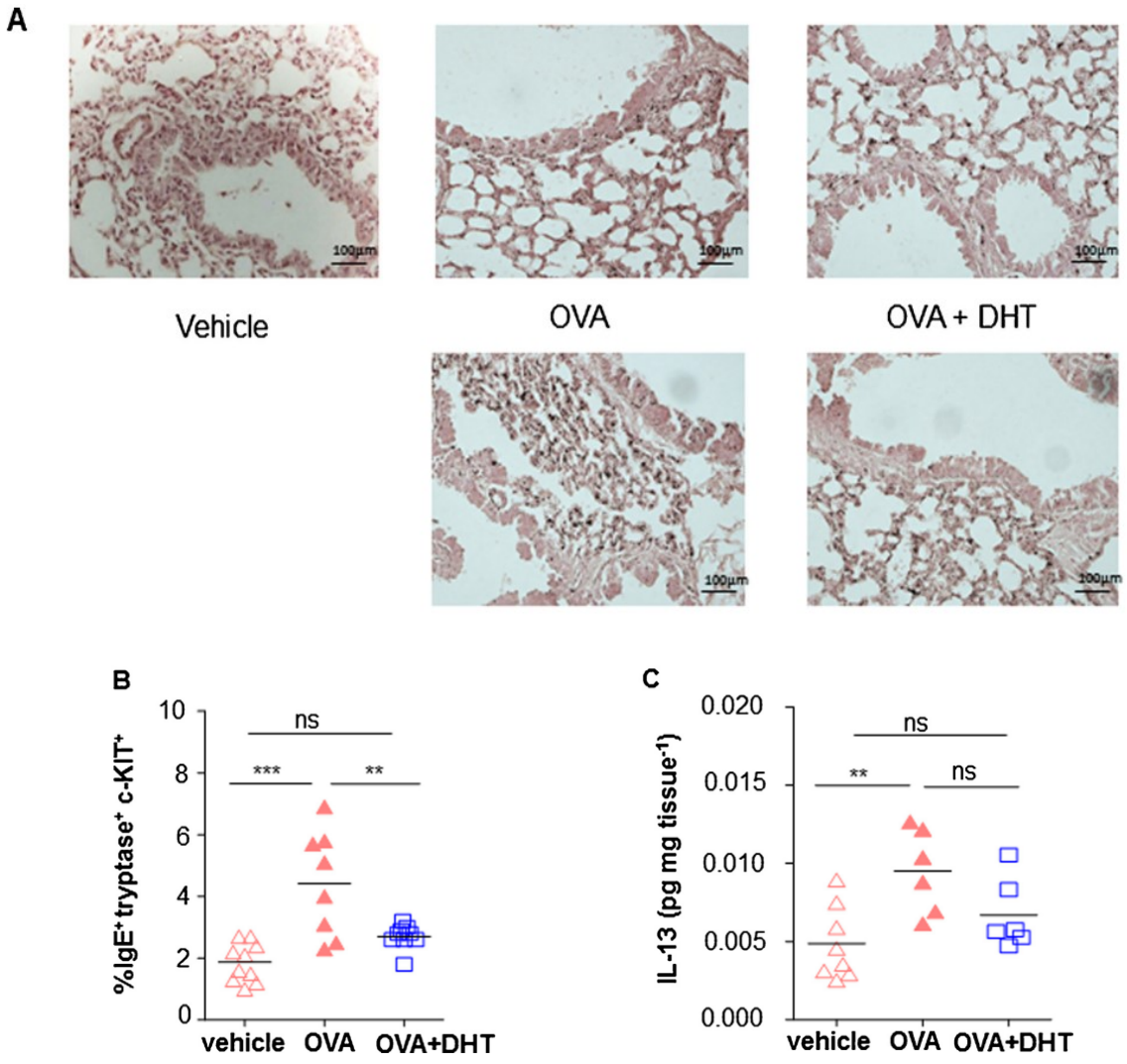


Figure 5. 13 Mast cells activation following OVA sensitization

Tryptase lung immunohistochemistry (**A**), percentage of $IgE^{+}tryptase^{+}$ $c\text{-}KIT^{+}$ cells (**B**) and pulmonary levels of IgE (**C**) carried out following 14 days of sensitization on female BALB/c mice. Data shown as a single value and means; n=6 mice per group. Statistical analysis used: one-way ANOVA plus Bonferroni; **P<0.01; ***P<0.001.

Effect of 5α-DHT on mast cell degranulation

Mast cell degranulation was evaluated using mast-cell like RBL-2H3 cell line. These cells were pretreated with the antigen DNP-HAS to induce degranulation that was evaluated as hexosaminidase release. This antigen-induced degranulation was inhibited by DHT and

cromolyn *(Fig. 5.14 A)*. To further confirm the activity of DHT on mast cell degranulation, the experiment was repeated using Ca^{2+}-ionophore A23187 as a degranulation stimulus. A similar effect of DHT was observed using A23187 *(Fig. 5.14 B)*.

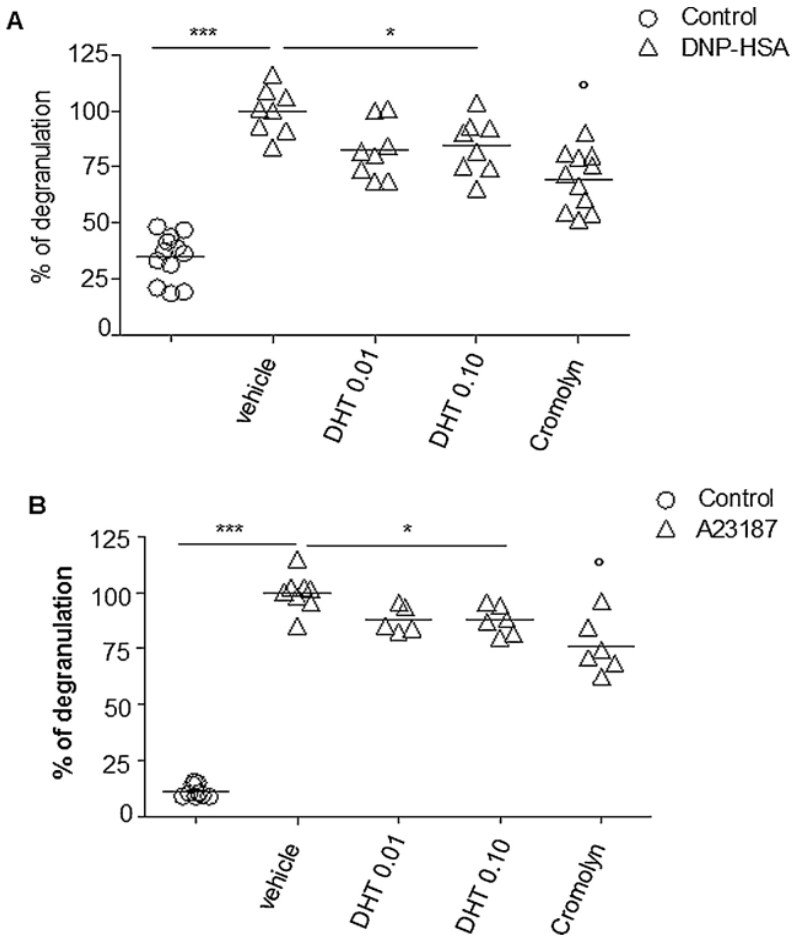


Figure 5. 14 DHT effect on RBL-2H3 degranulation

This cell line was incubated with the antigen DNP-IgE for 24h. Following time incubation, cells were incubated for 5 minutes with DHT (0.01 and 0.10 nM), cromolyn (1mM) or the vehicle (DMSO 0.1%). Triton-X-100 was added to induce cell lysis and the release of hexosaminidase, that was measured at 405nm. Data shown as a single value and means**.** Statistical analysis used: one-way ANOVA plus Bonferroni; *P<0.05; ***P<0.001; °P<0.001 vs. vehicle DNP-HAS (A) and vehicle A23187 (B).

5.2.6 Discussion and Conclusion

The performed experiments confirmed the sex differences in airway reactivity to carbachol following OVA sensitization between male and female BALB/c mice. Intraperitoneal DHT administration during the sensitization phases abrogated OVA-induced hyperreactivity to carbachol in female mice, cancelling the sex differences in carbachol-induced-reactivity. The DHT activity was comparable to ZIL activity in airway function, both reduced airway hyperreactivity induced by OVA. Moreover, DHT administration decreased the LTs pulmonary levels in female OVA-sensitized mice while, as we have already seen in previous experiments, there was no increase in the LTs pulmonary levels in male OVA-sensitized mice. Thus, DHT showed a protective role in airway reactivity and this correlates with LTs pathways. The protective role of DHT in airway function was confirmed by immunohistological analysis, DHT administration reduced the cell infiltration and mucus production in lung harvested from OVA-sensitized female mice. It is well known that OVA-induced sensitization creates Th2 environment with an increase of all Th2 mediators, for instance, IL-4 and 13. For this reason, the effect of DHT on the Th2 environment was evaluated. Anyway, as the experiments reported, DHT had no significant effect on Th2 mediator levels, indicating that DHT did not affect TH2 immune response.

Mast cells are the main cells involved in the release of proinflammatory mediators following OVA-sensitization and are associated with the increase of IgE levels in the lung. The immunohistological analysis demonstrated that DHT administration reduced the mast cell activation in lung tissue and this correlated with the reduction of the percentage of IgE^{+} -$tryptase^{+}$-$cKIT^{+}$. To further analyse the effect of DHT on mast cells, RBL-2H3 cell line were pretreated with DHT and then exposed to two different stimuli, antigenic stimuli, or calcium ionophore, in both cases DHT treatment reduce mast-cells degranulation. These experiments suggested that the effect of DHT was mediated by the activation and the degranulation of mast cells. Thus, the protective role of DHT on airway function and inflammation could be mediated by the non-genomic effect. Also, the activity of DHT on LTs production could be mediated by the non-genomic effect through the phosphorylation of ERK2 (Pergola C., 2008).

Thus, in conclusion, our study confirmed the protective role and the capacity of DHT to abrogate sex differences in airway reactivity and inflammation. In accordance with the previous experiments, this study strongly suggested that sex-hormones fluctuations

should be considered for targeted therapy. Since DHT showed a protective role also in LTs production and these mediators were widely involved in airways diseases, anti-LTs therapy could be suggested in adult women and old men because in both cases there is a decrease in androgens levels.

Chapter 6

Chapter 6

6.Role of Sex in β_2-agonists activity

6.1 Rationale

Different studies have pointed out that sex hormones could affect airway relaxation and β_2-agonists activity (Machuki J.O., 2019) (Zhu B., 2016), anyway, the mechanisms which underline this difference are still unclear.

Testosterone (TES) can enhance the relaxation induced by β_2-agonists showing a synergistic effect (Suárez L, 2011). TES induces the bronchial relaxation through the blockage of L-type voltage-dependent Ca^{2+} channels (L-VDCC) and PGE_2 seems to be involved in this process since indomethacin partially prevents TES-induced relaxation (Perusquía M, 2015).

6.2 Aim

Herein, we have investigated the mechanisms that underlie the sex differences in airway relaxation and the β_2-receptor activity.

6.3 Material and Methods

Material and methods related to these experiments are reported in Chapter 3.

6.4 Statistical Analysis

Statistical analysis related to these experiments is reported in Chapter 3. Details of each analysis and statistic parameters are explained in the description of the graphs.

6.5 Results

Analysis of sex-differences in airway relaxation

Sex difference in β_2-receptor-mediated relaxation was analysed in different strains of mice (BALB/c, CD1, and C57/Bl6). Bronchi were harvested and subjected to a concentration-response curve of salbutamol.

The analysis of airway relaxation in physiological conditions showed significant results. Indeed, in *Figure 6.1*, bronchi harvested from male mice (BALB/c, CD1, and C57/Bl6) showed a significant increase in β_2 -receptor activity induced by β_2-agonist salbutamol ex-vivo stimulation.

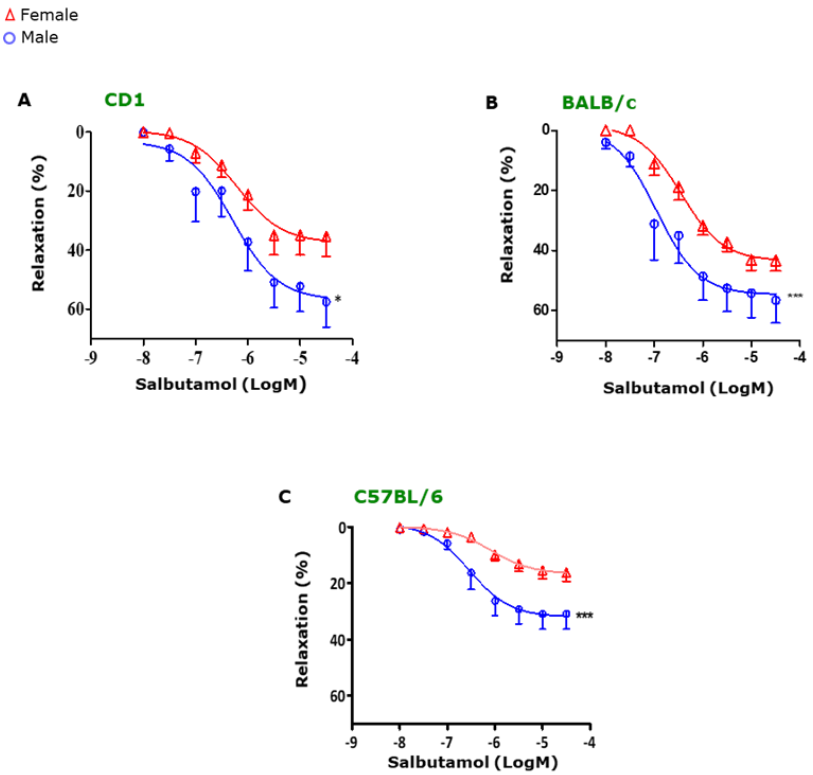


Figure 6. 1 Analysis of airway relaxation in physiological condition

Bronchi harvested from male and female CD1 (A), BALB/c (B) and C57BL/6 (C) mice were placed in isolated organ bath to analyse airway relaxation to β_2-agonist salbutamol. Data represent mean ± S.E.M. and significant is represented by *P<0.005 and ***P<0.001. Statistical analysis used is Two-way ANOVA plus Bonferroni.

Prolonged stimulation induced by β_2-agonists reduces the β_2-receptors activity

Adult male and female BALB/c and CD1 mice were sacrificed to analyse a prolonged β_2-receptors-induced relaxation through isolated organ baths. Two successive concentration-response curves of salbutamol (0.01 mM-0.03 μM) were performed at 15 minutes from each other. The first concentration-response curve of salbutamol induced around 40% of relaxation both in male and female CD1 mice (Figure 6.2A and B). Prolonged stimulation of β_2-receptors with the second concentration-response curves of salbutamol significantly reduced the activity of the receptor both in male and female CD1 mice (***P<0.001) (Fig. 6.2 A and B).

The same effect was observed in male and female BALB/c mice (Fig. 6.2 C and D). Particularly, male mice showed a β_2-receptors-induced relaxation around 60% (Fig. 6.2 C) while female around 40% (Fig. 6.2 D) following the first concentration-response curve of salbutamol. Herein, as for the CD1 mice, prolonged stimulation by β_2-agonists induced a complete loss of β_2-receptors activity both in male and female BALB/c mice (***P<0.001) (Fig.6.2 C and D), demonstrating that prolonged stimulation induced by β_2-agonists induces the β_2-receptors desensitization process in male and female mice at the same manner.

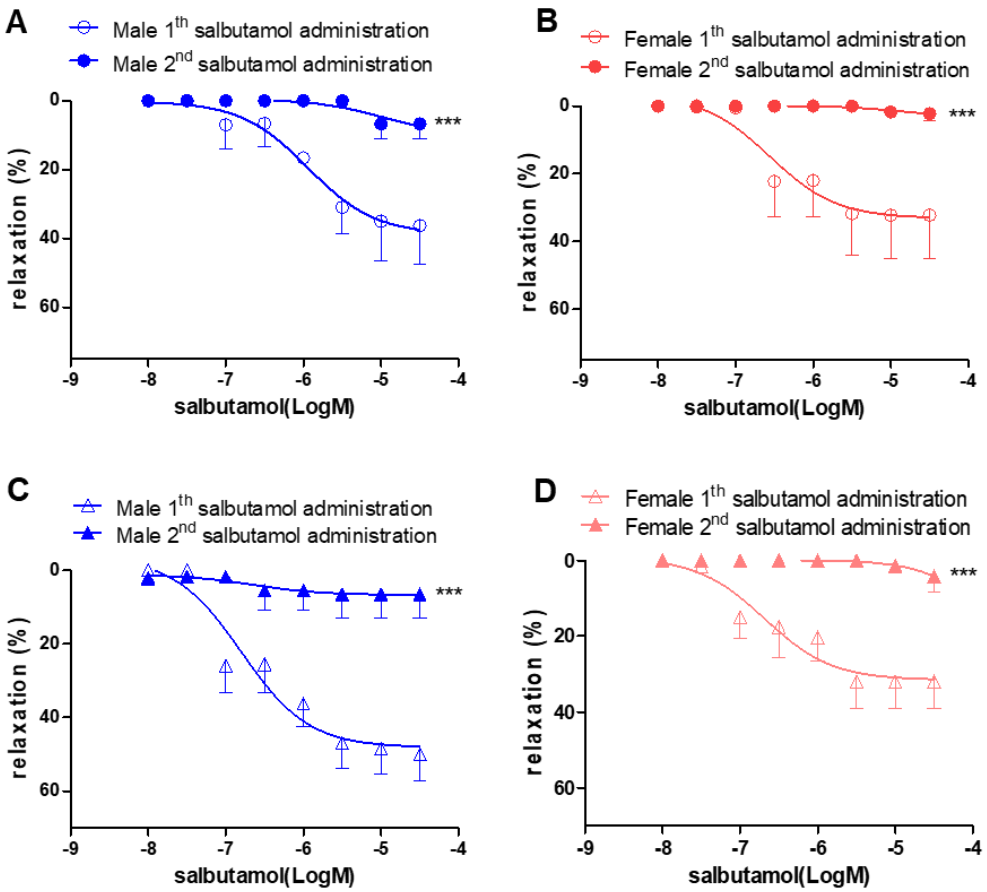


Figure 6. 2 Airway relaxation in CD1 and BALB/c mice

Prolonged stimulation of β_2-receptors in male **(A)** and female **(B)** CD1 mice and in male **(C)** and female **(D)** BALB/c mice. Data represent mean ± S.E.M. n= 4 for each group; Statistical analysis used: two-way ANOVA plus Bonferroni; ***P<0.001.

