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**PH.D. THESIS**

# Role of Thymic Stromal Lymphopoietin (TSLP) in Asthma


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**Abbreviations**

ACQ, asthma control questionnaire

ACT, asthma control test

ASM, airway smooth muscle

CAF, cancer-associated fibroblasts

CRTH2, G-protein-coupled chemokine receptor-homologous molecule

DC, dendritic cell

ECP, eosinophil cationic protein

FeNO, fractional exhaled nitric oxide

$FEV_1$, forced expiratory volume in the 1st second

GINA, global initiative for asthma

HRQoL, health-related quality of life

ICS, inhaled glucocorticoid

IL, interleukin

IL-7Rα, interleukin 7 receptor-α

ILC1, innate lymphoid type 1

ILC2, innate lymphoid type 2

ILC3, innate lymphoid type 3

IFN-γ, interferon gamma

IgE, immunoglobulins E

i.v., intravenous

LABA, long-acting stimulant $\beta_2$ agonist

lfTSLP, long form TSLP

LPS, lipopolysaccharide

LTRA, leukotriene receptor antagonist

mAb, monoclonal antibody

MBP, major basic protein

NKT, natural killer T cell

PAR2, protease-activated receptor 2

PEF, peak expiratory flow

$PGD_2$, prostaglandin $D_2$

SABA, short-acting $\beta_2$ stimulant

s.c., subcutaneous

sfTSLP, short form TSLP

Tfh, T follicular helper cell

TLR, toll-like receptor

TNF-$\alpha$, tumor necrosis factor $\alpha$

Tregs, regulatory T cell

TSLP, thymic stromal lymphopoietin

TSLPR, thymic stromal lymphopoietin receptor

$V_H$, variable heavy chain

$V_L$, variable light chain

**ABSTRACT**

Thymic Stromal Lymphopoietin (TSLP) is a pleiotropic cytokine originally isolated from a murine thymic stromal cell line and characterized as a lymphocyte growth factor. TSLP is predominantly expressed by lung and gut epithelial cells, keratinocytes, and by dendritic cells (DCs). TSLP can be produced also by airway smooth muscle (ASM) cells, mast cells, monocytes, macrophages and granulocytes, and cancer-associated fibroblasts (CAFs). There are two variants for TSLP in human tissues: the main isoform expressed in steady state is the short form (sfTSLP), which presumably plays a homeostatic role, whereas the long form (lfTSLP) is upregulated in inflammatory conditions. TSLP activates a specific heterodimeric receptor, identified on the majority of immune cells. The presence of TSLP receptor on a vast repertoire of cells indicates the relevance of this cytokine in various pathophysiological conditions. Indeed, TSLP has been implicated in several allergic diseases (e.g., bronchial asthma, atopic dermatitis, eosinophilic esophagitis), in chronic inflammatory (e.g., chronic obstructive pulmonary disease and celiac disease) and autoimmune (e.g., psoriasis, rheumatoid arthritis) disorders and in cancer. In this Ph.D. research project, we have investigated the role of TSLP in bronchial asthma. Our results suggest that circulating total TSLP is increased in patients with asthma compared to healthy controls. Plasma concentrations of total TSLP are not correlated to changes in forced expiratory volume in the 1$^{st}$ second ($FEV_1$) and asthma control test (ACT) score in asthma patients. Collectively, these results indicate that TSLP could represent a promising relevant therapeutic target in asthma.

# 1. INTRODUCTION

## 1.1. Thymic Stromal Lymphopoietin (TSLP)

Thymic stromal lymphopoietin (TSLP) is a pleiotropic cytokine originally isolated from a murine thymic stromal cell line (Friend, Hosier et al. 1994, Varricchi, Pecoraro et al. 2018) and characterized as a lymphocyte growth factor (Sims, Williams et al. 2000). A human homolog was identified using *in silico* methods (Ray, Furlonger et al. 1996, Reche, Soumelis et al. 2001). The human TSLP gene is located on chromosome 5q22.1 next to the atopic cytokine cluster on 5q31 (Quentmeier, Drexler et al. 2001). Differently, the murine TSLP is mapped on chromosome 18 (Sims, Williams et al. 2000).

TSLP is highly expressed by lung (Kato, Favoreto et al. 2007, Lee and Ziegler 2007, Allakhverdi, Comeau et al. 2009, Calven, Yudina et al. 2012, Lee, Headley et al. 2012, Nagarkar, Poposki et al. 2012) and intestinal epithelial cells (Harada, Hirota et al. 2009, Iliev, Spadoni et al. 2009, Cultrone, de Wouters et al. 2013, Collison, Sokulsky et al. 2015, Fornasa, Tsilingiri et al. 2015, Biancheri, Di Sabatino et al. 2016), keratinocytes (Soumelis, Reche et al. 2002, Li, Hener et al. 2006, Vu, Baba et al. 2010, Bjerkan, Schreurs et al. 2015, Fornasa, Tsilingiri et al. 2015), and by DCs (Kashyap, Rochman et al. 2011). TSLP can be produced also by airway smooth muscle (ASM) cells (Zhang, Shan et al. 2007), mast cells (Soumelis, Reche et al. 2002, Allakhverdi, Comeau et al. 2007, Allakhverdi, Comeau et al. 2009, Okayama, Okumura et al. 2009), eosinophils (Ebbo, Crinier et al. 2017), monocytes (Kashyap, Rochman et al. 2011), macrophages, granulocytes (Ying, O'Connor et al. 2008), basophils (Sokol, Barton et al. 2008, De Monte, Reni et al. 2011), and cancer-associated fibroblasts (CAFs) (Figure 1).

## 1.2. TSLP and its Receptor

Human TSLP exerts its biological activities by binding to a high-affinity heteromeric complex composed of thymic stromal lymphopoietin receptor (TSLPR) and interleukin 7 receptor-α (IL-7Rα). TSLP initiates signaling by establishing a ternary complex with its specific receptor, TSLPR, and then with IL-7Rα (Pandey, Ozaki et al. 2000, Park, Martin et al. 2000) (Figure 2). TSLP, positively charged, binds to TSLPR, which is negatively charged, with high affinity and fast kinetics (Verstraete, Peelman et al. 2017). Then, IL-7Rα binds to the TSLP: TSLPR binary complex. The formation of the ternary complex, TSLPR:TSLP: IL-7Rα, initiates signaling in cells co-expressing TSLPR and IL-7Rα. Figure 3 shows the structure of the TSLP:TSLPR:IL-7Rα complex determined by X-ray.

Tezepelumab is a human monoclonal antibody (mAb) that binds with high affinity to TSLP. TSLP possesses two distinct binding sites: one for TSLPR and another for IL-7Rα (Verstraete, Peelman et al. 2017). Figure 4 shows that the variable heavy chain ($V_H$) of Tezepelumab binds with high affinity to TSLP, while the variable light chain ($V_L$) fragment does not interact with TSLP (Verstraete, Peelman et al. 2017). Therefore, Tezepelumab binds to TSLP and inhibits intracellular signaling in human immune cells.

## 1.3. Isoforms of TSLP

Harada and coworkers identified two isoforms (short and long) for TSLP in human bronchial epithelial cells (Harada, Hirota et al. 2009). The short form TSLP (sfTSLP) was constitutively expressed in all normal tissues examined, including human lung fibroblasts. The long form TSLP (lf TSLP),

was upregulated in inflammatory conditions (Harada, Hirota et al. 2009). These investigators demonstrated that poly I:C, a toll-like receptor 3 (TLR3) ligand, induced the upregulation of lfTSLP (Allakhverdi, Comeau et al. 2007, Kato, Favoreto et al. 2007).

sfTSLP is constitutively expressed in all normal tissues examined, including lung fibroblasts and its sequence of the 63 amino acids is homologous to the C-terminus of the lfTSLP. Initially, short and long TSLP variants were considered the result of alternative splicing (Harada, Hirota et al. 2009). Subsequently, the same group identified two distinct 50-untranslated regions resulting in two different open reading frames for TSLP in the human genome (Harada, Hirota et al. 2011). The concept that two isoforms are not related to the same transcript but are rather controlled by two different promoter regions was confirmed by Rescigno and collaborators, who examined the differential expression and biologic activities of the two isoforms *in vitro* and *in vivo* (Fornasa, Tsilingiri et al. 2015). They also found that in healthy barrier surfaces, such as human intestinal and skin tissues, sfTSLP is the main transcript variant. sfTSLP inhibits *in vitro* the production of several cytokines (i.e., TNF-α, IL-1β, IL-6), whereas lfTSLP increases the release of IFN-γ. Importantly, they reported that lfTSLP activates the canonical TSLPR on human immune cells, whereas sfTSLP induces or inhibits signaling through an unknown receptor (Fornasa, Tsilingiri et al. 2015).

Dong *et al.* confirmed that several inflammatory stimuli increase only the expression of lfTSLP. In a mouse model of asthma, sfTSLP reduced inflammation and inhibited bronchial hyperreactivity (Dong, Hu et al. 2016). Finally, it has been demonstrated that some allergens markedly increase the production of 1fTSLP in primary human keratinocytes (Kuroda, Yuki et al. 2017).

Specific antibodies anti-sfTSLP are not commercially available and none of previous studies had used tools to identify expression or functions of sfTSLP and lfTSLP.

Despite increasing evidence of a dichotomy for the two isoforms of TSLP in humans, the pathophysiological roles of the short and long isoforms of TSLP in bronchial asthma are largely unknown. Therefore, it would be of paramount importance to examine the differential expression of the two isoforms in peripheral blood from patients with different phenotypes of asthma. Unfortunately, it is not known whether the plasma concentrations of total TSLP are really increased in patients with asthma. Moreover, and perhaps more importantly, it is unknown whether plasma concentrations of TSLP correlate with asthma severity.