Montelukast activity on β_2-receptors mediated relaxation

Since cys-LTs are widely involved in the β_2-receptor desensitization process, Montelukast activity was evaluated on bronchi exposed to β_2-agonist administration. Bronchi from male and female CD1 mice were subjected to a first concentration-response curve of salbutamol and then preincubated with Montelukast (1µM) for 10 minutes (Figure 6.3). After this incubation, bronchi were exposed to a second concentration-response curve of salbutamol.

Following previous experiments, prolonged stimulation of β_2-receptor with two successive curves of salbutamol decreased the receptor activity both in male and female mice (Figure 6.3). Anyway, Montelukast incubation restored the β_2-receptor activity in both sexes (Figure 6.3) but the effect was enhanced in male than female mice.

Also, the prolonged stimulation of β_2-receptor with a long-acting β_2-agonist Formoterol significantly decreased the receptor activity in both sexes (figure 6.4 A, B). Pre-incubation of Montelukast 0.1 µM for 5 minutes on carbachol tone restored β_2-receptor activity.

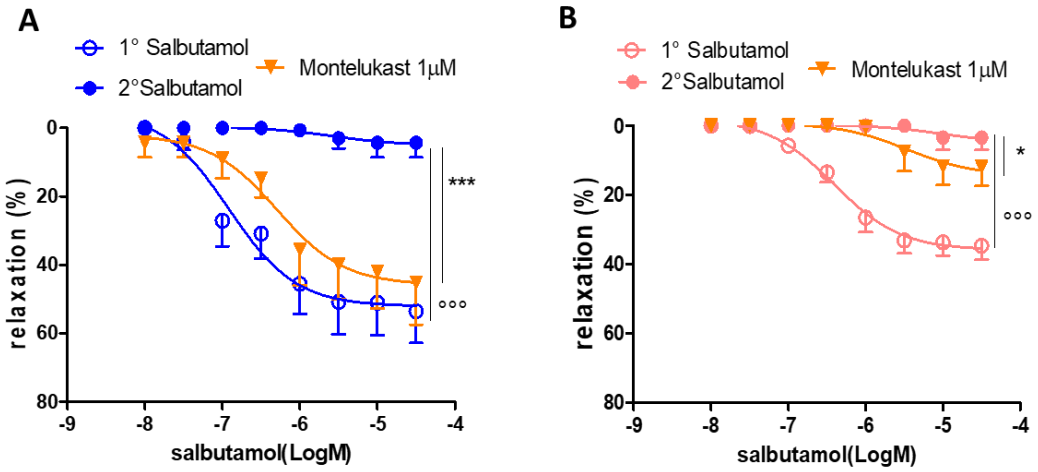


Figure 6. 3 Montelukast activity on salbutamol induced-relaxation

Effect of Montelukast on β_2-receptors mediated relaxation in **(A)** male and **(B)** female CD1 mice. Bronchi were preincubated with Montelukast (1µM) for 10 minutes and then subjected to a second salbutamol concentration-response curve. Data represent mean ± S.E.M. Number of mice: **(A)**11 mice for 1°Salbutamol, 3mice for 2°salbutamol, 6 mice for Montelukast 1 µM; **(B)**15 mice for 1°Salbutamol, 4mice for 2°salbutamol, 6 mice for Montelukast 1 µM. Statistical analysis used: two-way ANOVA plus Bonferroni; ***P<0.001 vs Montelukast 1 µM, °°°P<0.001 (1°Salbutamol vs 2° salbutamol). *P<0.05 vs Montelukast 1 µM.

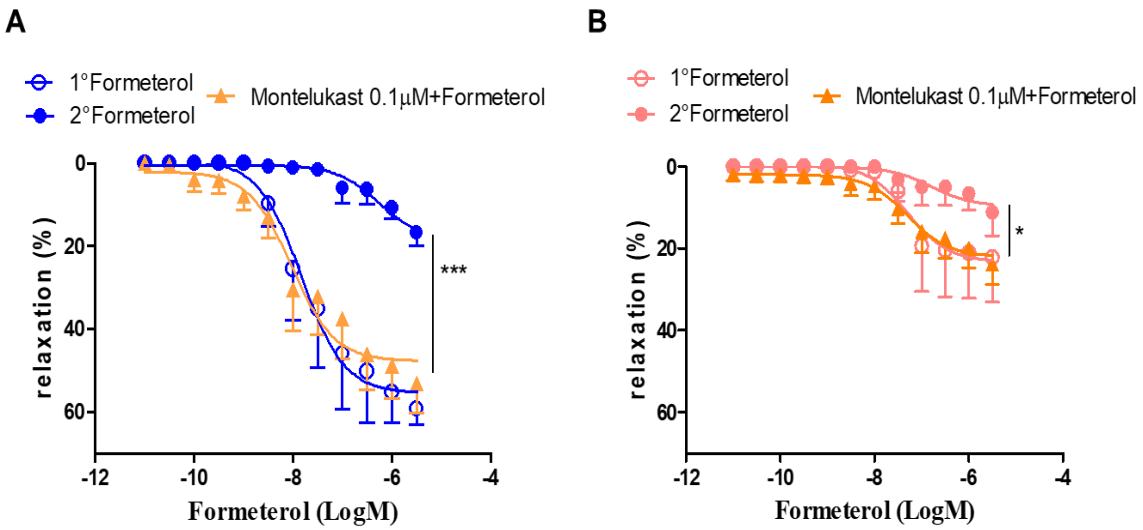


Figure 6. 4 Montelukast activity on formoterol induced-relaxation

Effect of Montelukast on formoterol induced relaxation in **(A)** male and **(B)** female CD1 mice. Bronchi were preincubated with Montelukast (0.1µM) for 5 minutes on carbachol tone and then subjected to a second formoterol concentration-response curve. Data represent mean ± S.E.M. Number of mice: **(A)**5 mice for 1°formeterol, 5 mice for 2°formeterol, 8 mice for Montelukast 0.1µM; **(B)** 5 mice for 1°formeterol, 5 mice for 2°formeterol, 11 mice for Montelukast 0.1 µM. Statistical analysis used: two-way ANOVA plus Bonferroni; ***P<0.001, *P<0.05 vs Montelukast 0.1 µM.

β_2-receptors expression in male and female BALB/c mice

To further analyse sex differences in airway relaxation, Western Blot was performed on bronchial lysate to evaluate the β_2-receptors expression in male and female BALB/c mice. Bronchial tissues were harvested from male and female BALB/c mice and homogenate according to the above protocol.

Sex differences in β_2-receptors-induced relaxation between male and female mice were not linked to sex differences in β_2-receptors expression (Figure 6.5 A).

Moreover, the PDE4a expression in bronchial tissue was evaluated to further investigate the mechanism involved in sex differences in bronchial relaxation (Figure 6.5 B). Western blot analysis demonstrated that PDE4a expression was higher in female than male

BALB/c mice, suggesting that different amounts of cyclic-AMP could be involved in the bronchial relaxation between male and female BALB/c mice.

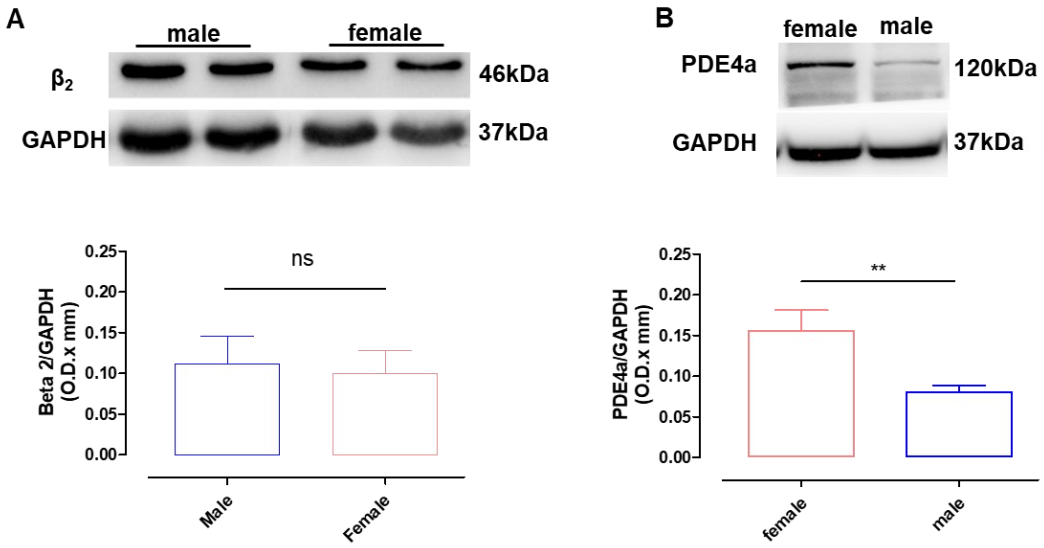


Figure 6. 5 Sex difference in β2-receptors and PDE4a expression

Western Blot analysis on bronchial tissue for **(A)** β_2-receptors and **(B)** PDE4a expression in male and female BALB/c mice. Bronchial tissues were harvested and homogenate in RIPA buffer to perform Western Blot analysis. Data represent mean ± S.E.M. n=10-12 for each group. Statistical analysis used: t-test (non-parametric, Mann-Whitney test); **P<0.001.

PDE inhibition restores β2-receptors- induced relaxation in female mice

Since female mice showed an increase in PDE4a expression in bronchial tissue, bronchi from male and female mice were preincubated with 3-isobutyl-1-methylxanthine (IBMX), a PDE inhibitor, to investigate the role of PDE on β2-receptors induced relaxation.

Following previous experiments, prolonged stimulation of β2-receptors with salbutamol significantly reduced the bronchial relaxation both in male and female mice (figure 6.6). Anyway, the IBMX pretreatment (0.1μM; 10 minutes) restored the β2-receptors activity in female but not in male mice (figure 6.6).

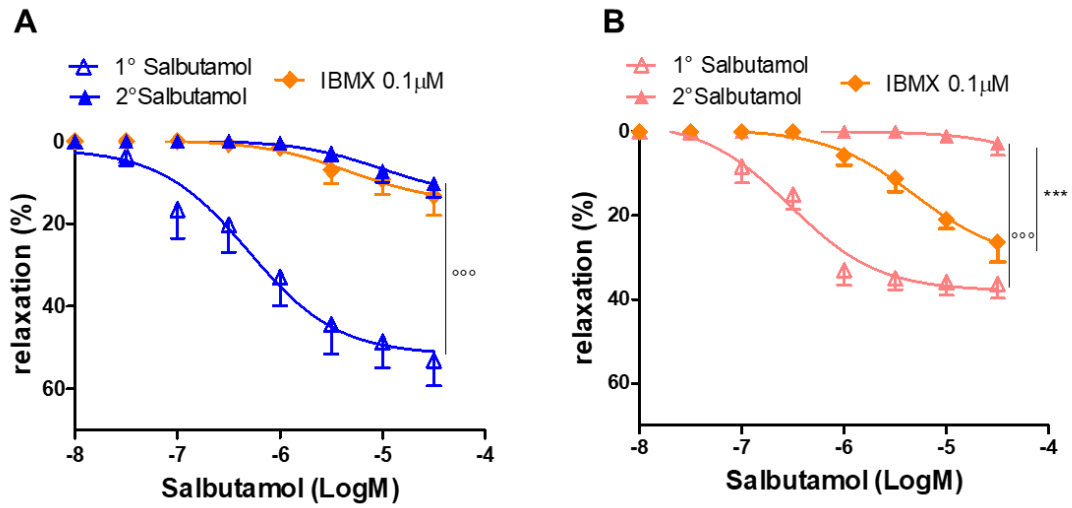


Figure 6. 6 IBMX effect on bronchial relaxation

Bronchi from male **(A)** and female **(B)** mice were exposed to IBMX 0.1µM for 10 minutes (vehicle received krebs solution) and then salbutamol concentration-response curve was performed. Data represent mean ± S.E.M. n= 10 for 1° salbutamol (male and female); n=4 for 2° salbutamol male group; n=6 for 2° salbutamol female group; n=6 for IBMX 0.1 µM male group; n=4 for IBMX 0.1 µM female group. Statistical analysis used: two-way ANOVA plus Bonferroni; ***P<0.001.

Role of Nitric Oxide (NO) in β2-receptors induced relaxation

As we have already seen above, male, and female mice showed different β_2-receptors-induced relaxation in physiological conditions. Trying to understand the mechanisms involved in this sex difference, the role of NO was analysed in bronchial relaxation.

To investigate if there is any sex difference in NO effect on β_2-receptors- induced relaxation, bronchial tissues were pre-incubated with a Nitric-oxide-synthase inhibitor (NOS inhibitor) L-NAME 100µM for 15 minutes, and then salbutamol curves was performed. L-NAME pretreatment did not affect the bronchial reactivity of male mice (Fig. 6.7 A) while significantly increased the β_2-receptors- induced relaxation in female mice (figure 6.7 B) cancelling sex differences in bronchial relaxation.

Moreover, to further analyse PDE activity and the correlation between NO and PDE, bronchial tissues were preincubated with Dybutiryl-cAMP (db-cAMP), a cAMP analogue that is also a PDE inhibitor, 100µM for 5 minutes. Both in bronchial tissues harvested from male and female mice db-cAMP increased the β_2-receptors relaxation (***P<0.001) (figure 6.8 A, B). Anyway, the effect is enhanced in bronchi harvested from female mice, this data correlates with L-NAME data.

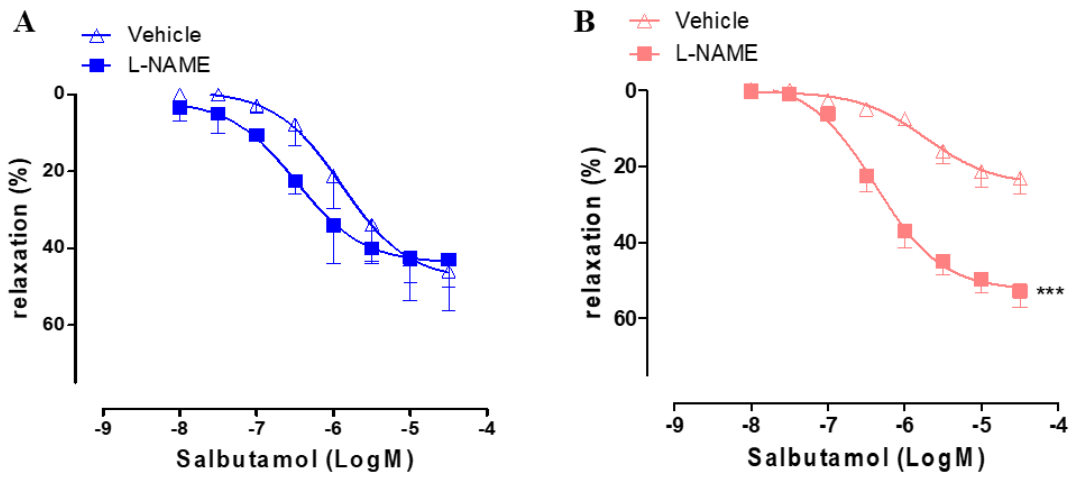


Figure 6. 7 NO effect on bronchial relaxation

Bronchi from male **(A)** and female **(B)** BALB/c mice were exposed to L-NAME 100µM for 15 minutes (vehicle received krebs solution) and then salbutamol concentration-response curve was performed. Data represent mean ± S.E.M. n= 3-5 for each group. Statistical analysis used: two-way ANOVA plus Bonferroni; ***P<0.001.

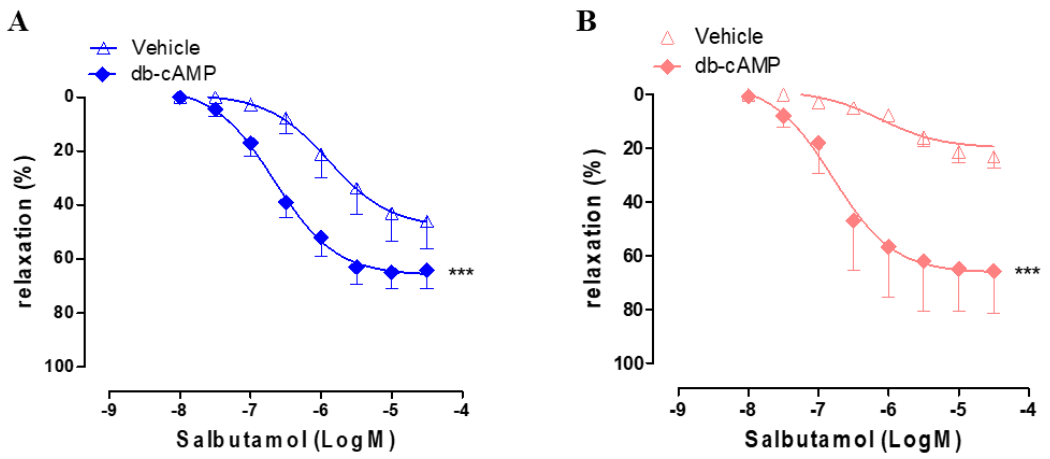


Figure 6. 8 db-cAMP effect on bronchial relaxation

Bronchi from male **(A)** and female **(B)** BALB/c mice were exposed to db-cAMP 100μM for 5 minutes (vehicle received krebs solution) and then salbutamol concentration-response curve was performed. Data represent mean ± S.E.M. n= 3-5 for each group. Statistical analysis used: two-way ANOVA plus Bonferroni; ***P<0.001.

6.6 Discussion and Conclusion

As we have already seen in airway contraction, gender plays an important role also in β_2-receptors induced relaxation. Indeed, the experiments demonstrated that male mice, regardless of strain, showed a greater bronchial relaxation than female mice. Gender involvement was visible not only in the β_2-receptors induced relaxation but also in mechanisms involved in the β_2-receptor desensitization. Pretreatment of bronchi with montelukast restored the bronchial relaxation mainly in male mice suggesting that LTs could be involved in β_2-receptor desensitization playing a different role between males and females.

Moreover, we have investigated the β_2-receptor response in male and female mice to confirm that this sex difference in β_2-receptors activity was not related to the sex difference in the β_2-receptors expression, thus western blot analysis was performed on bronchi.