Several studies have shown an increased tissue expression of TSLP in patients with atopic dermatitis and ulcerative colitis (Fornasa, Tsilingiri et al. 2015), whereas decreased levels of sfTSLP have been observed in patients with celiac disease (Collison, Sokulsky et al. 2015, Biancheri, Di Sabatino et al. 2016).

There is also evidence that TSLP can be cleaved by endogenous proteases in pathological conditions. Bianchieri and collaborators demonstrated that the furin protease, overexpressed in celiac patients, cleaves lfTSLP into different fragments (Biancheri, Di Sabatino et al. 2016). Other studies have reported that proteases expressed at the site of the inflammation cleave TSLP in two fragments showing a more intense pro-Th2 activity than the native form of lfTSLP (Kabata, Moro et al. 2013, Nagarkar, Poposki et al. 2013).

## 1.4. TSLP Activates Several Immune Cells Involved in Asthma

TSLP has several pleiotropic properties mediated by the activation of a broad range of immune and non-immune cells (Varricchi, Pecoraro et al. 2018). The expression of TSLP is induced by a wide spectrum of stimuli involved in the pathogenesis of different phenotypes/endotypes of asthma, such as allergens (Kuroda, Yuki et al. 2017), cytokines (e.g., TNF-α, IL-1β) (Allakhverdi, Comeau et al. 2007, Lee and Ziegler 2007, Cultrone, de Wouters et al. 2013, Collison, Sokulsky et al. 2015), respiratory viruses (Kato, Favoreto et al. 2007, Calven, Yudina et al. 2012, Lee, Headley et al. 2012, Nagarkar, Poposki et al. 2012, Xie, Takai et al. 2012), bacterial (Vu, Baba et al. 2010) and fungal products (Kouzaki, O'Grady et al. 2009), cigarette smoke extracts (Nakamura, Miyata et al. 2008, Smelter, Sathish et al. 2010) and tryptase (Wilson, The et al. 2013). Such a broad pathophysiological profile has motivated therapeutic targeting of TSLP-mediated signaling (Zhang, Huang et al. 2011, Borowski, Vetter et al. 2013, Verstraete, Peelman et al. 2017, Ye, Mou et al. 2017).

TSLP-activated DCs prime $CD4^+$ T cells to polarize toward Th2 cells (e.g., production of IL-4, IL-5, and IL-13) (Soumelis, Reche et al. 2002, Omori and Ziegler 2007). TSLP can cause goblet cell hyperplasia and mucus production when produced by activated lung epithelial cells (Allakhverdi, Comeau et al. 2007, Angkasekwinai, Park et al. 2007, Drake and Kita 2017). TSLP targets group 2 innate lymphoid cells (ILC2s) (Halim, Krauss et al. 2012, Kabata, Moro et al. 2013, Martin and Martin 2016, Stier, Zhang et al. 2018) and drives the development of Th2 cells (He, Oyoshi et al. 2008, Ochiai, Jagot et al. 2018). In addition, TSLP provides critical signals for T follicular helper cells (Tfh) differentiation (Varricchi, Harker et al. 2016, Pattarini, Trichot et al. 2017) and human B cell proliferation (Milford, Su et al. 2016).

Borriello and coworkers have demonstrated that stimulation of human monocytes with lipopolysaccharide (LPS) does not express TSLPR and IL-7Rα but rather induces the expression of TSLPR complex on a percentage of monocytes (Borriello, Iannone et al. 2017).TSLP activates human eosinophils through the engagement of TSLPR and IL-7Rα (Wong, Hu et al. 2010, Cook, Stahl et al. 2012, Morshed, Yousefi et al. 2012, Noh, Shin et al. 2016).

Siracusa and coworkers reported that TSLP promoted peripheral basophilia in mice and that TSLPR-expressing basophils play a role in Th2-dependent immunity (Siracusa, Saenz et al. 2011). It has been reported that approximately 10% of human basophils express TSLPR and that TSLP increases histamine release from basophils (Salter, Oliveria et al. 2015). By contrast, a collaborative study demonstrated that human basophils do not express IL-7Rα and do not respond to TSLP (Salabert-Le Guen, Hemont et al. 2018). Similar findings were also reported by Schroeder and collaborators using highly purified human basophils (Schroeder and Bieneman 2017). These observations emphasize the immunological differences between human and murine basophils (Varricchi, Raap et al. 2018, Marone, Schroeder et al. 2020).

$CD34^+$ progenitor mast cells and human lung mast cells express both TSLPR and IL-7Rα (Allakhverdi, Comeau et al. 2007). TSLP, alone or in combination with IL-1β or TNF-α, does not induce mast cell degranulation but induces cytokines/chemokines release (Allakhverdi, Comeau et al. 2007, Kaur, Doe et al. 2012, Han, Oh et al. 2014). TSLP in combination with IL-33 causes prostaglandin $D_2$ ($PGD_2$) production by human cord blood derived mast cells and by human peripheral blood-derived mast cells (Buchheit, Cahill et al. 2016). Recently, it has been demonstrated that although TSLP has no effect on histamine release from human skin mast cells, it potentiates substance P-induced mediator release (Babina, Wang et al. 2021). Collectively, these findings demonstrate that TSLP might orchestrate a plethora of immune cells involved in the pathogenesis of different phenotypes of asthma.

## 1.5. Bronchial Asthma and GINA Guidelines

Bronchial asthma is a highly prevalent inflammatory airways disease affecting 300-400 million people worldwide (Peters and Wenzel 2020) (http://www.globalasthmareport.org/). This disorder accounts for 1-2% of the healthcare budget in several developed countries (Serebrisky and Wiznia 2019). Asthma is a heterogeneous disease (mild, intermediate, severe) characterized by chronic inflammation of the airways and is defined by a clinical history of respiratory symptoms (wheezing, dyspnea, chest tightness and cough) that vary over time and intensity. The majority of asthmatic patients have mild disease, while approximately 5–10% have severe asthma (Chung, Wenzel et al. 2014, Backman, Jansson et al. 2019). The symptoms of asthma are triggered by various etiological factors, such as allergens and respiratory tract infections. The pathogenesis of asthma is not fully known. Therefore, treatment of different phenotypes of asthma is far from ideal.

Bronchial asthma is associated with hyperreactivity of the bronchial smooth muscle to harmless stimuli and chronic low-grade inflammation of the airways persistent even in the asymptomatic phases (Global Strategy for Asthma Management and Prevention from www.gina.org (2021). Once the bronchial asthma diagnosis has been confirmed, it is fundamental to detect the risk factors and co-morbidities (e.g., chronic rhinosinusitis, nasal polyposis, gastroesophageal reflux disease, obesity).

According to GINA guidelines (Figure 5), therapies include control drugs (Controllers) to prevent exacerbations and drugs to treat acute asthma attacks (Relievers). Controller drugs are not required if symptoms arise less than twice a month, $FEV_1$ (forced expiratory volume in the 1st second) is > 80% predicted and/or variability in peak expiratory flow (PEF) are < 20% (mild disease).

Only if necessary, a therapy with short-acting stimulant $\beta_2$ (SABA) and inhaled glucocorticoids (ICS) is indicated (Step 1). When symptoms arise more than twice a month, the daily use of low doses of ICS is recommended (Step 2). If necessary, the leukotriene receptor antagonists (LTRA) and the association of ICS with SABA are also included in the therapy. If a good control of symptoms is not achieved, it is advisable to add a long-acting stimulant β2 (LABA), thus administering low doses of ICS in combination with the β2 receptor agonists. In the subsequent Steps (3 and 4), the doses of ICS-LABA should be progressively increased and in case of lack of symptom control, the use of drugs such as theophylline, anticholinergics and oral glucocorticoids are recommended. GINA guidelines (Step 5) also include biological drugs (e.g., mAbs directed against specific molecular or receptorial targets) indicated in those patients in whom the disease remains uncontrolled despite maximum therapy. Biological drugs [mAbs anti-IgE (Omalizumab), anti-IL-5 (Mepolizumab, Reslizumab), anti-IL-5Rα (Benralizumab), and anti-IL-4Rα (Dupilumab)] are indicated in patients with severe uncontrolled asthma [Global Strategy for Asthma Management and Prevention from www.gina.org (2021)].

### 1.6. Heterogeneity of Asthma and Different Phenotypes

Asthma does not indicate a single disease, but rather a group of clinical disorders associated with low-grade inflammation, a reversible limitation of airflow and/or bronchial hyperreactivity (Varricchi, Bagnasco et al. 2016, McDowell and Heaney 2020, Sze, Bhalla et al. 2020). In the international guidelines, the presence of a set of specific inflammatory features has also been added among the criteria of the pathophysiology.

The pathogenetic model of asthma based on a central role played by Th2 lymphocytes, which determined hyperreactivity and reversible bronchial obstruction is considered outdated (Wenzel 2006). In fact, the clinical evidence of failure of therapies based on this simplistic assumption appears obsolete observing the variability of phenotypes and the presence of non-Th2 pathogenetic mechanisms (McDowell and Heaney 2020, Sze, Bhalla et al. 2020).

Actually the term “asthma” includes a set of clinical (symptoms, exacerbations, lung functions) and inflammatory features on the basis of which different phenotypes have been distinguished according to the complex interactions among genetic and environmental factors (Wenzel 2012).

It has been proposed to distinguish:

• Clinical phenotypes.

• Phenotypes related to triggers.

• Phenotypes related to the type of inflammation.