The PDE activity seems to be involved in β_2-receptor-induced relaxation. Indeed, higher PDE4a expression was found in bronchi from female mice, and pretreatment of bronchi

with IBMX, a non-selective PDE inhibitor, restored the β_2-receptor relaxation in female mice eliminating the sex difference. Thus, gender could affect PDE4a expression in bronchi and this could modify the amount of cAMP in females resulting in a different airway relaxation.

To further investigate the connection between gender and the PDE pathway, tissues were preincubated with L-NAME, a NO synthesis inhibitor. In literature is already known that NO, particularly NO-sensitive guanylyl cyclase (NO-GC), increases the PDE expression and activity in SMC increasing cAMP levels (Dünnes S, 2018). This could explain the activity of L-NAME in bronchi from female mice. Inhibiting NO synthesis, L-NAME could affect the PDE pathway, which is higher in females, and this leads to an increase in cAMP intracellular level cancelling the sex difference in bronchial relaxation. Moreover, cAMP analogue db-cAMP, which acts through the inhibition of PDE, was incubated on bronchi inducing an increase in β_2-receptors mediated relaxation mainly in female mice.

To conclude, our study demonstrated that sex affects the β_2-receptors activity and bronchodilators response. Moreover, this sex difference was also present in mechanisms involved in receptor desensitization in which LTs play an important role. Anyway, LTs appeared to be involved in the desensitization process differently in males and females. Indeed, Montelukast treatment was influenced by sex and it showed a greater effectiveness in restoring airway relaxation in males compared to females. A mechanism involved in this sex difference in airway relaxation was the PDE-NO pathway. PDE4a seems to have a down-regulatory activity on β_2-receptors in females resulting in a reduction of airway relaxation. Acting on this mechanism, through PDE inhibitor or NO-synthase inhibitor, the sex difference in β_2-receptors activity was deleted. Taken together, all these data might induce to think that targeted bronchodilatory therapy may be necessary and this could involve the use of PDE inhibitor in female rather than in the male.

Chapter 7

Chapter 7

COLLABORATIONS

7.1Collaboration with Pharmaceutical Company

During my Ph.D. course, I had the opportunity to spend a period at the pharmaceutical company Genetic S.p.A. (Fisciano, SA), where I spent six months under the supervision of Dr. Antonio Petti (QP) and the head of R&D Laboratory Dr. Ivan Trerotola (QA).

Genetic S.p.A. is a company that deals with research and development, manufacturing, and marketing of innovative therapeutic solutions in different therapeutic areas, such as airways diseases, inflammatory diseases, and heart failure. Moreover, Genetic S.p.A. offers to third parties its licenses and concessions for the production and packaging of specialty pharmaceuticals.

During my time at this company, I witnessed the development of generic inhaler drugs by carrying out qualitative and quantitative analysis.

7.1.2 Stability Analysis on the pivotal biobatch

The key step in the development of generic products is the analysis of the reference product (Lionberger R. A., 2008) to develop the generic product and all the processes used for the development. Successively, the bioequivalent studies are performed on the pivotal biobatch to demonstrate that generic product is therapeutically equivalent to the original branded drug (Lavorini F., 2013) (figure 7.1).

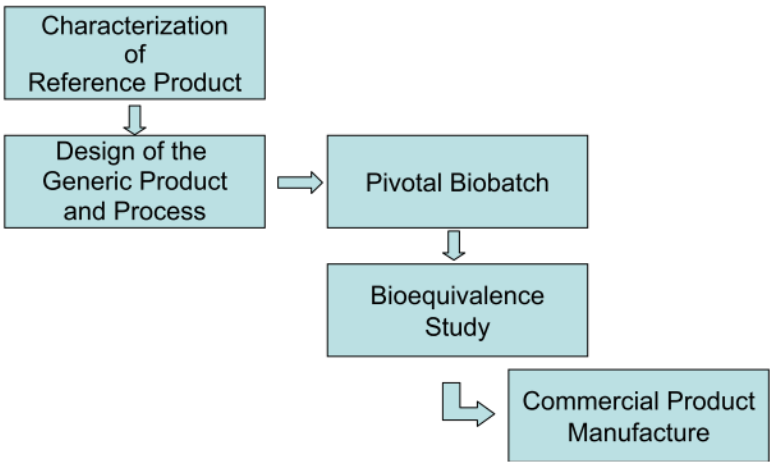


Figure 7. 1 Development of generic drugs

(Lionberger R. A., 2008)

I studied the stability of inhaler products stored at different temperatures and humidity conditions (pivotal biobatch: 25°C- 60% humidity; 30°C-65% humidity). Inhaler testing and quantitative analysis, with NGI (Next Generation Pharmaceutical Impactor) and HPLC (High-performance liquid chromatography) respectively, were performed on inhaler product stored at different temperature to evaluate the correct method of storage to avoid damage to the product. Another inhalation test I performed was the breathing test to simulate the child and adult breath and evaluate the number of inhaled particles according to the different breath between adults and children.

7.1.3 Inhaled devices

The principal strategy to treat obstructive lung disease, like asthma and COPD is the inhaled drug delivery. Compared to the oral therapy, this strategy has three main benefits, the first one is the rapid onset of action, second, the high local concentration, and the third one is the absence of a needle (Pleasants RA., 2018). However, the effectiveness of inhalation drugs varies not only concerning the formulation but also according to the characteristics of the device used. There are different types of devices used for inhalation therapy, and these are commonly divided into three categories: pressurized metered-dose inhalers (pMDIs); dry powder inhalers (DPIs); and nebulizers (Martin AR., 2014).

The pMDIs are the first used multi-dose portable inhalation device (Pleasants RA., 2018), and all inhaled drugs are available in this form as single or combination products. This device is composed of an aluminium canister that contains the gas phase and liquid phase, the formulation, which is either solution, suspension, and contains a propellant, suspending agents and surfactants (Pleasants RA., 2018). The main advantages of this device are the portability, compactness, and availability during emergencies but what mainly limits its use is the coordination of inspiration and actuation, this is the main reason why this device is not suitable for children and the elderly.

DPIs have been developed later to overcome the limit regarding the coordination of inspiration and actuation. This device delivers the drug in the form of a dry powder and often has lactose as an excipient to improve the flow-ability of the powder (Pleasants RA., 2018). Compared to pMDI, this device is activated with the breath indeed, the patient must take a deep breath to mobilize the powder anyway, this could be a limit in the use of this device for some patients.

Nebulizers contain the drug in aqueous solution or suspension and then this is atomized in aerosol droplets (Martin AR., 2014). The main advantage of this device is that it does not require a special breathing technique, thus this is suitable also for children and the elderly. Anyway, also this device has a disadvantage indeed, the administration of the same amount of the active agent takes longer with the nebulizer rather than MDIs and DPIs.

7.1.4 Next Generation Pharmaceutical Impactor

NGI (Copley Scientific) is a device designed for the pharmaceutical industry for testing MDI, DPI, and nebulizers. This device has seven stages (five with cut-offs between 0.54 and 6.12 microns) and can operate at any flow rate between 30 and 100L/min (Marple VA., 2003). NGI is made up of two sections, the lower part with the collection cups while the upper part consists of the lid and the stages. The nozzle stages are coupled in one plate called the “seal body” (figure 7.2) which is located on the cup tray with cups where the particles are deposited during the test. The cup tray rests on a structure with hooks, bottom frame with a locking handle, that allows closing of the entire instrument. Thus, the lid contains the air passages, the seal body contains the nozzle while the bottom contains the collection cups in a tray (Marple VA., 2003). When the entire instrument is closed, the

induction port (IP) is located on the top of the device to allow the introduction of the aerosol dose through the device. The holes of the stages have different sizes, starting from stage 1 with one hole of 14.3mm until stage 7 with 630 holes of 0.206mm (figure 7.2). The last plate is for the micro-orifice collector (MOC) which has 4032 holes of 70micron. This size is lower than most of the inhaler particles, and this allows to capture of many of the inhaler particles inside the plate for the analysis. Thus, the inhaler dose passes through the IP, and depending on the size, it settles in different cups passing through the stages. After the inhaler test, all the inhaler particles are collected from the cups to perform the quantitative analysis with HPLC to evaluate where most of the inhaler particles are deposited. Particles deposited in the first stages will be able to settle in the upper tract of the airways, for instance, oropharynx, while the ones deposited in the final stages and the MOC will be able to settle in the lower tract of the airways, like alveoli and terminal bronchioles. The inhaler drugs that reach the airways exerting a local effect have a size between 1 and 5µm (Pleasants RA., 2018). Deposition of particles 2-8mm occurs in bronchi which correspond approximately to stage 2 and 3, while particles with 0.6mm diameters settle in terminal bronchioles corresponding approximately to stage 5 (figure 7.3). Smaller particles with a diameter of 200 micrometers are deposited in the alveolar sacs corresponding approximately to stage 7 ***3*** (Pleasants RA., 2018) (figure 7.3).

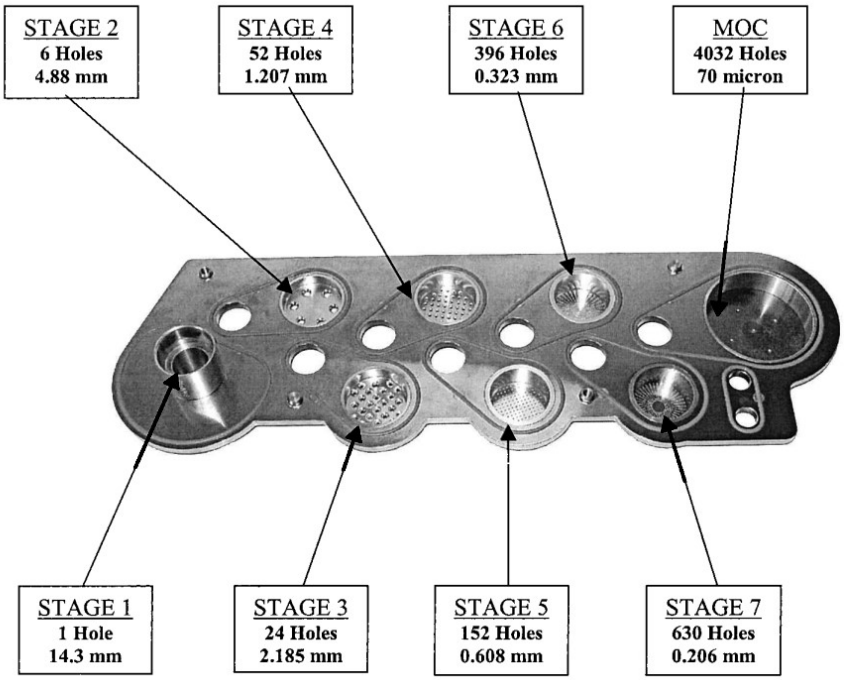


Figure 7. 2 NGI "seal boby" (top view)

(Marple VA., 2003)

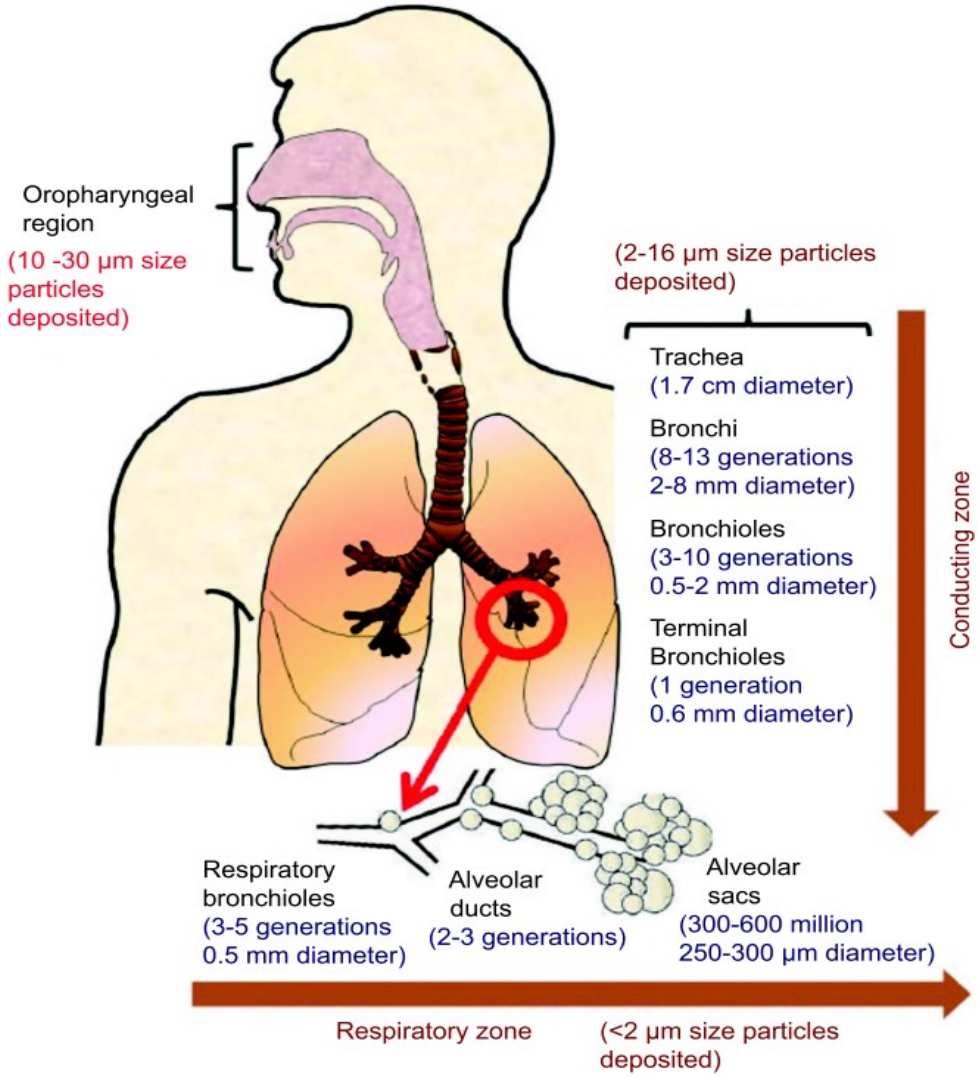


Figure 7. 3 Dimension of the airways

(Pleasants RA., 2018)

7.1.5 Breathing Simulator

The breathing simulator (Copley Scientific) is an instrument specifically designed for generating different types of breath, that of infants, children, adults, and the elderly. Thus, this device can mimic the human breath, and this is useful for orally inhaler product testing. The effectiveness of the pMDIs and DPIs depends not only on the formulation

but on the respiratory profile that varies with age. For this reason, the breathing simulators are becoming a routine part of orally inhaled product testing (Copley, 2014).

To evaluate the inhaled particles, it is necessary to set the volume required, which is approximately 500mL for adults, 155mL for the child, 50mL for infants, and 25mL for the neonate, and then, pMDIs, DPIs, or nebulizer can be connected to the device (figure 7.4). The breathing Simulator can be connected to the NGI to evaluate the deposition of inhaler particles considering the breathing profile.

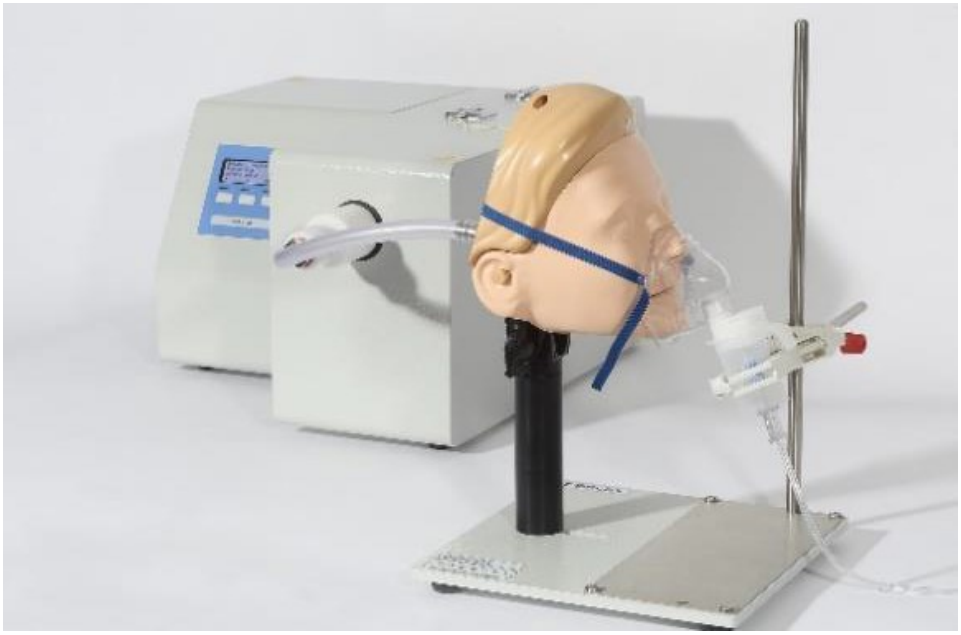


Figure 7. 4 Breathing simulator

(Driving Results in inhaler testing, Copley 2020 Edition)

7.2 Imperial College of London

During the last year of my Ph.D. course, I spent 7 months at the Imperial College of London. I carried out a research activity as a visiting student in the group of Professor Jane A. Mitchell, under the supervision of Prof. Mitchell, Dr. Nicholas Kirkby, and Dr. Maria Lopes Pires. During this period, I studied the constrictor and dilator pathways in airways and pulmonary arteries in parallel to evaluate if there is a possible correlation between bronchi and vessels.

7.2.1 Introduction

Bronchial and Pulmonary circulation

The structure of the lung is particular and allows an efficient exchange of respiratory gas, which happens between the airways and vascular tree (Petersson J., 2014). The lung is the only organ with two circulation: the bronchial and pulmonary circulation. The pulmonary circulation differs from the systemic one because the pulmonary arteries carry mixed venous blood (Suresh K., 2020). The main pulmonary artery divides into the right and left branches, which enter in the right and left lung, respectively. After entering in the lung, the pulmonary artery divides further following the bronchial ramification up to the alveoli. This proximity between bronchi and pulmonary artery induces a relationship between ventilation and perfusion (Suresh K., 2020). Moreover, the pulmonary artery bifurcation into arterioles and capillaries covers about the 95% of the alveolar surface, inducing the diffusion of the gases thanks to a thin membrane (1µm) which separate capillaries from the alveoli (Petersson J., 2014). This large surface area allows the close relationship between the air space and the blood capillaries (Hsia C.C.W., 2016). In literature is reported that this close relationship between bronchi and pulmonary artery starts during the fetal development (Suresh K., 2020). Indeed, around 38 days of gestation, pulmonary arterial SMCs (PASMCs) derive from the bronchial smooth muscle from the adjacent airways (Suresh K., 2020). The bronchial circulation has many roles that are critical in airway and lung function (McCullagh A, 2010). This circulation receives only 1% of the left ventricular output, starting from the pulmonary artery, and supplies oxygenated blood to the airways, nerves, and regional lymph nodes. Moreover, this

circulation is involved in the clearance of particulate matter from the airways and provide nutrients to the airway and other structures (McCullagh A, 2010). In addition, the bronchial circulation is also involved in the release of different mediators, like NO and PDE, which can act on bronchi and vessels.