Clinical phenotypes can be differentiated by severity, presence of airflow limitation and age of onset. The 2021 GINA guidelines classify asthma into three different severity phenotypes: mild, moderate and severe. Mild, moderate and severe asthma are treated according to GINA guidelines previously mentioned: mild asthma according to Steps 1 or 2; moderate asthma according to Step 3; and severe asthma according to Steps 4 or 5.

The age of onset is explanatory to distinguish two asthma phenotypes: "early-onset" and "late-onset". The latter form is very heterogeneous: patients have more than 12 years, a worse lung function and no family history of asthma (Miranda, Busacker et al. 2004, Wenzel 2006). In patients with late onset of severe asthma, persistent eosinophilic or neutrophilic inflammation of the airways was often found, suggesting the presence of pathogenetic mechanisms other than moderate asthma (Amelink, de Groot et al. 2013). In patients with late onset of the disease, the analysis of the sputum frequently shows high levels of neutrophils (> 40%). In this particular phenotype of

neutrophilic asthma, there is an association with frequent exacerbations, nocturnal asthma and glucocorticoid-refractory asthma.

Another set of phenotypes can be identified by observing the triggers of asthma attacks, searching for the cause-effect relationship between exposure and symptoms. The most frequent phenotype is allergic or extrinsic asthma. In this case, the main stimulus for the development of the disease is environmental and the interaction with a genetically predisposed host leads to the development of an immune response mediated by various cytokines (i.e., IL-4, IL-5, IL-13) produced by the pulmonary epithelium, tissue mast cells and peripheral blood basophils.

Different mechanisms characterize and support eosinophilic and neutrophilic asthma. In eosinophilic asthma there is a prevalence of Th2 pattern driven by the activation of Th2 cells and ILC2, leading to the production of IgE and mast cell/basophil activation. Neutrophilic inflammation is usually induced by pathogens, such as viruses, bacteria or environmental pollutants that trigger the production of interleukins (e.g., CXCL8). This difference between eosinophilic and neutrophilic asthma has pharmacologic implications. Glucocorticoids are highly effective in eosinophilic inflammation, whereas they have limited effects in human neutrophils (Simpson, Grissell et al. 2007). It is important to note that in some patients there is a combination of an eosinophilic and neutrophilic inflammation. This phenotype, defined as mixed inflammatory asthma, is associated with severe and difficult airway symptoms (Hastie, Moore et al. 2010). Figure 6 illustrates a simplified scheme of three different types of chronic airway inflammation in asthma patients.

Eosinophilic phenotypes are characterized by elevated blood eosinophil counts, typically defined as at least 150 or 300 cells/μL, and/or elevated percentage of sputum eosinophil of at least 2–3% (Carr and Kraft 2018). In the case of allergic asthma, patients also exhibit a serum IgE level of at least 30 IU/mL, in addition to sensitivity to a perennial aeroallergen (Carr and Kraft

2018). Fractional exhaled nitric oxide (FeNO) levels may also be elevated in patients with eosinophilic or allergic phenotypes, although there is not yet a consensus on the threshold that constitutes elevated FeNO.

Predominantly neutrophilic inflammation with normal eosinophil counts or elevated counts of eosinophils and neutrophils (mixed granulocytic inflammation) describe other phenotypes of asthma (Moore, Hastie et al. 2014, Carr and Kraft 2018, Tliba and Panettieri 2019). In addition to the heterogeneity of immune cells and biomarkers, there is variability among patients in other pathophysiological features of asthma, such as airway hyperresponsiveness and remodeling, which may be related to or separate from inflammatory events in the airways (Saglani and Lloyd 2015).

Another phenotype of asthma is characterized by a significant loss of $FEV_1$ every year (physiologically of 15 / 20 ml) and occurs mainly in patients with severe disease. The risk factors for this phenotype are smoking agents, chronic mucosal hypersecretion, low initial $FEV_1$, age of onset > 12 years, frequent exacerbations, blood or sputum hypereosinophilia and bronchial hyperreactivity (ten Brinke, Zwinderman et al. 2001). Among the most important mediators, there is IL-5 which favors the infiltration of eosinophils with the release of powerful mediators such as the major basic protein (MBP) and eosinophil cationic protein (ECP).

Recently, a new classification of phenotypes/endotypes of asthma has been proposed. This includes two major forms: T2 low asthma and T2 high asthma (Figure 7). The latter form is prevalent and it is mainly characterized by eosinophilic inflammation and activation of mast cells, and basophils, ILC2, and Th2 cells (Varricchi, Bagnasco et al. 2016, McDowell and Heaney 2020, Sze, Bhalla et al. 2020). T2 low asthma is less characterized and includes forms with prevalent neutrophilic or paucigranulocytic inflammation. In T2 low asthma, neutrophils and their mediators play a major pathogenetic role. In addition, ILC1/3, Th1 and Th17 cells are predominant immune cells.

It is important to note that severe asthma is an uncontrolled pathology despite standard-of-care therapy (Chung, Wenzel et al. 2014, Chen, Golam et al. 2018), which severely impacts health-related quality of life (HRQoL) with persistent symptoms and frequent and life-threatening exacerbations (Chen, Gould et al. 2007, McDonald, Hiles et al. 2018). Severe asthma has a high asthma-related healthcare and medication costs (Chastek, Korrer et al. 2016, Zeiger, Schatz et al. 2016). Co-morbidities of severe asthma are allergic rhinitis, nasal polyposis, chronic rhinosinusitis, gastroesophageal reflux disease, and obesity (Porsbjerg and Menzies-Gow 2017).

## 1.7. TSLP and Asthma

Different groups have found an association of polymorphisms in TSLP with asthma (Gudbjartsson, Bjornsdottir et al. 2009, He, Hallstrand et al. 2009, Harada, Hirota et al. 2011). Additional evidence for the relevance of TSLP in airway inflammation has been provided by genetic studies of mice. TSLPR-deficient mice are resistant to the development of ovalbumin-induced inflammation in mice (Al-Shami, Spolski et al. 2005, Zhou, Comeau et al. 2005). On the other side, mice overexpressing TSLP in the airway epithelium develop an inflammatory disease with characteristics of asthma (Zhou, Comeau et al. 2005). Intranasal delivery of TSLP and antigen lead to the onset of severe disease (Headley, Zhou et al. 2009). Asthmatic patients have higher concentrations of TSLP in their lungs (Ying, O'Connor et al. 2005, Ying, O'Connor et al. 2008) and in peripheral blood (Chauhan, Singh et al. 2015).

## 1.8. Tezepelumab: a Monoclonal Antibody Anti-TSLP

Tezepelumab is a first-in-class human mAb that blocks TSLP activity, thus inhibiting its interaction with TSLP receptor complex. Figure 8 schematically illustrates the X-ray structure of the variable heavy ($V_H$) and light ($V_L$) fragments of Tezepelumab and TSLP. The $V_H$ region of Tezepelumab binds with high affinity to TSLP. By contrast, the $V_L$ chain does not interact with TSLP. Tezepelumab binds to TSLP and blocks the formation of TSLPR:TSLP:IL-7Rα ternary complex on effector cells. Gavreau and collaborators demonstrated that Tezepelumab (700 mg i.v. on days 1, 29 and 57) inhibited the early and late asthmatic response, reduced sputum eosinophils and FeNO levels in patients with moderate atopic asthma (Gauvreau, O'Byrne et al. 2014). In the phase 2b PATHWAY study (NCT02054130), Tezepelumab reduced asthma exacerbations by up to 71% compared to placebo in patients with severe, uncontrolled asthma across the spectrum of inflammatory phenotypes, and improved lung function and asthma control.

Corren and collaborators have demonstrated that Tepezelumab (70, 210 or 280 mg s.c. every 4 weeks) reduced asthma exacerbations, peripheral blood eosinophil levels, FeNO, and improved both $FEV_1$ and asthma control questionnaire-6 (ACQ-6) scores in patients with different phenotypes of asthma (Corren, Parnes et al. 2017). Collectively, these studies indicate that TSLP represents a novel therapeutic target in different phenotype of asthma.

Several phase 3 trials of Tezepelumab in asthma patients are underway. A pivotal exacerbation study, NAVIGATOR (NCT03347279) has the aim to assess the possible efficacy of Tezepelumab in patients with different severe asthma phenotypes, including those with low blood eosinophil counts.

The study SOURCE (NCT03406078) aims to evaluate the oral glucocorticoid-sparing potential of Tezepelumab. DESTINATION (NCT03706079), a long-term extension study, and CASCADE (NCT03688074), an ongoing phase 2 bronchoscopy study, seek to evaluate the effect of Tezepelumab on airway inflammation and airway remodeling in patients across the spectrum of type 2 airway inflammation (Menzies-Gow, Wechsler et al. 2020).

## 2. AIMS OF THIS THESIS

The studies previously discussed demonstrate that TSLP, or more likely TSLP isoforms (sfTSLP and lfTSLP), are involved in the pathophysiology of experimental models of airway inflammation (Dong, Hu et al. 2016, Kuroda, Yuki et al. 2017) and of bronchial asthma (Gauvreau, O'Byrne et al. 2014, Corren, Parnes et al. 2017). There is compelling evidence that asthma is a chronic inflammatory disorders characterized by tissue remodeling (Varricchi, Rossi et al. 2019, Spadaro, Giurato et al. 2020). Macrophages represent the predominant cells in human lung parenchyma (Staiano, Loffredo et al. 2016, Lavin, Kobayashi et al. 2017) and play a central role in the chronic evolution of asthma. Therefore, the purpose of this study was fourfold. In particular:

1) we evaluated the plasma concentrations of TSLP in patients with asthma compared to healthy controls.
2) We have evaluated the plasma concentrations of TSLP in patients with different severity of asthma.
3) We have correlated the plasma concentrations of TSLP with asthma control test (ACT) score in patients with different severity of asthma.
4) We have correlated the plasma concentrations of TSLP with peripheral blood eosinophils in patients with asthma.