Is there a correlation between asthma and PAH?

As described above, there seems to be a correlation between bronchial and pulmonary circulation already during the fetal development. During the gas exchange between pulmonary circulation and alveoli, different mediators seem to be released and this could affect both the bronchial and vascular tone. This correlation between bronchi and vessels in the lung is reported also in pathological condition. Indeed, it has been reported that severe structural changes and inflammatory processes were found in a wall of pulmonary arteries of a patient's that died after an asthma attack (Saetta M., 1991). Another study suggests that pulmonary circulation is affected by allergic airway inflammation (Rabinovitch M, 2015).

In 2005 Tormanen (Tormanen KR, 2005) demonstrated that allergic inflammation induced by Ovalbumin (OVA) increases the distribution of eosinophils both in peri-bronchial and perivascular tissue, demonstrating that airway inflammation, following by allergen exposure, is supported by PA inflammation. Moreover, both in pulmonary vessels and in bronchial tissue, OVA-challenge increases the number of proliferating endothelial cells. The structural changes following OVA-challenge include an increase in smooth muscle cells, epithelial and endothelial cells, and extracellular remodelling both in the airway and PA. An interesting study by Tormanen (Tormanen KR, 2005) showed pulmonary vascular remodelling and inflammation presented a similar time course as bronchial remodelling from the first OVA administration. In particular the study showed an increase in smooth muscle and endothelial cell proliferation-inducing a remodelling process following 24h after 7 days of OVA-sensitization.

T-helper type 2 (Th2) cytokines and mediators are involved in airway diseases and drive both airway inflammation, hyperresponsiveness and remodelling. The main Th2 cytokines involved in the pathophysiology of asthma are interleukin-4 and 13 (IL-4, 13), which promote B-cell isotype switching and mucus production. The severity of

pulmonary arterial remodelling, following OVA-challenge, is correlated with the levels of Th2-induced serum IgE and pulmonary cytokines (Daley E. E. C., 2008).

In addiction, the OVA-challenge enhances the remodelling process both in the bronchi anad pulmonary artery, and the same process was reported in BALB/c mice after a chronic exposure of house dust mite (Said SI., 2010).

During airway inflammation both cellular infiltration and remodelling process may spread from the site of origin to surrounding tissue, then from the bronchi to the pulmonary artery. Although evidences suggest that there is a close relationship between the response of airway and pulmonary smooth muscle cells which starts in the embryological phase, involving the migration of airway smooth muscle cells and mesenchymal cells (McCullagh A, 2010), the mechanism involved in this relationship is still unclear.

One pathway that could explain the shared pathological features between asthma and PAH is the Nuclear factor of the activated T-cells (NFAT) pathway. NFAT is a family of transcription factors involved in the immune response (Said SI., 2010). There are four NFAT proteints (NFATc1, NFATc2, NFATc3, and NFATc4) that are cytoplasmic. The dephosphorylation by the Ca^{2+}-responsive phosphatase calcineurin activates these proteins inducing the translocation to the nucleus. After the translocation, NFAT interacts with other transcription factors to exert different regulatory actions (Said S.I., 2010). The increasing levels of NFAT were found in pulmonary vessels and bronchi in both diseases, promoting smooth muscle cell proliferation and remodelling process (Bonnet S, 2007). This increase in NFAT levels is associated with a lack of Vasoactive Intestinal Peptide (VIP), both in asthma and PAH (Wu D., 2011). The effects of this peptide are mediated by two types of G-protein-coupled receptors: VPAC1 and VPAC2. The stimulation of these receptors induces an increase of cAMP inducing smooth muscle relaxation. VIP is also involved in the regulation of the immune system through the activation of the VPAC2 receptor. This receptor is expressed in innate immune cell types, human mast cells, neutrophils, and macrophages. Particularly VIP regulates the balance between T-helper type 1 and T-helper type 2 cells (Wu D, 2011).

Another mediator which is widly involved both in airway and pulmonary inflammation is the Endothelin-1 (ET-1). ET-1 is a peptide secreted by ECs, and there are three isoforms, ET-1,2 and 3, but the most expressed both in airway and pulmonary artery is ET-1. This is one of the most potent endogenous vasoconstrictor and bronchoconstrictor,

and binding the ET_A and ET_B induces SMCs contraction, proliferation and migration. ET-1 is involved in airway and vascular inflammation, indeed the up-regulation of endothelin receptors was found in vascular and airway hyperreactivity (Zhang Y., 2010). The ET_A and ET_B receptor antagonists are approved in the treatment of pulmonary arterial hypertension, like mecitentan (Belge C., 2019).

Therefore, there are many evidences that airways and pulmonary arteries share some common features in disease. In fact, the pulmonary inflammation and smooth muscle remodelling, and proliferation are the key processes involved both in pulmonary arterial hypertension and asthma. Moreover, pulmonary vessels are affected in asthma, and drugs used in the treatment of both diseases aim to reduce the remodelling process and increase the smooth muscle relaxation. Thus, there is scientific merit in studying constrictor and dilator pathways in airways and pulmonary arteries in parallel.

7.2.2 Analysis of the bronchial and vascular reactivity

Rationale

As we have seen above, there seems to be a correlation between bronchi and pulmonary arteries in physiological condition, and this correlation starts during the fetal development. This correlation is also found in pathological conditions. Moreover, the bronchial circulation plays an important role in airway and lung both in physiological and pathological conditions (McCullagh A, 2010) offering a significant contribution in the gas exchange and releasing different mediators, like NO and prostaglandins. Thus, this study aims to evaluate the constrictor and dilator pathways in airways and pulmonary arteries in parallel.

Material and Methods

Material and methods related to these experiments are reported in Chapter 3.

Aim

The aim of this study is to analyse a possible correlation between bronchial and vascular reactivity to different contracting agents. The role of nitric oxide, prostaglandins and leukotrienes has been analysed in bronchi, PA, aorta, and carotid artery. Aorta and carotid artery were used as a representative of systemic vessels.

Statistical Analysis

Statistical analysis related to these experiments is reported in Chapter 3. Details of each analysis and statistic parameters are explained in the description of the graphs.

Results

Analysis of vascular and bronchial reactivity

To analyse the vascular reactivity, 5-HT (1µM-0.01M) was administrated to vessels from C57/Bl6 mice while to evaluate the bronchial contractility a concentration-response curve of Carbachol (1µM-0.01M) was performed on bronchiole.

Aorta rings from C57/Bl6 mice were used as examples of systemic vessels to confirm the activity of drugs on vessels. Vasoconstrictor activity of 5-HT was higher in bronchial tissue compared to pulmonary arterial tissue (Figure 7.5B). The increased activity of 5-HT on bronchial tissue than pulmonary arterial tissue was confirmed by EC50 and Emax analysis (Figure 7.6B).

As expected, carbachol, a muscarinic agonist, induced a significant increase in bronchial contraction ($p<0.0001$) compared to vessels, both aorta and PA (Figure 7.5C). Carbachol did not induce vessels contraction and as is already known in the literature, this lack of contraction on vessels by carbachol could be due to muscarinic receptor activation that stimulates endothelial to release Nitric Oxide (NO) inducing dilatation of vessels (R. F. Furchgott, 1980). Emax analysis confirmed the effectiveness of the action of carbachol on bronchial tissues compared to PA and aorta (Figure 7.6C).

U46619, a thromboxane A_2 agonist, induced contraction both in vessels and bronchi. U46619 effect was enhanced in bronchial tissue ($p<0.0001$), Figure 7.5D. Anyway, there was not a significant difference in Ec50 and Emax analysis (Figure 7.6D).

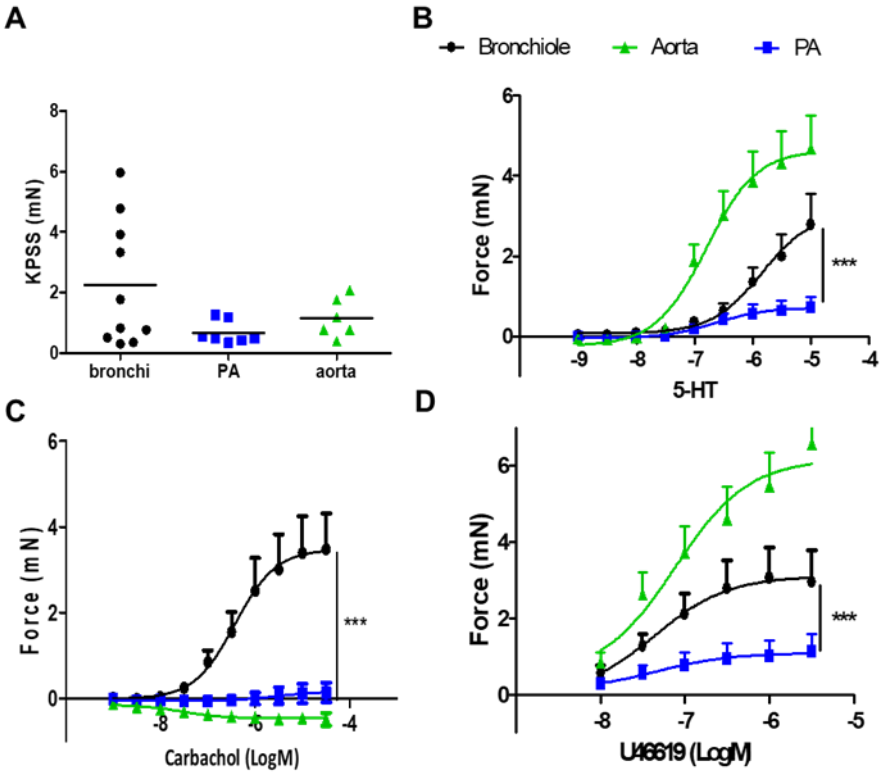


Figure 7. 5 Analysis of bronchial and vascular reactivity

Bronchi, pulmonary artery, and aorta were harvested from C57/BL6 mice and exposed to 5-HT (B), carbachol (C) and U46619 (D) concentraction-response curve using the myograph. (A) KPSS precontraction in bronchi, PA, and aorta Data represent mean ± S.E.M. n=11 for each group and significant. differences are represented by *P<0.005, **P<0.01 and ***P<0.001. Statistical analysis used is Two-way ANOVA plus Bonferroni.

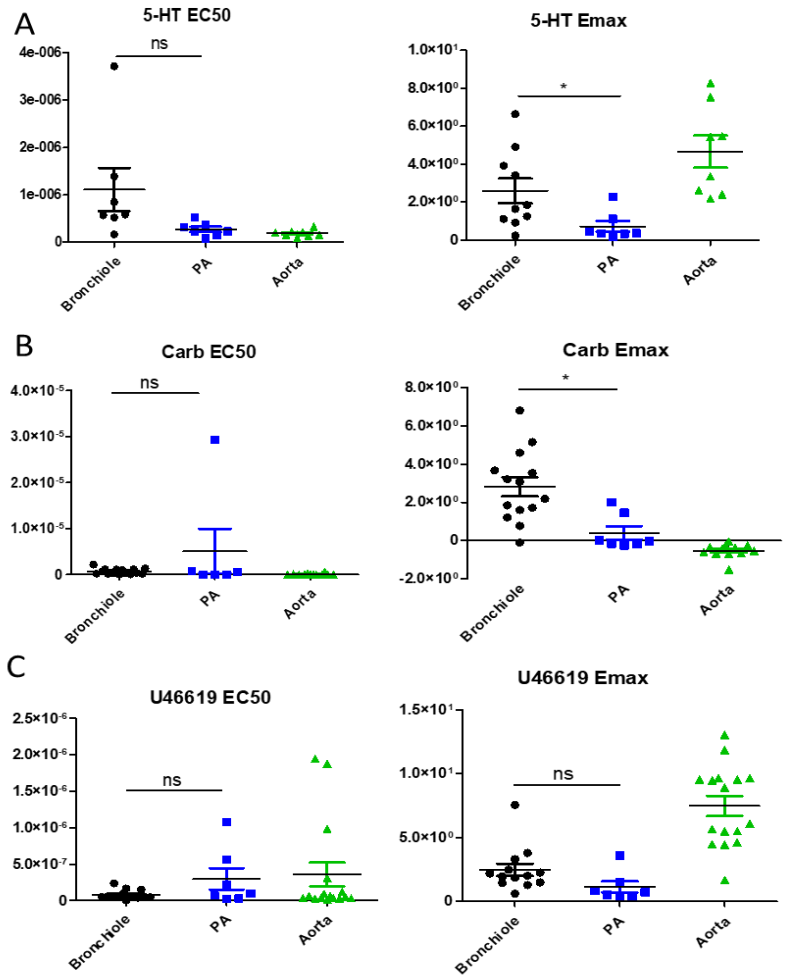


Figure 7. 6 Analysis of EC50 and Emax in bronchi, PA and aorta

EC50 and Emax analysis following (A) 5-HT, (B) carbachol, and (C) U46619 administration. Data represent mean with single data and significant is represented by n.s., not significant ($P>0.05$); *$P<0.005$. Statistical analysis used is two-tailed Student t-test.

Role of COX on bronchial and vascular reactivity

Cyclooxygenase (COX) pathway plays a critical role both in airway and vasculature diseases (Gye Young Park, 2006). Here we analysed the role of Diclofenac, a non-selective COX inhibitor, in bronchi, PA, aorta, and carotid in physiological conditions.

Before analysing COX activity, KPSS precontraction was measured in tissues used as a control and tissues used for the Diclofenac incubation protocol (Figure 7.7A).

The analysis of data demonstrated that COX-inhibition with Diclofenac (1µM) did not affect the bronchial reactivity following carbachol administration (Figure 7.7C), but significantly enhanced the bronchoconstriction induced by 5-HT (Figure 7.7B) and U46619 (Figure 7.7D).

Diclofenac had an opposite effect on the pulmonary artery compared to that observed in bronchial tissue (Figure 7.8). The inhibition of the prostaglandins pathway significantly decreased the vasoconstriction induced by U46619 (Figure 7.8 D) and reduced the activity of carbachol (Figure 7.8 C). 5-HT vasoconstriction on the pulmonary artery was not abrogated by Diclofenac (Figure 7.8B).

On aorta tissue, the inhibition of the prostaglandin's pathway did not affect the vasoconstriction induced by 5-HT (Figure 7.9B) and U46619 (Figure 7.9C).

As observed on aorta tissue, Diclofenac preincubation did not affect vascular reactivity induced by serotonin (Figure 7.10 B), and U46619 (Figure 7.10C) in the carotid tissues.

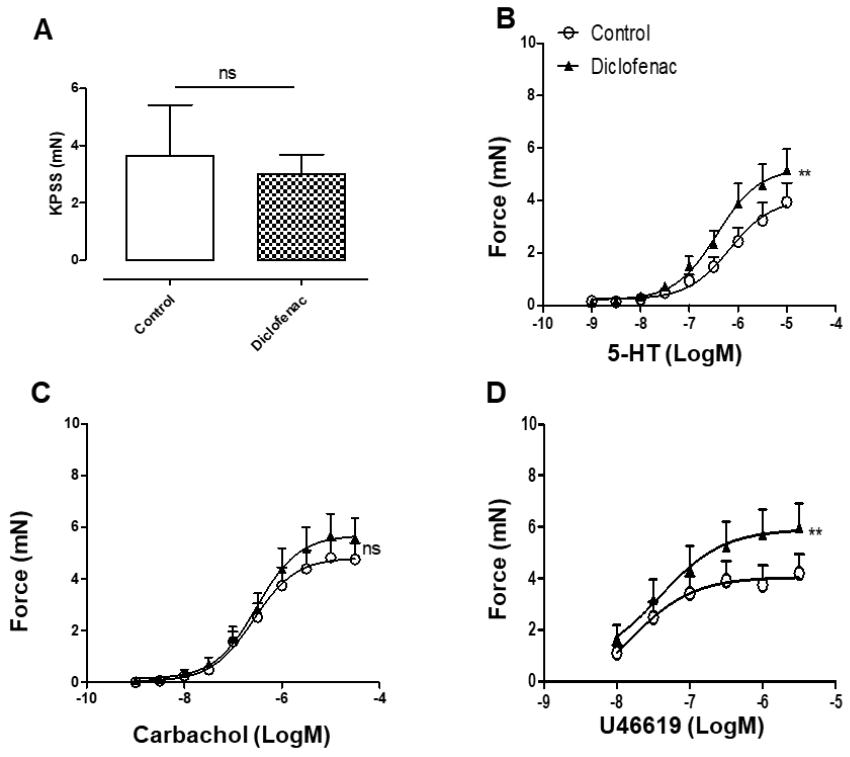


Figure 7. 7 Diclofenac activity on bronchial reactivity

Bronchi from C57/BL6 mice were preincubated with Diclofenac 1µM for 15 minutes and then exposed to 5-HT(B), carbachol (C) and U46619 (D), while

control group was preincubated with PSS for 15 minutes. (A)Analysis of KPSS precontraction in control and Diclofenac group. n=11 for each group and significant Data represent mean ± S.E.M. and significant is represented by ns: non-significative, **P<0.01. Statistical analysis used is Two-way ANOVA plus Bonferroni.