## 3. MATERIALS AND METHODS

### 3.1. Study Population

We studied 30 adult patients with asthma (10 males and 20 females; age range: 19–65 years; median age: 50 years) followed at the Outpatient Asthma Clinics directed by Professor Giuseppe Spadaro at the University of Naples Federico II. Thirty healthy individuals (10 males and 20 females; age range: 27–65 years; median age: 47 years) were enrolled as control group. Asthma diagnosis and severity assessment were based on the Global Initiative for Asthma (GINA). All patients underwent routine clinical evaluation that included medical history, physical examination, pulmonary function test ($FEV_1$), total and differentiated leukocyte count in peripheral blood. Healthy donors in the control group had a normal spirometry and negative history of asthma and chronic obstructive pulmonary disease.

### 3.2. Blood Sampling

The Ethics Committee of University of Naples Federico II approved that plasma obtained during routine diagnostics could be used for research investigating the pathophysiology of asthma and written informed consent was obtained from patients according to the principles expressed in the Declaration of Helsinki. The controls had been referred for routine medical check-up and volunteered for the study. Blood samples were collected by means of a clean venipuncture and minimal stasis. The researchers who performed the experiments were blinded to the patient history.

### 3.3. ELISA Assay

TSLP concentration in peripheral blood and cell pellets and supernatants was measured using commercially available ELISA kit (2000-31.3 pg/ml) (R&D System, MN, USA). This assay has been calibrated and validated (Varricchi, Pecoraro et al. 2018).

### 3.4. Statistical Analysis

The data are expressed as mean values ± SEM. Statistical analysis was performed in Prism 6 (GraphPad Software). Statistical analysis was performed by Student's T-test or one-way analysis of variance followed by Dunnett's test (when comparison was made against a control) by means of Analyse-it for Microsoft Excel, version 2.16 (Analyse-it Software, Ltd.). Statistically significant differences were accepted when the $p$ value was at least $< 0.05$.

# 4. RESULTS

## 4.1. TSLP Plasma Concentrations in Asthma Patients Compared to Healthy Controls

TSLP is a member of the IL-2 family of cytokines that binds to a heterodimeric receptor composed of TSLP receptor (TSLPR) and IL-7Rα (CD127), and mainly signals through STAT5 phosphorylation (Ziegler 2012, Varricchi, Pecoraro et al. 2018, Markovic and Savvides 2020). TSLP concentrations in bronchial lavage fluid and biopsies are increased in patients with asthma compared to healthy controls (Ying, O'Connor et al. 2005, Ying, O'Connor et al. 2008, Shikotra, Choy et al. 2012, Mitchell, Salter et al. 2018). TSLP drives various elements of asthma pathophysiology, including airway hyperresponsiveness, mucus overproduction and airway remodelling, *via* effects triggered downstream (Varricchi, Pecoraro et al. 2018). TSLP activates DCs, which prime naïve T helper cells to produce IL-4, IL-5 and IL-13 (Soumelis, Reche et al. 2002). TSLP also activates several other immune cells, including mast cells (Allakhverdi, Comeau et al. 2007), eosinophils (Wong, Hu et al. 2010, Cook, Stahl et al. 2012, Morshed, Yousefi et al. 2012, Noh, Shin et al. 2016) and ILC2 (Camelo, Rosignoli et al. 2017).

In a first series of experiments we measured the concentration of total TSLP in plasma obtained from 30 asthmatic patients and 30 healthy donors matched by age and sex. Total TSLP was measured by a commercial ELISA (R&D System, Italy). Figure 9 shows that the circulating concentrations of TSLP in healthy controls greatly varied from 0 to 36,817 pg/ml. Similarly, the plasma levels of TSLP in asthmatic patients ranged from 0 to 44,672 pg/ml. There was a trend of increase of circulating levels of TSLP in asthmatics (8,872 ± 2,345 pg/ml) to be higher than controls (3,435 ± 1,435 pg/ml).

Plasma levels of TSLP were not affected by gender in both controls (Figure 10A) and in asthmatic patients (Figure 10B). There was no significant

correlation between age and TSLP concentration in both healthy controls (r = 0.05; NS) and in asthmatic patients (r = 0.01; NS) (Figure 11).