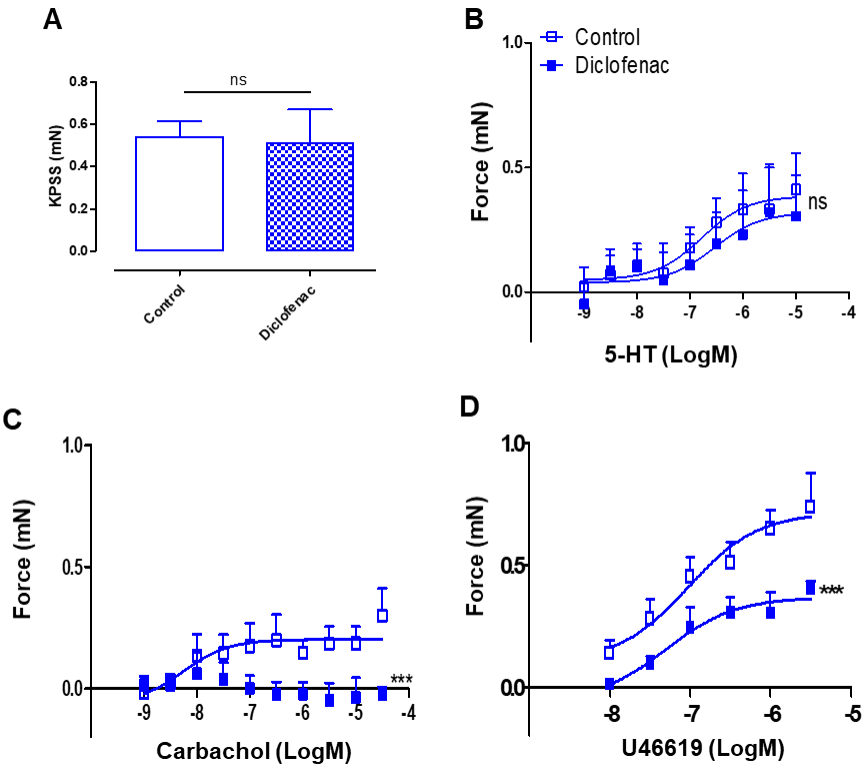


Figure 7. 8 Diclofenac effect on pulmonary arterial reactivity

Pulmonary artery from C57/BL6 mice was preincubated with Diclofenac 1µM for 15 minutes and then exposed to 5-HT(B), carbachol (C) and U46619 (D), while control group was preincubated with PSS for 15 minutes. (A)Analysis of KPSS precontraction in control and Diclofenac group. n=11 for the control group and n=6 for diclofenac group. Data represent mean ± S.E.M. and significant is represented by ns: non-significative, ***P<0.001. Statistical analysis used is Two-way ANOVA plus Bonferroni.

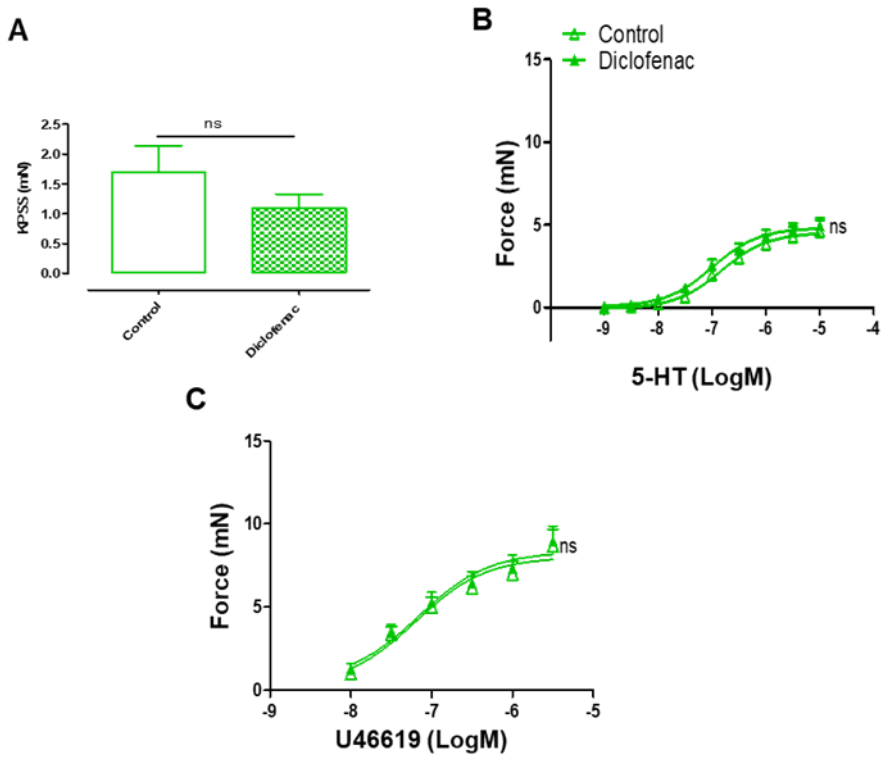


Figure 7. 9 Diclofenac effect on the reactivity of the aorta

Aorta from C57/BL6 mice was preincubated with Diclofenac 1µM for 15 minutes and then exposed to 5-HT(B), and U46619 (C), while control group was preincubated with PSS for 15 minutes. (A)Analysis of KPSS precontraction in control and Diclofenac group. n=11 for each group. Data represent mean ± S.E.M. and significant is represented by ns: non-significative. Statistical analysis used is Two-way ANOVA plus Bonferroni.

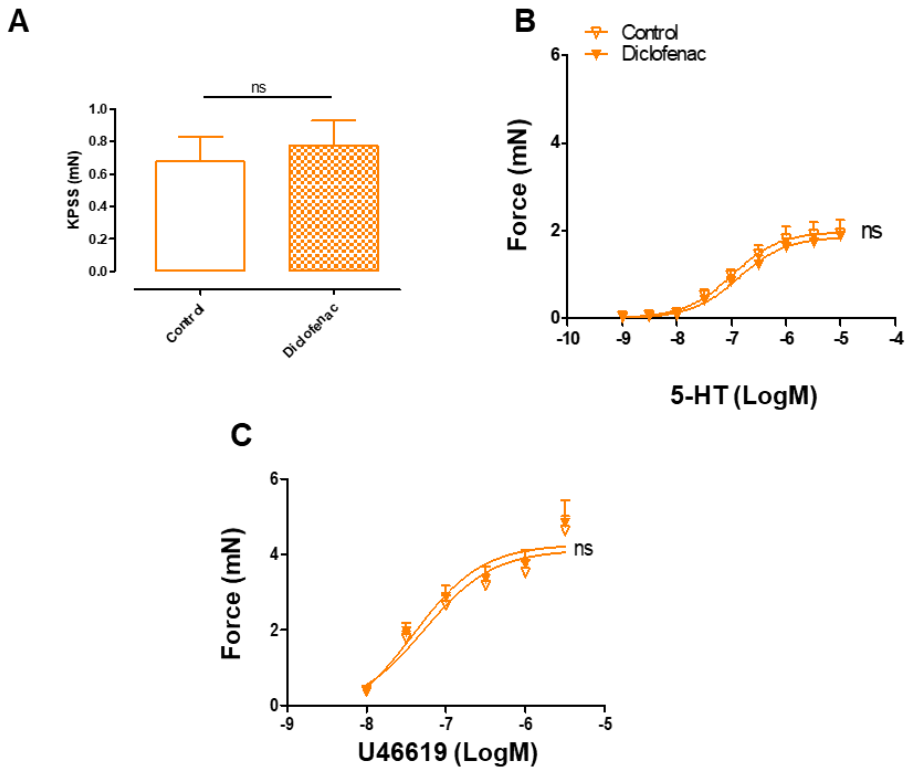


Figure 7. 10 Diclofenac effect on the reactivity of the carotid artery

Carotid from C57/BL6 mice was preincubated with Diclofenac 1µM for 15 minutes and then exposed to 5-HT(B), and U46619 (C), while control group was preincubated with PSS for 15 minutes. (A)Analysis of KPSS precontraction in control and Diclofenac group. n=11 for each group. Data represent mean ± S.E.M. and significant is represented by ns: non-significative, **P<0.01. Statistical analysis used is Two-way ANOVA plus Bonferroni.

Role of Nitric Oxide (NO) in bronchial and vascular reactivity

Bronchial and vascular reactivity were analysed following the incubation of NOS-synthase inhibitor, L-NAME, to investigate the role of the NO pathway in bronchi and vessels.

KPSS precontraction was the same in tissues used as a control and tissues incubated with L-NAME (figures 7.11 A, 7.12 A, 7.13 A, 7.14 A). The inhibition of the NO pathway had an important effect on bronchial reactivity, increasing the contractility response to 5-HT (figure 7.11 B), carbachol (figure 7.11 C), and U46619 (figure 7.11 D), and this could suggest the involvement of the NO pathway in the balance between contracting and relaxing agents in bronchial tone.

L-NAME incubation seems to have a different activity on vascular tone following carbachol incubation, reducing the vascular reactivity of the pulmonary artery to carbachol (figure 7.12 C). The NO inhibition significantly increased the reactivity induced by u46619 (figure 7.12 D). The same trend of increasing contractility seems to be also in the L-NAME group following the serotonin concentration-response curve (figure 7.12 B), anyway, there was no statistical significance.

NO inhibition did not affect vascular reactivity of the aorta following serotonin (figure 7.13 B) and U46619 administration (figure 7.13 D) but increased the reactivity to serotonin (figure 7.14 B) and U46619 (figure 7.14 D) in carotid tissue. Both in the aorta and carotid, the inhibition of the NO pathway reverted the effect of carbachol administration (figures 7.13 C and 7.14 C).

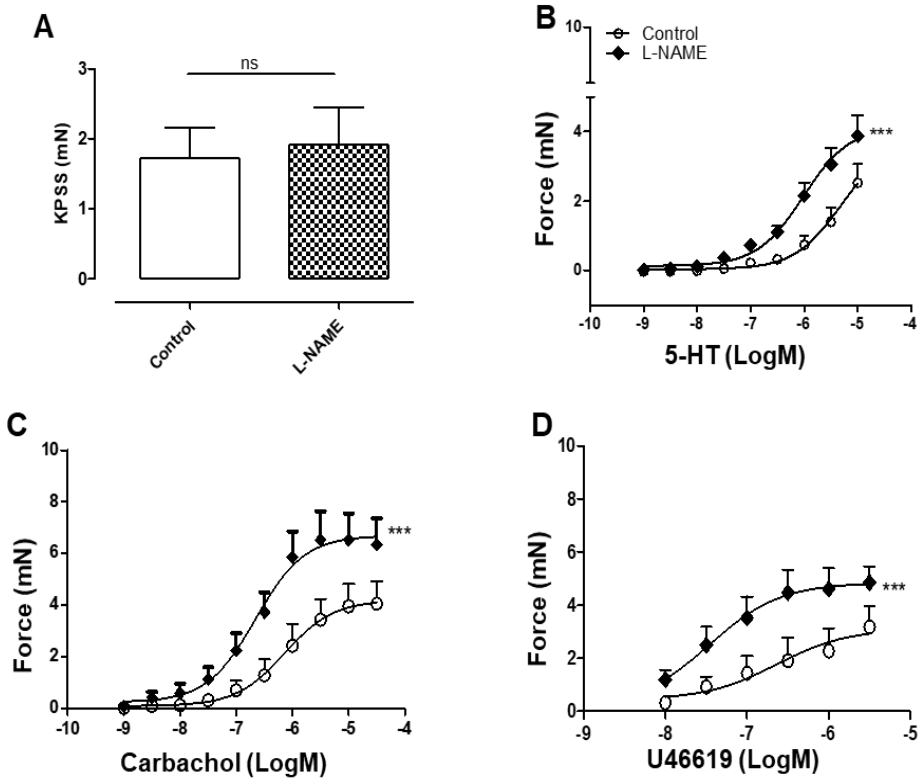


Figure 7. 11 L-NAME effect on bronchial reactivity

Bronchi from C57/BL6 mice were preincubated with L-NAME 1mM for 15 minutes and then exposed to 5-HT(B), carbachol (C) and U46619 (D), while control group was preincubated with PSS for 15 minutes. (A)Analysis of KPSS precontraction in control and L-NAME group. n=6 for each group. Data represent

mean ± S.E.M. and significant is represented by ns=non- significative; ***P<0.001. Statistical analysis used is Two-way ANOVA plus Bonferroni test.

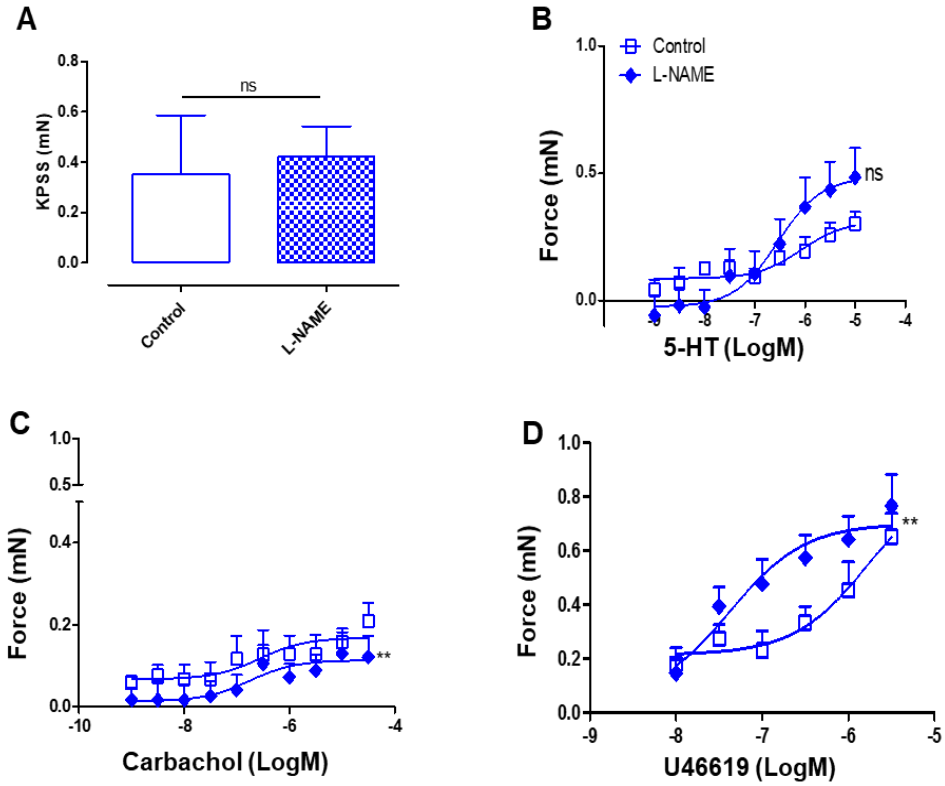


Figure 7. 12 L-NAME effect on vascular reactivity of the PA

Pulmonary artery from C57/BL6 mice were preincubated with L-NAME 1mM for 15 minutes and then exposed to 5-HT(B), carbachol (C) and U46619 (D), while control group was preincubated with PSS for 15 minutes. (A)Analysis of KPSS precontraction in control and L-NAME group. n=6 for each group Data represent mean ± S.E.M. and significant is represented by ***P<0.001. Statistical analysis used is Two-way ANOVA plus Bonferroni test.

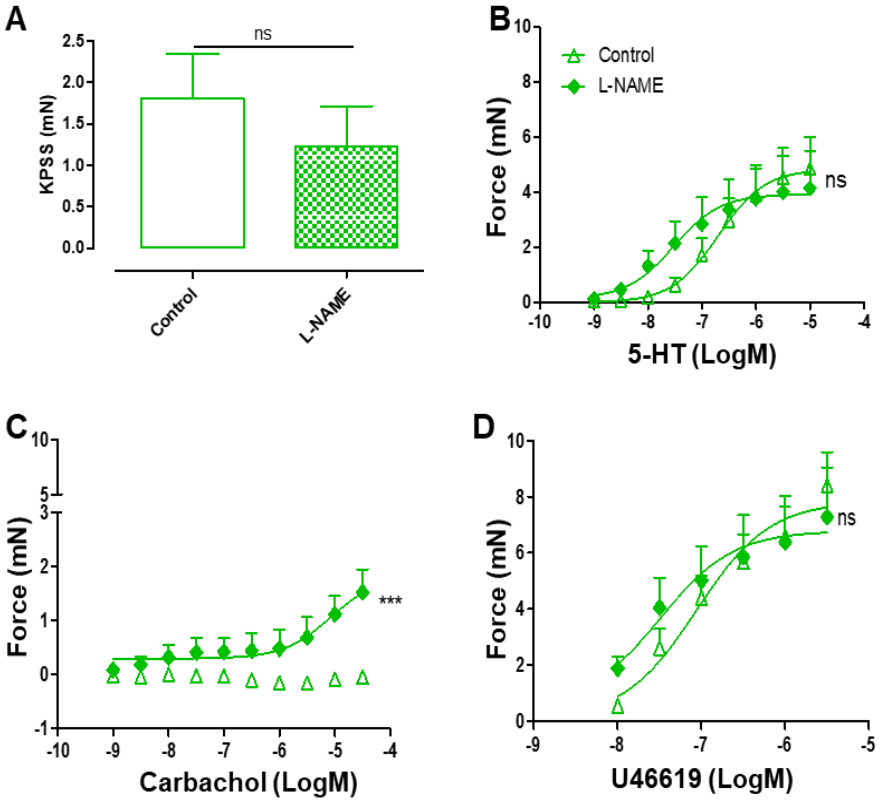


Figure 7. 13 L-NAME effect on vascular reactivity of the aorta

Aorta from C57/BL6 mice were preincubated with L-NAME 1mM for 15 minutes and then exposed to 5-HT(B), carbachol (C) and U46619 (D), while control group was preincubated with PSS for 15 minutes. (A)Analysis of KPSS precontraction in control and L-NAME group. n=6 for each group Data represent mean ± S.E.M. and significant is represented by ***P<0.001. Statistical analysis used is Two-way ANOVA plus Bonferroni test.

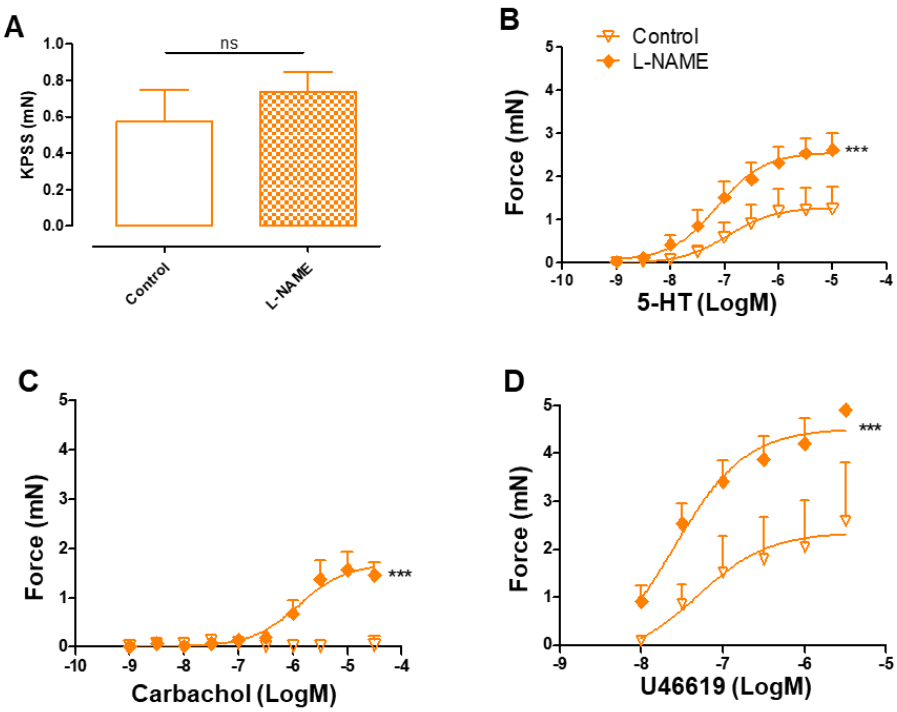


Figure 7. 14 L-NAME effect on vascular reactivity of the carotid artery

Carotid from C57/BL6 mice were preincubated with L-NAME 1mM for 15 minutes and then exposed to 5-HT(B), carbachol (C) and U46619 (D), while control group was preincubated with PSS for 15 minutes. (A)Analysis of KPSS precontraction in control and L-NAME group. n=6 for each group. Data represent mean ± S.E.M. and significant is represented by ***P<0.001. Statistical analysis used is Two-way ANOVA plus Bonferroni test.

Role of Leukotrienes in bronchial and vascular reactivity

As seen above, Diclofenac incubation affected the bronchial reactivity following 5-HT, carbachol, and U46619 administration. This could be because diclofenac inhibits the bronchodilator effects of prostanoids or because arachidonic acid is shunted to the lipoxygenase pathway. Thus, to investigate the effect of diclofenac, bronchial tissue was preincubated with a Cysteinyl-leukotrienes antagonist, Montelukast, at the concentration of 1µM for 15 minutes to inhibit LTs activity.

KPSS administration induced the same contractility in all bronchi used for different protocols (figure 7.15A). Following previous experiments, the inhibition of the prostaglandin's pathway increased the bronchial reactivity to serotonin (figure 7.15 B)

and U46619 concentration-response curves (figure 7.15 D) while the effect of diclofenac on carbachol administration was not statistically significant (figure 7.15 C). Anyway, this contracting activity of Diclofenac on airways was not reverted by the co-incubation with Montelukast (figure 7.15 B, C, and D).

The inhibition of cys-LTs with montelukast incubation did not affect the bronchial reactivity to serotonin, carbachol, and U46619 (figure 7.15 B, C, and D). Anyway, the association of montelukast and diclofenac enhanced the bronchial reactivity resulting in a synergistic effect (figure 7.15 B, C, and D).

On aorta rings, the effect of Montelukast was different from that observed on bronchial rings. Montelukast incubation on the aorta enhanced the vascular reactivity to serotonin and U46619 (figure 7.16 B, D) but had no significant effect on carbachol activity (figure 7.16 C). This contracting activity of Montelukast on the aorta tissue was abrogated by the co-incubation of Diclofenac (figure 7.16 B, D).

Diclofenac incubation, as we have already seen above, did not affect vascular reactivity induced by serotonin, carbachol, and U46619 administration (figure 7.16 B, C, and D).

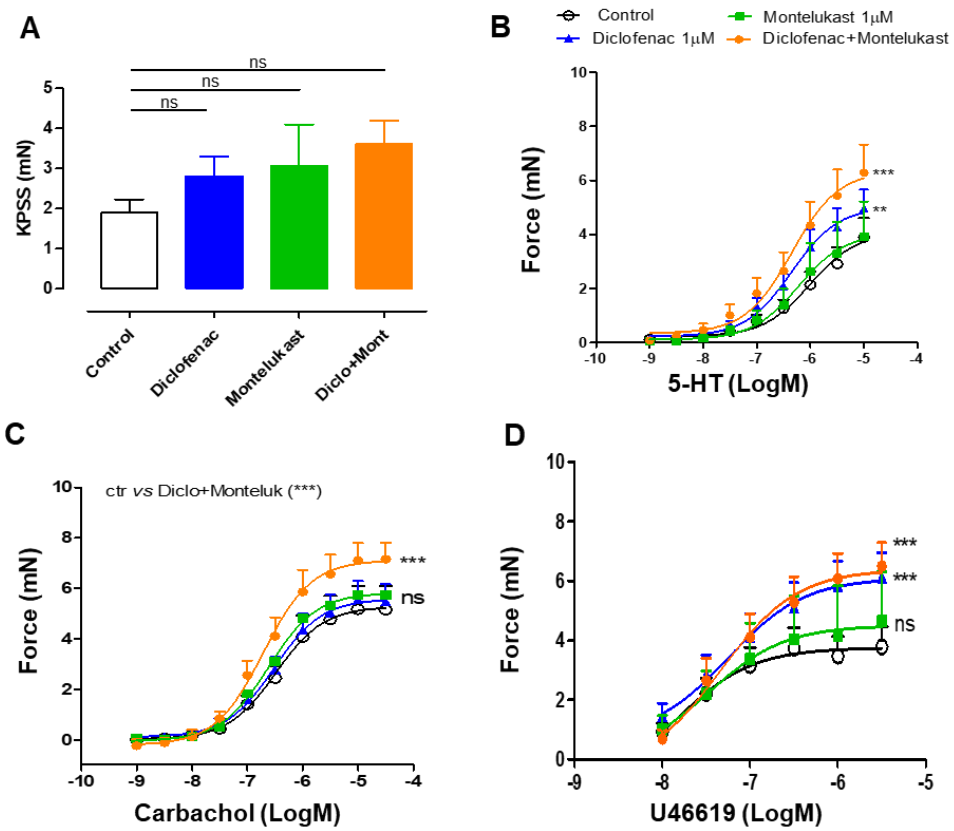


Figure 7. 15 Analysis of LTs pathway on bronchial reactivity

Bronchi from C57BL/6 mice were preincubated with Montelukast 1µM or Diclofenac 1µM or with the association of both for 15 minutes and then exposed to (B) 5-HT, (C) carbachol or (D) U46619. (A) Analysis of KPSS precontraction in bronchial tissues. n=11 for the control and diclofenac groups, n=6 for montelukast and Diclofenac+Montelukast groups. each group Data represent mean ± S.E.M. and significant is represented by ***P<0.001; *P<0.05; **P<0.01. Statistical analysis used is Two-way ANOVA plus Bonferroni test.

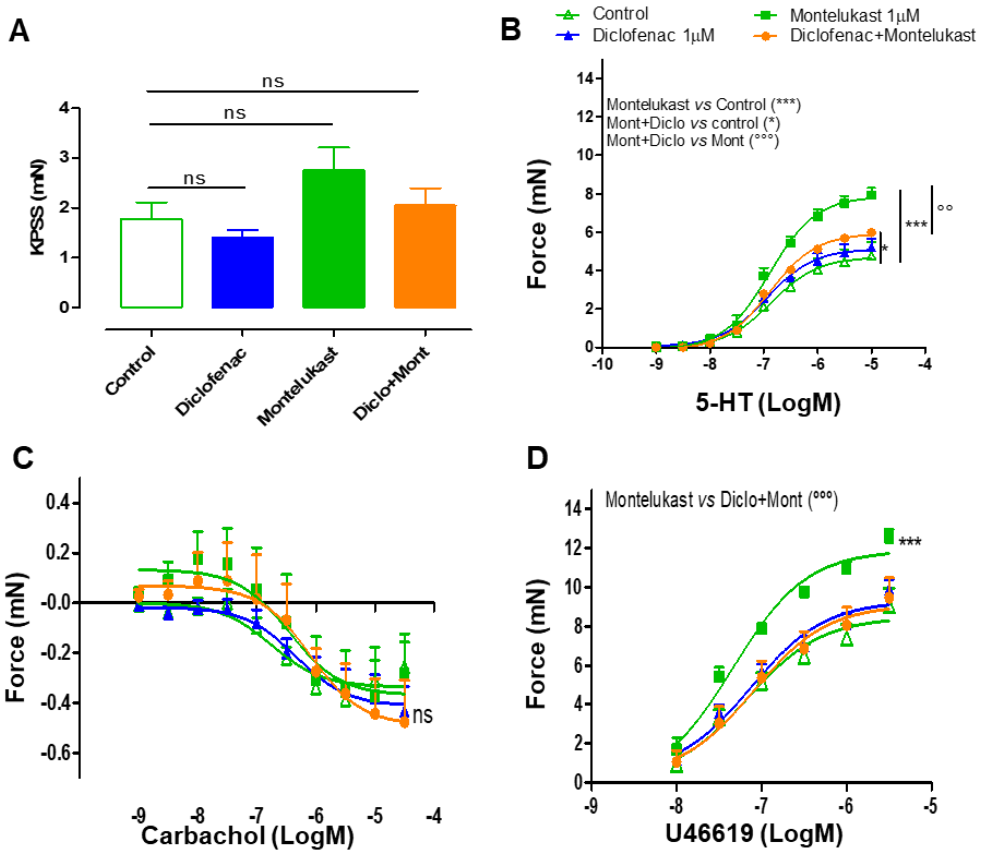


Figure 7. 16 Analysis of LTs pathway on vascular reactivity

Aorta from C57BL/6 mice were preincubated with Montelukast 1µM or Diclofenac 1µM or with the association of both for 15 minutes and then exposed to (B) 5-HT, (C) carbachol and (D) U46619. (A) Analysis of KPSS precontraction in all bronchi used. n=11 for the control and diclofenac groups, n=6 for montelukast and Diclofenac+Montelukast groups. Data represent mean ± S.E.M. and significant is represented by ***P<0.001; *P<0.05; **P<0.01. Statistical analysis used is Two-way ANOVA plus Bonferroni test.

Vascular COX1 affects bronchial reactivity to contracting agents

The experiments above with diclofenac showed that the COX pathway limits the bronchoconstriction. To test if this could be due to COX activity in endothelial cells in vessels within the airway, bronchi were harvested from COX1-Tie2 and COX1-Fps KO mice (where COX-1 is deleted from endothelial cells) and were exposed to 5-HT, carbachol, and U46619 concentration-response curves to evaluate the role of the endothelial COX1 in bronchial reactivity.

COX1-Tie2 HO/TG mice showed higher bronchial reactivity to carbachol and U46619 in vitro administration than COX1-Tie2 HO/WT mice (figure 7.17 B, C). A similar trend was also in bronchi harvested from COX1-Tie2 HO/TG and then exposed to a serotonin curve, anyway there was no statistical significance (figure 7.17 A).

In figure 7.18 data is reported for the bronchial reactivity of COX1-Fps KO mice (which lack COX-1 in fibroblasts) compared to WT mice. Bronchi harvested from the transgenic mice had the same bronchial reactivity as the WT group (figure 7.18 A, B, and C).

Thus, just the vascular endothelial-COX1 and not fibroblast-COX-1 seems to affect bronchial reactivity.

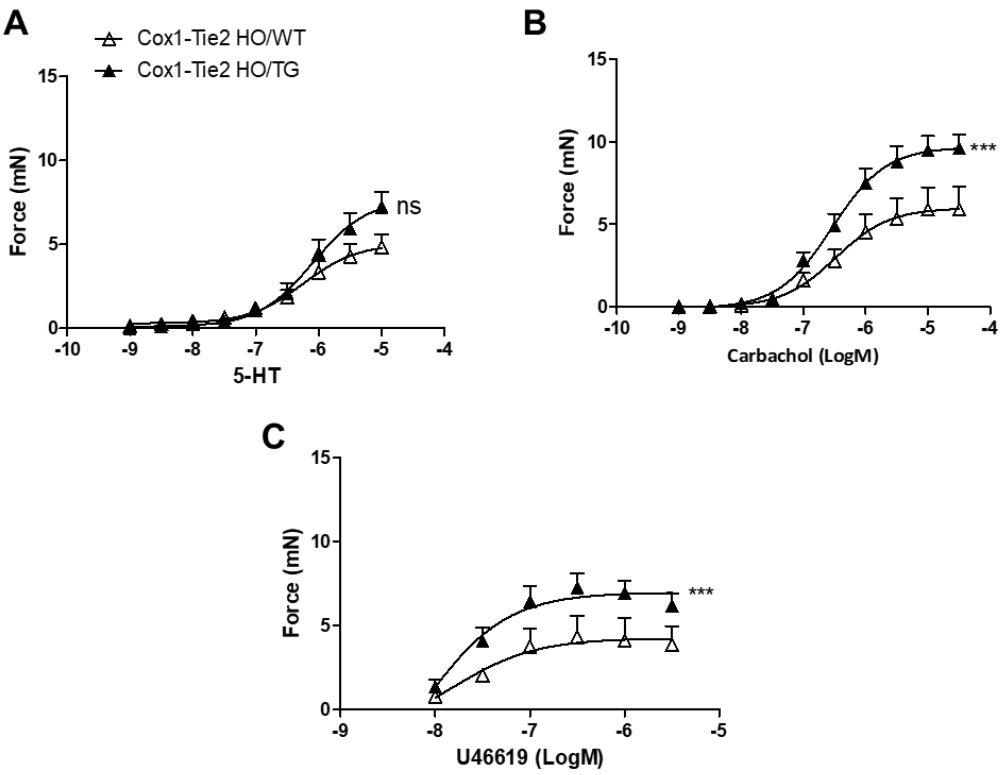


Figure 7. 17 Analysis of bronchial reactivity in COX1-Tie2 KO mice

*Bronchi from C57BL/6 COX1-Tie2 KO mice were exposed to (A) 5-HT, (B) carbachol and (C) U46619 concentration-response curves. n=5 for each group. Data represent mean ± S.E.M. and significant is represented by ***P<0.001. Statistical analysis used is Two-way ANOVA plus Bonferroni test.*

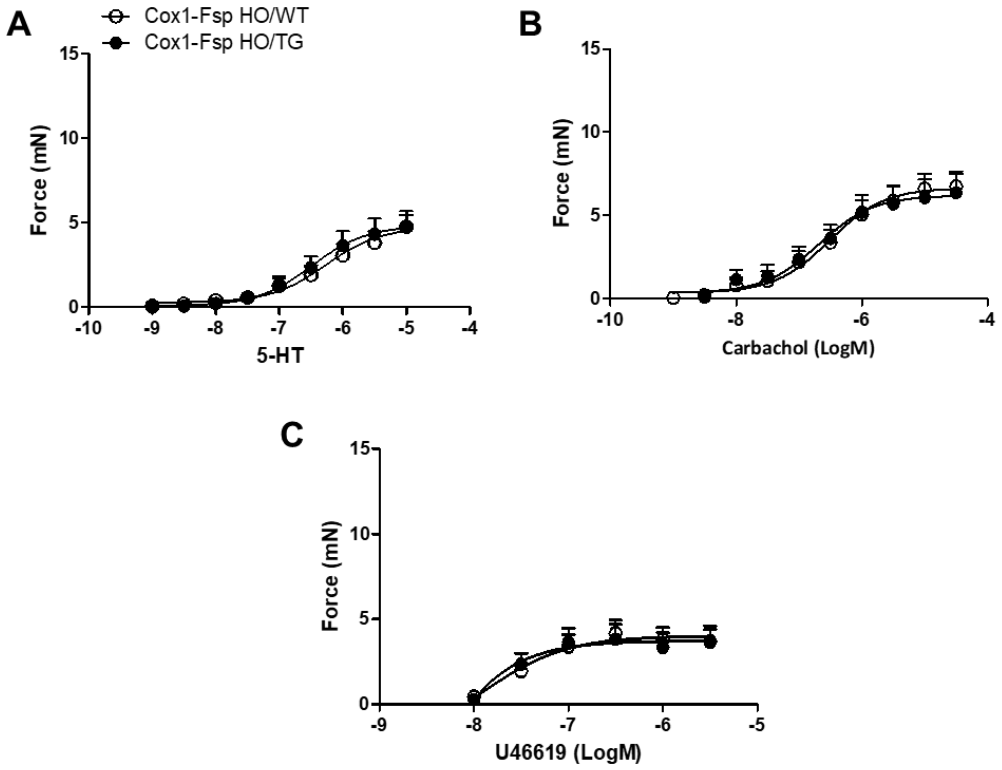


Figure 7. 18 Analysis of bronchial reactivity in COX1-Fps KO mice

Bronchi from C57BL/6 COX1- Fps KO mice were exposed to (A) 5-HT, (B) carbachol and (C) U46619 concentration-response curves. n=5 for each group. Data represent mean ± S.E.M. Statistical analysis used is Two-way ANOVA plus Bonferroni test.

Discussion and Conclusion

These experiments demonstrated that bronchi and vessels show different reactivity to serotonin, carbachol, and U46619, a TXA_2 analogue, in physiological conditions. Since COX and NO pathways are widely involved both in bronchial and vascular tone, herein the involvement of COX and NO pathways was analysed on bronchi and vessels to evaluate differences in prostaglandins and nitric oxide activity.

The enhanced bronchial reactivity observed in tissues preincubated with Diclofenac (figure 7.3) could be attributed to a direct or indirect effect. The first effect involves the inhibition of relaxant prostanoids, particularly PGI_2 and PGE_2, which interact with IP (PGI_2) and $EP_{2,4}$ (PGE_2) inducing smooth muscle relaxation (Zaslona Z., 2015) while the indirect effect involves the LTs pathway because of the shift in the arachidonic acid metabolism.

The COX pathway plays a critical role also in vascular tone, and it is involved in both smooth muscle cell contraction and relaxation by releasing different mediators (Vanhoutte P. , 2009). Prostanoids involved in the vasoconstriction are produced by the endothelial COX. Indeed, the endothelial release of $TXA_{2,}$ and the binding with TP receptor, is the main constrictor pathway on smooth muscle cells. The proper functioning of the prostanoids pathway allows to maintain a balance between contracting and relaxing agents but the alteration of this pathway with the Diclofenac incubation induced a reduction in the vascular reactivity to carbachol and U46616 (figure 7.4) decreasing all the contracting agents of the COX pathway and shifting the balance to relaxing agents.

It is already known that NO has a relaxing effect on smooth muscle by increasing the amount of intracellular cGMP which activates the protein kinase G causing the displacement of calcium from the cytoplasm to the sarcoplasmic reticulum (Klinger JR., 2013). One possible pathway could be the release of NO from the airway epithelium. The NO release from the airway epithelium is stimulated by the activation of bradykinin which binds the B_2-kinin receptor, when the NO is released from the epithelial cells it can stimulate the production of PGI_2 by the intermediate cells. The increased amount of PGI_2 have led to airway smooth muscle relaxation increasing the production of cAMP (Vanhoutte P. M., 2013).

Therefore, the inhibition of the eNOS activity, using L-NAME, revealed the contracting function in the bronchi, pulmonary artery, and carotid (figure 7.7; 7.8; 7.10). Moreover, L-NAME also revealed the contracting function in vessels exposed to carbachol concentration-response curve.

Other mediators involved in maintaining balance in vascular and bronchial tone are LTs. The inhibition of LTs activity using a cys-LTs receptor antagonist, Montelukast, enhances the vascular reactivity to serotonin and U46619. Anyway, this effect is abrogated by the

co-incubation with Diclofenac, demonstrating that the shift of the AA metabolism could be the cause of the increased vascular reactivity induced by Montelukast.

In contrast, on the bronchial tissue, the inhibition of LTs activity did not affect the reactivity while the inhibition of the prostaglandin pathway enhanced the bronchial reactivity to carbachol, serotonin, and U46619. The co-incubation of Diclofenac and Montelukast did not revert the effect of Diclofenac indicating that the shift of the arachidonic acid is not the cause of the hyperreactivity induced by Diclofenac. Thus, this effect could be attributed to a direct effect of Diclofenac on airways by inhibiting bronchodilator prostanoids.

Moreover, to further analyse the association between bronchi and vessels, bronchial reactivity of vascular endothelial-COX1 KO mice (Cox 1-Tie2) and fibroblast-COX1 mice (Cox 1-Fsp) was analysed. The increased bronchial reactivity observed in Cox 1-Tie2 HO/TG mice compared to Cox 1-Tie2 HO/WT demonstrated that the inhibition of COX1 from the endothelium of vessels affects the bronchial reactivity. The results obtained from the analysis of bronchial reactivity in Cox1-Fsp KO mice further confirmed this hypothesis, demonstrating that the prostanoid pathway in the vascular endothelium, but not in fibroblasts, plays a key role in the bronchial reactivity.

In conclusion, taken together all these data demonstrated that Diclofenac has an opposite effect on vessels and bronchi, decreasing vascular response and increasing the bronchial reactivity. The bronchial hyperreactivity induced by Diclofenac was not related to LTs activity but it could be given to a direct effect on prostacyclin activity. For this reason, the next experiment that we are planning to do is to incubate the bronchial tissue with Diclofenac plus a prostacyclin receptor-antagonist to evaluate if the hyperreactivity induced by Diclofenac is related to a prostacyclin activity. Moreover, since the vascular endothelium-COX1 pathway affects the bronchial response, it might be interesting to evaluate if this pathway is also involved in the bronchial hyperreactivity induced by Diclofenac.

Chapter 8

Chapter 8

8.Discussion

Chronic respiratory diseases, like asthma and COPD, are still one of the main causes of disability and death, these diseases are not curable but different treatments can be used to control the symptoms improving patients' quality of life. These treatments involve the use of bronchodilators and anti-inflammatory drugs, anyway, as mentioned above, the chronic use of these drugs show different limits. Also, an additional limit in the use of these drugs is represented by the different response of the patient based on gender.

Thus, this study aimed to investigate the pathological mechanisms involved in airway hyperreactivity using a murine model of airway inflammation. Gender has been considered in the analysis of mechanisms involved in both airway contraction and relaxation.

The first objective has been that to evaluate the role of the S1P pathway in airway inflammation and hyperreactivity induced by chronic exposure to cigarette smoke. We found that S1P is involved in bronchial hyperreactivity induced by cigarette smoke and its activity is mediated by $S1P_2$ and $S1P_3$ receptors. Indeed, smoking mice for 11 months showed an increased expression of both receptors in the airways and an increased level of S1P in the lung. This discovery allows us to say that S1P could be a therapeutic target for the treatment of airway hyperreactivity and inflammation in smokers.

Then, we focused our attention on the sex differences in bronchial reactivity. We found that gender affected the airway contraction in physiological conditions and this sex difference was enhanced in a murine model of asthma-like features and it was coupled to a sex difference in the LTs production in the lung. These sex differences in airway function and inflammation were abrogated by the intraperitoneal administration of DHT in female mice. Besides, DHT administration also cancelled the sex difference in LTs production. This protective role of DHT was mediated by the activation and degranulation of mast cells, suggesting that this is a non-genomic effect.

Gender also affects the β_2-receptors induced relaxation and the mechanisms involved in β_2-receptor desensitization. This sex difference in airway relaxation was not coupled to a different β_2-receptor expression between males and females but a different PDE4a expression in sexes was observed. To further investigate the connection between PDE and β_2-receptor relaxation, bronchi were preincubated with IBMX. The inhibition of PDE restored β_2-receptor relaxation in female mice eliminating the sex difference. Thus, gender affected PDE4a expression in bronchi and this could modify the amount of cAMP in females resulting in a different airway relaxation. This hypothesis was confirmed by the activity of L-NAME and db-cAMP, which deleted the sex difference in airway relaxation.

The last experiments were conducted at the Imperial College of London and aimed to analyse the bronchial and vascular reactivity in parallel. These experiments showed that the inhibition of prostanoids pathway with the diclofenac incubation had an opposite effect in bronchi and vessels, increasing the bronchial reactivity and reducing the vascular reactivity. This increased bronchial reactivity after diclofenac incubation was not coupled to the LTs pathway. In addition, the experiments demonstrated that the prostanoids pathway from the endothelium, but not from the fibroblast, affected the bronchial reactivity.

Publications

- *"Functional contribution of sphingosine - 1 - phosphate to airway pathology in cigarette smoke - exposed mice"* G. De Cunto et al. British Pharmaceutical Society 2019

- *"Leukotriene-mediated sex dimorphism in murine asthma-like features during allergen sensitization."* Rossi A. et al. Pharmacol Res. 2019 Jan; 139:182-190.

- *"5α-dihydrotestosterone abrogates sex bias in asthma like features in the Mouse"* Ida Cerqua *et al.* Pharmacological Research 158 (2020) 104905

- *"The Inhibition of Caspase-1- Does Not Revert Particulate Matter (PM)-Induced Lung Immunesuppression in Mice."* Colarusso C. et al. Front Immunol. 2019 Jun 21; 10:1329

- *"Exacerbation of Allergic Airway Inflammation in Mice Lacking ECTO-5 9 - Nucleotidase (CD73)"* E. Caiazzo *et al.* Frontiers in Pharmacology 2020

- *Chapter "XV Dermatologia" of the Book "Manuale di Farmacoterapia", IDELSON-GNOCCHI, 2019*

References

Alangari AA. (2014). Corticosteroids in the treatment of acute asthma. *Ann Thorac Med.*

Busse W.W. (2007). Expert Panel Report 3 (EPR-3): Guidelinesfor the Diagnosis and Management of Asthma–Summary Report 2007. *J ALLERGY CLIN IMMUNOL.*

Cohn L. (2006). Mucus in chronic airway diseases: sorting out the sticky details. *The Journal of Clinical Investigation.*

Johansson MW. (2017). Eosinophil activation status in separate compartments and association with asthma. *Frontiers in Medicine.*

Kelloway JS. (1997). Zafirlukast: The First Leukotreene-Receptor Antagonist Approved for the Treatment of Asthma. *Ann Pharmacother*.

Lionberger R. A. (2008). FDA Critical Path Initiatives: Opportunities for Generic Drug Development. *The AAPS Journal*.

Peebles Jr. R.S. (2019). Prostaglandins in Asthma and Allergic Diseases. *Pharmacol Ther*.

Wenzel S.E. (2003). The role of leukotrienes in asthma. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 145-155.

Borak J., L. R. (2016). Bronchial hyperresponsiveness. *Occupational Medicine*.

A. Keselman, N. H. (2015). Estrogen Signaling Modulates Allergic Inflammation and Contributes to Sex Differences in Asthma. *Frontiers in Immunology*.

Amrani Y., B. P. (2017). Beta2-adrenoceptor function in asthma. *Advances in Immunology*.

Antonietta Rossi, F. R. (2018). Leukotriene-mediated sex dimorphism in murine asthma like features during allergen sensitization. *Pharmacological Research*.

Athanazio, R. (2012). Airway disease: similarities and differences between asthma, COPD and bronchiectasis. *CLINICS*, 1335-1343.

Austin E.D., L. J. (2014). The Genetics of Pulmonary Arterial Hypertension. *Circ Res*.

Bacerra-Diaz M., W.-K. M. (2017). New prospectives on the regulation of type II inflammation in asthma . *F1000Research* .

Bagdonas E, R. J. (2015). Novel aspects of pathogenesis and regeneration mechanisms in COPD. *International Journal of COPD*.

Barnes, P. J. (2010). Glucocorticosteroids:current and future directions. *British Journal ofPharmacology*.

Barnes, P. J. (2010). Inhaled Corticosteroids. *Pharmaceuticals*.

Bartal, M. (2005). COPD and tobacco smoke. *Monaldi Arch Chest Dis*.

Baxi S. N., P. W. (2010). The Role of Allergen Exposure and Avoidance in Asthma. *Adolesc Med State Art Rev*.

Belge C., D. M. (2019). Treatment of pulmonary arterial hypertension with the dual endothelin receptor antagonist macitentan: clinical evidence and experience. *Therapeutic Advances in Respiratory Diseas*.

Bergeron C, T. M. (2010). Airway remodeling in asthma: from benchside to clinical practise. *Can Respir J*.

Billington C.K., P. R. (2017). β2-agonists. *Handb Exp Pharmacol*.

Bloomfield S. F., R. G.-S. (2016). Time to abandon the hygiene hypothesis: new perspectives on allergic disease, the human microbiome, infectious disease prevention and the role of targeted hygiene. *Royal Society for Public Healt*.

Bonnet S, R. G. (2007). The nuclear factor of activated T cells in pulmonaryarterial hypertension can be therapeutically targeted. *PNAS*.

Boonpiyathad T., S. Z. (2019). Immunologic mechanisms in asthma. *Seminars in immunology*.

Bujarsky S., P. A. (2015). The Asthma–COPD Overlap Syndrome. *The new england journalof medicine*.

Busse W. W., L. J. (2010). Role of viral respiratory infections in asthma and asthma exacerbations. *Lancet*.

Byoungjae K., T. H. (2018). Fundamental role of dendritic cells in inducing Th2 responses. *The Korean Association of Internal Medicine*.

C. Draijer, P. R. (2013). Characterization of Macrophage Phenotypes in Three Murine Models of House-Dust-Mite-Induced Asthma. *Mediators of Inflammation*.

C. Pergola, A. R. (2011). Testosteros suppresses phospholipase D, causing sex differences in leukotriene biosynthesis in human monocytes. *The FASEB journal*.

Caiazzo E., C. I. (2020). Exacerbation of Allergic AirwayInflammation in Mice LackingECTO-59-Nucleotidase (CD73). *Frontiers in Pharmacology* |.

Carlsen K.H., C. K. (2005). Parental smoking and childhood asthma: clinical implications. *Treat Respir Med*.

Castro M., C. J. (2018). Dupilumab Efficacy and Safety in Moderate-to-Severe Uncontrolled Asthma. *The new england journal of medicine*.

Cavarra E., B. B. (2001). Effects of Cigarette Smoke in Mice with Different Levels of. *Am J Respir Crit Care Med*.

Cazzola M, P. C. (2019). *Pharmacology and Therapeutics of Bronchodilators.*

Cazzola M., P. C. (2012). Pharmacology and Therapeutics of bronchodilators. *Pharmacological Reviews*.

Cerqua I, T. M. (2020). 5α-dihydrotestosterone abrogates sex bias in asthma like features in the mouse. *Pharmacological Research*.

Chai O.H., H. E. (2011). Mast cells play a key role in Th2 cytokine-dependent asthma model through production of adhesion molecules by liberation of TNF-α. *Exp. Mol. Med*.

Chalfant C.E., S. S. (2005). Sphingosine 1-phosphate and ceramide 1-phosphate: expanding roles in cell signaling. *Journal of Cell Science*, 4605-4612.

Chaplin D.D. (2010). Overview of the Immune Response. *J Allergy Clin Immunol.*

Chen H. (2018). Role of thromboxane A 2 signaling in endothelium-dependent contractions of arteries. *Prostaglandins and Other Lipid Mediators*.

Chiba Y, M. M. (2005). Effect of Cigarette Smoke ExposureIn Vivoon BronchialSmooth Muscle ContractilityIn Vitroin Rats. *Am J Respir Cell Mol Biol V*.

Christman BW, M. C. (1992). An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. *N Engl J Med*.

Colarusso C., D. F. (2019). The Inhibition of Caspase-1- Does Not Revert Particulate Matter (PM)-Induced Lung Immunesuppression in Mice. *frontiers in immunology*.

Copley, M. (2014). Using breathing simulators to enhance inhaled product testing . *Copley Scientific*.

Cruz A.A., B. E. (2010). The social determinants of asthma. *Eur Respir J*.

Daley E., E. C. (2008). Pulmonary arterial remodeling induced by a Th2 immune response. *The journal of experimental Medicine*.

D'Amato G., L. G. (2005). Enviromental risk factors and allergic bronchial asthma. *Clin Exp Allergy*, 1113-1124.

De Cunto G, B. V. (2019). Functional contribution of sphingosine - 1 - phosphate to airway pathology in cigarette smoke - exposed mice. *he British Pharmacological Society*.

Del Giacco S. R., B. A. (2016). Allergy in severe asthma. *European Journal of Allergy and Clinical Immunology*.

Dharmage SC., P. J. (2019). Ephydemiology of asthma in children and adults. *Frontiers in Prdiatrics*.

Dholia N, Y. U. (2018). Lipid mediator Leukotriene D 4 -induces airway epithelial cells proliferation through EGFR/ERK1/2 pathway. *Prostaglandins and Other Lipid Mediators*.

Dixon A.E., P. U. (2018). The effect of obesity on lung function. *Expert Rev Respir Med*.

Dodson MW., B. L. (2018). Pulmonary Arterial Hypertension. *Heart Failure Clin*.

Domingo C, P. O. (2018). The prostaglandin D 2 receptor 2 pathway in asthma: a key player in airway inflammation. *Respiratory Research*.

Dongmei Wu, D. L. (2011). Prospect of vasoactive intestinal peptide therapy for COPD/PAH and asthma: a review. *Respiratory Research*.

Dünnes S, V. B. (2018). Phosphodiesterase 3A expression and activity in the murine vasculature is influenced by NO-sensitive guanylyl cyclase. *European Journal of Physiology*.

Dusser D., D. F. (2019). Safety of tiotropium in patients with asthma. *Therapeutic Advances in Respiratory Diseas*.

Erdem SB., N. H. (2015). Side Effects of Leukotriene Receptor Antagonists in Asthmatic Children. *Iran J Pediatr.* .

F Roviezzo, R. S. (2015). S1P-induced airway smooth muscle hyperresponsiveness and lung inflammation in vivo: molecular and cellular mechanisms. *British Journal of Pharmacology*.

Fahy J. V. (2015). Type 2 inflammation in asthma — present in most, absent in many. *Nat Rev Immunol.*

Fang L., S. Q. (2020). Immunologic and Non-Immunologic MechanismsLeading to Airway Remodeling in Asthma. *Internationa Journal of Molecular Science* .

Farjadian S, M. M.-P. (2018). ADAM33 gene polymorphisms in Southwestern Iranian patients with asthma . *Iran J Basic Med Sci*.

Fehrenbach H., W. C. (2017). Airway remodeling in asthma: what really matters. *Cell Tissue Res.*

Fuseini H., N. D. (2017). Mechanisms driving gender differences in asthma. *Curr Allergy Asthma Rep*.

García-Menaya JM., C.-D. C.-M. (2019). Pharmacogenetic Factors Affecting Asthma Treatment Response. Potential Implications for Drug Therapy. *Front Pharmacol.*

(n.d.). *GINA 2019.*

Golden MP, H. W. (2007). Leukotrienes. *The new engl a nd jour na l o f medicine*.

Guarnieri M., B. J. (2014). Outdoor air pollution and asthma. *Lancet.*

Guo M, P. R. (2005). Cytokines Regulate,-2-Adrenergic Receptor Responsiveness in Airway SmoothMuscle via Multiple PKA- and EP2 Receptor-Dependent Mechanisms. *Biochemistry*.

Gye Young Park, J. W. (2006). Involvement of cyclooxygenase-2 and prostaglandins in the molecular pathogenesis of inflammatory lung diseases. *Am J Physiol Lung Cell Mol Physiol.*

Hedi H., N. G. (2004). 5-Lipoxygenase Pathway, Dendritic Cells,and Adaptive Immunity. *Journal of Biomedicine and Biotechnology*.

Henriksen DP., B. U. (2020). Efficacy of omalizumab in children, adolescents, and adults with severe allergic asthma: a systematic review, meta-analysis, and call for new trials using current guidelines for assessment of severe asthma. *Allergy Asthma Clin Immunol* .

Hogan AD., B. J. (2020). GINA updated 2019. *Ann Allergy Asthma Immunol 124*.

Hsia C.C.W., H. D. (2016). Lung Structure and the Intrinsic Challenges of Gas Exchange . *Compr Physiol.* , 827–895.

Hsu E., B. T. (2020). Beta 2 Agonists. *StatPearls*.

Imani D., E. M.-S. (2020). Interleukin-4 gene polymorphism (C33T)and the risk of the asthma: a meta-analysisbased on 24 publications. *BMC Medical Genetics*.

Ito I, F. E. (2009). Platelet-derived growth factor and transforming growth factor-bmodulate the expression of matrix metalloproteinases and migratoryfunction of human airway smooth muscle cells. *Clinical & Experimental Allergy,*.

J. Borak, R. Y. (2016). Bronchial hyperresponsiveness. *Occupational Medicine*.

Jacquet A. (2013). Innate Immune Responses in House Dust Mite Allergy. *ISRN Allergy*.

Jane A. M, K. N.-S. (2020). Cyclooxygenases and the cardiovascular system. *Pharmacology & Therapeutics*.

Jo-Watanabe A., O. T. (2019). The Role of Leukotrienes as Potential TherapeuticTargets in Allergic Disorders. *International Journal ofMolecular Sciences*.

Kabesch M., T. I. (2003). A complete screening of the IL4gene:Novel polymorphisms and theirassociation with asthma and IgE inchildhood. *American Academy of Allergy, Asthma and Immunology*.

Kandathil A, C. M. (2018). Pulmonary vascular anatomy & anatomical variants. *Cardiovascular Diagnosis and Therapy*.

Keselman A., H. N. (2015). estrogen Signaling Modulates Allergic inflammation and Contributes to Sex Differences in Asthma. *Frontiers in Immunology*.

Kesimer M., F. A. (2017). Airway Mucin Concentration as a Marker of Chronic Bronchitis. *N Engl J Med.*

Kilkenny C, B. W. (2010). Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLOS BIOLOGY*.

Kirkby NS, M. J. (2018). Eicosanoids, prostacyclin and cyclooxygenase in the cardiovascular system. *British Journal of Pharmacology*.

Klinger JR., A. S. (2013). Nitric Oxide Deficiency and Endothelial Dysfunction in Pulmonary Arterial Hypertension. *Am J Respir Crit Care Med*.

Kobilka BK. (2007). G Protein Coupled Receptor Structure and Activation. *Biochim Biophys Acta*.

Kouloumenta V, H. A. (2006). Non-genomic effect of testosterone on airway smooth muscle. *British Journal of Pharmacology*.

Kousha A., G. A. (2020). Interleukin 4gene polymorphism (−589C/T)and the risk of asthma: a meta-analysis andmet-regression based on 55 studies. *BMC Immunology*.

Kudo M., I. Y. (2013). Pathology of asthma. *frontiers in microbiology*.

Kunio Yui1, G. I. (2015). Eicosanoids DerivedFrom Arachidonic Acid and Their Family Prostaglandins and Cyclooxygenase in Psychiatric Disorders. *Current Neuropharmacolog*.

L.M. Montano, E. S. (2018). Testosterone induces hyporesponsiveness by interfering with IP3 receptors in guinea pig airway smooth muscle. *Molecular and Cellular endocrinology* , 17-30.

L.M.Montaño, E.-S. S. (2015). Testosterone-induced relaxation involves L-type and store-operated Ca2+ channels blockade, and PGE2 in guinea pig airway smooth muscle . *Eur J Physiol*.

Laffont S., B. E. (2017). Sex Differences in Asthma: A Key Role of Androgen-Signaling in Group 2 Innate Lymphoid Cells. *Frontienrs in Immunology*.

Laidlaw T. M., B. J. (2012). Cysteinyl leukotriene receptors, old and new; implications for asthma. *Clin Exp Allergy*.

Lambrecht B.N., H. H. (2019). The Cytokines of Asthma. *Immunity 50*.

Lavorini F., N. V. (2013). Switching from branded to generic inhaled medications: potential impact on asthma and COPD. *Expert Opinion on Drug Delivery*.

Lee JC., K. E. (2009). Common and distinct pathways for cellular activities in fgf-2 signaling induced by il-1beta in corneal endothelial cells. *Investigative ophthalmology & visual science*.

Leopold J.A. (2016). Molecular Mechanisms of Pulmonary Vascular. *International Journal of Molecular Science*.

Li HF., Y. L. (2019). Association betweenADAM33polymorphisms and asthma risk: asystematic review and meta-analysis. *Respiratory Research*.

Liapakis G, M. M. (2017). Family B G Protein-coupled Receptors and their Ligands: From Structure to Function. *Current Medicinal Chemistry*.

Lin SC., S. L. (2019). Advanced Molecular Knowledge of Therapeutic Drugs and Natural Products Focusing on Inflammatory Cytokines in Asthma. *Cells*.

Lloyd C.M., H. E. (2010). Functions of T cells in asthma: more than just TH2 cells. *Nat Rev Immunol.*

Lloyd CM, R. D. (2017). Allergen-induced airway remodelling. *Eur Respir J*.

Lo CCW, M. S. (2018). The Regulation of Pulmonary Vascular Tone by Neuropeptides and the Implications for Pulmonary Hypertension. *Front. Physiol*.

Loftus P.A., M. a. (2015). Epidemiology and economic burden of asthma. *nternational Forum of Allergy & Rhinology*.

Machuki J.O., Z. H. (2019). Estrogen regulation of cardiac cAMP-L-type Ca 2+ channel pathway modulates sex differences in basal contraction and responses to β 2 AR-mediated stress in left ventricular apical myocytes. *Cell Communication and Signaling*.

Marple VA., R. D. (2003). Next Generation Pharmaceutical Impactor (A New Impactor for Pharmaceutical Inhaler Testing). Part I: Design. *JOURNAL OF AEROSOL MEDICINE*.

Martin AR., F. W. (2014). Nebulizers for drug delivery to the lungs. *Expert Opin. Drug Deliv*.

Matsuda M, T. Y. (2018). Increased expression of CysLT 2 receptors in the lung of asthmatic mice and role in allergic responses. *Prostaglandins, Leukotrienes and Essential Fatty Acids*.

McCullagh A, R. M. (2010). The Bronchial Circulation—Worth a Closer Look: A Review of the Relationship Between the Bronchial Vasculature and Airway Inflammation. *Pediatric Pulmonology*.

McGregor MC., K. J. (2019). Role of Biologics in Asthma. *Concise Clinical Review*.

Mitchell JA, A.-S. B. (2014). Role of prostacyclin in pulmonary hypertension. *Global Cardiology Science and Practice*.

Mitchell JA., K. N.-S. (2020). Cyclooxygenases and the cardiovascular system. *Pharmacology & Therapeutics*.

Montani D, G. S.-M. (2013). Pulmonary arterial hypertension. *Orphanet Journal of Rare Disease*.

Montani D, S. A. (2013). Drug-induced pulmonary arterialhypertension: a recent outbreak. *Eur Respir Rev*.

Montaño L.M., S. E. (2020). Androgens are effective bronchodilators with anti-inflammatory properties:A potential alternative for asthma therapy. *Steroids*.

Montuschi, P. (2008). Leukotrienes, Antileukotrienes and asthma . *Mini-reviews in Medicinal Chemistry*.

Montuschi, P. (2010). Role of Leukotrienes and Leukotriene Modifiers in Asthma . *Pharmaceuticals*.

Morianos I., S. M. (2020). Dendritic Cells: Critical Regulators of Allergic Asthma. *International Journal of Molecular Sciences*.

Mukherjee A. B., Z. Z. (2011). Allergic Asthma: Influence ofGenetic and EnvironmentalFactors. *THE JOURNAL OF BIOLOGICAL CHEMISTRY*.

Mumby S., C. K. (2019). Transcriptional effects of Ozone and impact on airway inflammation. *frontiers in immunology*.

Nunes C., A. M.-A. (2017). Asthma costs and social impact. *Nuneset al. Asthma Research and Practice*.

Ora J., C. L. (2020). Advances with glucocorticoids in the treatment of asthma: state of the art. *EXPERT OPINION ON PHARMACOTHERAPY* .

Orriols M., G.-P. M. (2017). BMP type II receptor as a therapeutic target in pulmonary arterial hypertension. *Cell. Mol. Life Sci*.

P.E., K. (2001). The role of antileukotrienes in the treatment of asthma. *Annals of Allergy, Asthma & Immunology*.

Pace S., S. L. (2017). Androgen-mediated sex bias impairs efficiency of leukotrienebiosynthesis inhibitors in males. *J Clin Invest.*

Page C, H. P. (2012). Sir David Jack: an extraordinary drug discovererand developer. *British Journal of ClinicalPharmacology*.

Page C., C. M. (2014). Bifunctional drugs for the treatment ofasthma and chronic obstructive pulmonarydisease. *Eur Respir J*.

Papi A, B. F. (2020). Treatment strategies for asthma: reshaping the concept of asthma management. *Allergy Asthma Clin Immunol*.

Parameswaran, F. H. (2006). Asthma, COPD and bronchitis are just component of airway disease. *Eur Respir J*, 267-267.

Pattenden S., A. T.-G. (2006). Parental smoking and children's respiratory health: independent effects of prenatal and postnatal exposure. *Tob Control*.

Pei G, S. P. (1994). A constitutively active mutant f32-adrenergic receptor is constitutively desensitized and phosphorylated. *Proc. Natl. Acad. Sci.*

Pelaia G., R. T. (2008). Molecular mechanisms underlying airway smooth muscle contraction and proliferation: Implications for asthma. *Respiratory Medicine*.

Pergola C., D. G. (2008). ERK-mediated regulation of leukotriene biosynthesisby androgens: A molecular basis for gender differences in inflammation and asthma. *PNAS*, 19881-19886.

Perusquía M, F.-S. E.-G.-V.-B. (2015). Testosterone-induced relaxation involves L-type and store-operated Ca channels blockade,and PGE 2 in guinea pig airway smooth muscle. *Eur J Physiol*.

Peters U., D. A. (2018). Obesity and Asthma. *J Allergy Clin Immunol*.

Petersson J., G. R. (2014). Gas exchange and ventilation–perfusion relationships in the lung. *Eur Respir J.*

Petrache I., N. V. (2005). Ceramide upregulation causes pulmonary cell apoptosis and emphysema. *Nat Med.*

Pleasants RA., H. D. (2018). Aerosol Delivery Devices for Obstructive Lung Diseases. *RESPIRATORY CARE*.

R. Aalbers, H.-S. P. (2017). Positioning of Long-acting muscarinic Antagonists in the management of asthma. *Allergy asthma Immunology Res*.

R. F. Furchgott, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine . *Nature*.

Rabinovitch M, G. C. (2015). Inflammation and Immunity in the Pathogenesis of Pulmonary Arterial Hypertension. *National Institute of Health*.

Raghavan D., J. R. (2016). Increasing awareness of sex differences in airway diseases. *Respirology*.

Real FG, S. C.-L. (2006). Hormone replacement therapy, body mass indexand asthma in perimenopausal women: a crosssectional survey. *Thorax*.

Ricciotti E., F. G. (2011). Prostaglandins and Inflammation. *Arterioscler Thromb Vasc Biol.*

Rodriguez A., B. E. (2019). Urbanisation and asthma in low-income and middle-income countries: a systematic review of the urban–rural differences in asthma prevalence. *Thorax* , 1020–1030.

Romanet-Manent S., C. D. (2003). Allergic vs nonallergic asthma: what makes the difference? *European Journal of allergy and clinical immunology*.

Rossi A, R. F. (2019). Leukotriene-mediated sex dimorphism in murine asthma-like features during allergen sensitization. *Pharmacological Research 139*.

Rovati G.E., B. M. (2006). Cysteinyl-leukotrienes in the regulation of beta2-adrenoceptor function: an in vitro model of asthma. *Respiratory Research*.

Roviezzo F, D. L. (2007). Sphingosine-1-Phosphate/Sphingosine Kinase Pathway Is Involved in Mouse Airway Hyperresponsiveness. *Am J Respir Cell Mol Biol*.

Roviezzo F., B. A. (2015). Hydrogen sulfide inhalation ameliorates allergen induced airway hypereactivity by modulating mast cell activation. *Pharmacological Research*.

Roviezzo F., D. B. (2009). Systemic Administration of Sphingosine-1-Phosphate Increases Bronchial Hyperresponsiveness in the Mouse. *American Journal of Respiratory Cell and Molecular Biology*.

Roviezzo F., S. R. (2014). S1P-induced airway smooth muscle hyperresponsiveness and lung inflammation in vivo: molecular and cellular mechanisms. *British Journal of Pharmacology*.

Roviezzo F., S. R. (2015). S1P-induced airway smooth muscle hyperresponsiveness and lung inflammation in vivo: molecular and cellular mechanisms. *British Journal of Pharmacology*.

Rumzhum N.N., R. M. (2016). Effect of Sphingosine-1-Phosphate on cyclo-oxygenase-2 expression, Prostaglandin E2 secretion and beta2-adrenergic receptor desensitization. *American Journal of Respiratory Cell and Molecular Biology*.

S.M., H. (2010). Environmental Epigenetic of Asthma – An update. *J Allergy Clin Immunol.*

Saetta M., D. S. (1991). Quantitative Structural AnalysisofPeripheral Airways. *AM REV RESPIR DIS*.

Said SI., H. S. (2010). Asthma and Pulmonar arterial hypertension: do they share a key mechanism of pathogenesis? *Eur Respir J.*

Schermuly RT., G. H. (2011). Mechanisms of disease: pulmonary arterial hypertension. *Nature reviews Cardiology*.

Sessa M., M. A.-P. (2018). Relationship Between Gender and the Effectiveness of Montelukast: An Italian/Danish Register-Based Retrospective Cohort Study. *frontiers in pharmacology*.

Sharma N., T. P. (2011). Role of ADAM33 gene and associated single nucleotidepolymorphisms in asthma. *Allergy & Rhinology*.

Sharon Mumby, K. F. (2019). Trascriptional effects of Ozone and Impact on airway inflammation. *frontiers in immunology*.

Shore S.A., M. P. (2003). Regulation of b-adrenergic responses in airway smooth muscle. *Respiratory Physiology & Neurobiology*.

Silverman E.K., S. F. (1996). Risk factors for the development of chronic obstructive pulmonary disease. *Med Clin North Am*.

Sommer B., F.-S. E.-A. (2017). Cellular Na+ handling mechanisms involved in airway smooth muscle contraction. *INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE*.

Sood A., S. S. (2013). Adiponectin, Leptin, and Resistin in Asthma: Basic Mechanismsthrough Population Studies. *Journal of Allergy*.

Steinke J.W., B. L. (2001). Th2 cytokines and asthmaInterleukin-4: its role in the pathogenesis of asthma, and targetingit for asthma treatment with interleukin-4 receptor antagonists. *Respir Res*.

Stephanie A. S., M. P. (2003). Regulation of b-adrenergic responses in airway smooth muscle. *Respiratory Physiology & Neurobiology*.

Stern J., P. J. (2020). Asthma epidemiology and risk factors. *Springer*.

Struba G. M., M. M. (2010). Extracellular and Intracellular Actions of Sphingosine-1-Phosphate. *Adv Exp Med Biol*.

Suárez L, P. M. (2011). Sex Hormones ModulateSalbutamol-Elicited Long-Term Relaxation in Isolated Bovine Tracheal Strips. *Pharmacology*.

Sun F.J., Z. L. (2017). Association between ADAM metallopeptidase domain33 gene polymorphism and risk of childhood asthma:a meta-analysis. *Brazilian Journal of Medical and Biological Research (2*.

Sun MY., G. M. (2017). IP3 Receptor Signaling and Endothelial Barrier Function. *Cell Mol Life Sci.*

Suresh K., S. L. (2020). Lung Circulation. *Compr Physiol*.

Tamada T., I. M. (2017). Leukotriene Receptor Antagonistsand Antiallergy Drugs. *Handb Exp Pharmacol*.

Tamimi A., S. D. (2012). The effects of cigarette smoke on airway inflammation in asthma and COPD: Therapeutic implications. *Respiratory Medicine*.

Tamosiuniene R, T. W. (2011). Regulatory t cells limit vascular endothelial injury and prevent pulmonary hypertension. *Circulation research*.

Theron A. J., S. H. (2014). Cysteinyl Leukotriene Receptor-1 Antagonists asModulators of Innate Immune Cell Function. *Journal of Immunology Researc*.

Thompson-Souza GA, G. I. (2017). Cysteynil leukotrienes in eosinophil biology: Functional roles and Therapeutic perspectives in eosinophilic disorders. *Frontiers in medicine*.

Tian W, J. X. (2014). Leukotrienes in pulmonary arterial hypertension. *Immunol Res*.

Tormanen KR, U. L. (2005). Allergen Exposure of Mouse Airways Evokes Remodeling. *AMERICAN JOURNAL OF RESPIRATORY AND CRITICAL CARE MEDICINE*.

Trinh HKT., L. S. (2019). Asthma pharmacotherapy: an update onleukotriene treatments. *Expert Review of Respiratory Medicine*.

Trivedi M., D. E. (2019). Asthma in children and adults-What are the differences and What can they tell us about asthma? *frontiers in pediatrics*.

Tsai H.C., H. M. (2016). Sphingosine-1-Phosphate (S1P) and S1P Signaling Pathway:Therapeutic Targets in Autoimmunity and Inflammation. *Springer International Publishing Switzerland*.

Tuder RM. (2017). Pulmonary Vascular Remodeling in Pulmonary Hypertension. *Cell Tissue Res*.

Vanhoutte, P. (2009). COX-1 and Vascular Disease. *CliniCal pharmaCology & TherapeuTiCs*.

Vanhoutte, P. M. (2013). Airway epithelium-derived relaxing factor: myth, reality, or naivety? *Am J Physiol Cell Physiol*.

Voelkel NF., T. R. (1996). Inhibition of 5-Lipoxygenase–activating Protein (FLAP) Reduces Pulmonary Vascular Reactivity and Pulmonary Hypertension in Hypoxic Rats. *The American Society for Clinical Investigation*.

Watanabe AJ, O. T. (2019). The Role of Leukotrienes as Potential Therapeutic Targets in Allergic Disorders. *International Journal of Molecular science*.

Watkins PB, D. L.-B. (2007). Clinical pattern of zileuton-associated liver injury: results of a 12-month study in patients with chronic asthma. *rug Saf.* .

Webb R. C. (2003). SMOOTH MUSCLE CONTRACTION AND RELAXATION. *ADVANCES IN PHYSIOLOGY EDUCATION*.

William W. Busse, M. R. (2010). The role of Viral respiratory infections in asthma and asthma exacerbations. *Lancet*.

Wu D., L. D. (2011). Prospect of vasoactive intestinal peptide therapy for COPD/PAH and asthma: a review. *Respiratory Research*.

Yatomia Y., O. Y. (2001). Sphingosine 1-phosphate: synthesis and release. *Prostaglandins & other Lipid Mediators*.

Yung J.A., F. H. (2018). Sex hormones, gender and asthma. *Ann Allergy Asthma Immunol.*

Zaslona Z., G. M. (2015). Prostanoids in Asthma and COPD. *AMERICAN COLLEGE OF CHEST PHYSICIANS*.

Zhang Y., E. L. (2010). Up-Regulation of Endothelin Receptors Induced by Cigarette Smoke —Involvement of MAPK in Vascular and Airway Hyper-Reactivity. *TheScientificWorldJOURNAL*.

Zheng H., W. D. (2018). Leptin Promotes Allergic Airway Inflammation through Targeting the Unfolded Protein Response Pathway. *Scientific Reports*.

Zhu B., L. K. (2016). Gender-related differences in β-adrenergic receptor-mediated cardiac remodeling. *Can J Physiol Pharmacol*.