## 4.2. Correlation between TSLP Plasma Concentrations and Asthma Severity

TSLP is an up-stream cytokine presumably involved in the pathogenesis of different phenotypes of asthma (Varricchi, Pecoraro et al. 2018, Marone, Spadaro et al. 2019). Therefore, we tried to correlate the severity of asthma with the plasma concentrations of TSLP in different group of asthmatic patients. These patients were divided in three groups according the severity of asthma evaluated by changes in $FEV_1$. Patients with severe asthma had a $FEV_1$ < 60% of normal subjects, whereas patients with $FEV_1$ > 80% were considered mild asthmatics. Patients with intermediate asthma had a $FEV_1$ between 60% and 80% of normal values. Figure 12 shows that the plasma concentrations of TSLP did not change among the three groups of asthmatic patients.

The Asthma Control Test (ACT) is a multi-point system to evaluate the severity of asthma. Patients with ACT < 15 have severe asthma, whereas patients with ACT > 19 are considered mild asthmatics. ACT between 15 and 19 refers to moderate asthma. Figure 13 shows that there was a trend for severe asthmatics to have lower concentrations of circulating TSLP.

### 4.3. Correlation between TSLP Plasma Concentrations and Circulating Eosinophils

There is compelling evidence that circulating and tissue eosinophils play a pivotal role in the pathogenesis of severe eosinophilic asthma (Bagnasco, Ferrando et al. 2017, Bagnasco, Ferrando et al. 2017, Varricchi, Senna et al. 2017). Figure 14 shows that asthmatic patients with higher levels of circulating eosinophils (> 500 μL) have lower concentrations of TSLP. This apparently surprising observation could be explained, at least in part, by suggesting that patients with hypereosinophilia have a more severe form of asthma and were treated with high doses of ICS or systemic glucocorticoids.

## 5. DISCUSSION

The results of this study demonstrate that there is a trend for circulating TSLP to be increased in asthmatic patients compared to healthy controls. In this cohort of patients, plasma concentrations of total TSLP are not correlated to changes in $FEV_1$ and ACT score, two independent parameters of asthma severity. Interestingly, asthmatic patients with higher levels of eosinophils have lower concentrations of TSLP.

Previous studies have demonstrated the involvement of TSLP in different mouse models of asthma (Headley, Zhou et al. 2009, Dong, Hu et al. 2016) and an association of TSLP polymorphisms with asthma (Gudbjartsson, Bjornsdottir et al. 2009, He, Hallstrand et al. 2009, Harada, Hirota et al. 2011). In addition, asthmatic patients have high concentrations of TSLP in their lung (Ying, O'Connor et al. 2005, Ying, O'Connor et al. 2008). In this study, we attempted to measure TSLP concentrations in peripheral blood of asthma patients and controls using an ELISA technique, which does not differentiate sfTSLP from lfTSLP. We found that in a large percentage of healthy controls and of asthma patients, TSLP was undetectable. This could be to low sensitivity of our assay or to cleavage of circulating TSLP by proteases. In fact, several groups of investigators have shown that endogenous TSLP can be cleaved into smaller fragments by several proteases at sites of inflammation (Kabata, Moro et al. 2013, Nagarkar, Poposki et al. 2013, Biancheri, Di Sabatino et al. 2016). The latter hypothesis is indirectly supported by the observation that plasma levels of total TSLP are apparently decreased in more severe patients characterized by lower ACT score and higher levels of eosinophils.

We would like to speculate that in patients with severe asthma, characterized by low ACT score and high eosinophil count, there is marked release of proteolytic enzymes from activated immune cells which can cleave circulating TSLP.

A technical limitation of this study derives from the use of ELISA, which does not distinguish sfTSLP from lfTSLP. Unfortunately, at present specific antibodies anti-sfTSLP and/or anti-lfTSLP are not available and none of previous studies had used tools to identify the expression and functions of the two isoforms in peripheral blood of normal subjects and asthma patients. Thus, despite the compelling evidence of a dichotomy for the two isoforms of TSLP in humans (Harada, Hirota et al. 2009, Fornasa, Tsilingiri et al. 2015), the pathophysiological roles of sfTSLP and lfTSLP in bronchial asthma remain largely unknown.

In conclusion, the results emerging from these studies widen the role of TSLP in bronchial asthma. We found that there is a trend for circulating total TSLP to be increased in patients with asthma compared to healthy controls. These results were obtained in relatively small cohorts of asthma patients and healthy controls. We cannot exclude the possibility that examining larger cohorts of patients and controls our findings might reach statistical significance.

Surprisingly but interestingly, concentrations of total TSLP are apparently lower in asthma patients with higher eosinophil counts and lower ACT score. We plan to investigate whether proteolytic enzymes present and released (e.g., tryptase, chymase) by activated immune cells relevant in asthma (e.g., mast cells, eosinophils) might cleave TSLP *in vitro* and *in vivo* to smaller fragments.

We would like to suggest that our results might have translational relevance, reinforcing the results of clinical studies (Gauvreau, O'Byrne et al. 2014, Corren, Parnes et al. 2017) that TSLP represents a novel therapeutic targets in bronchial asthma.

## 6. FIGURES

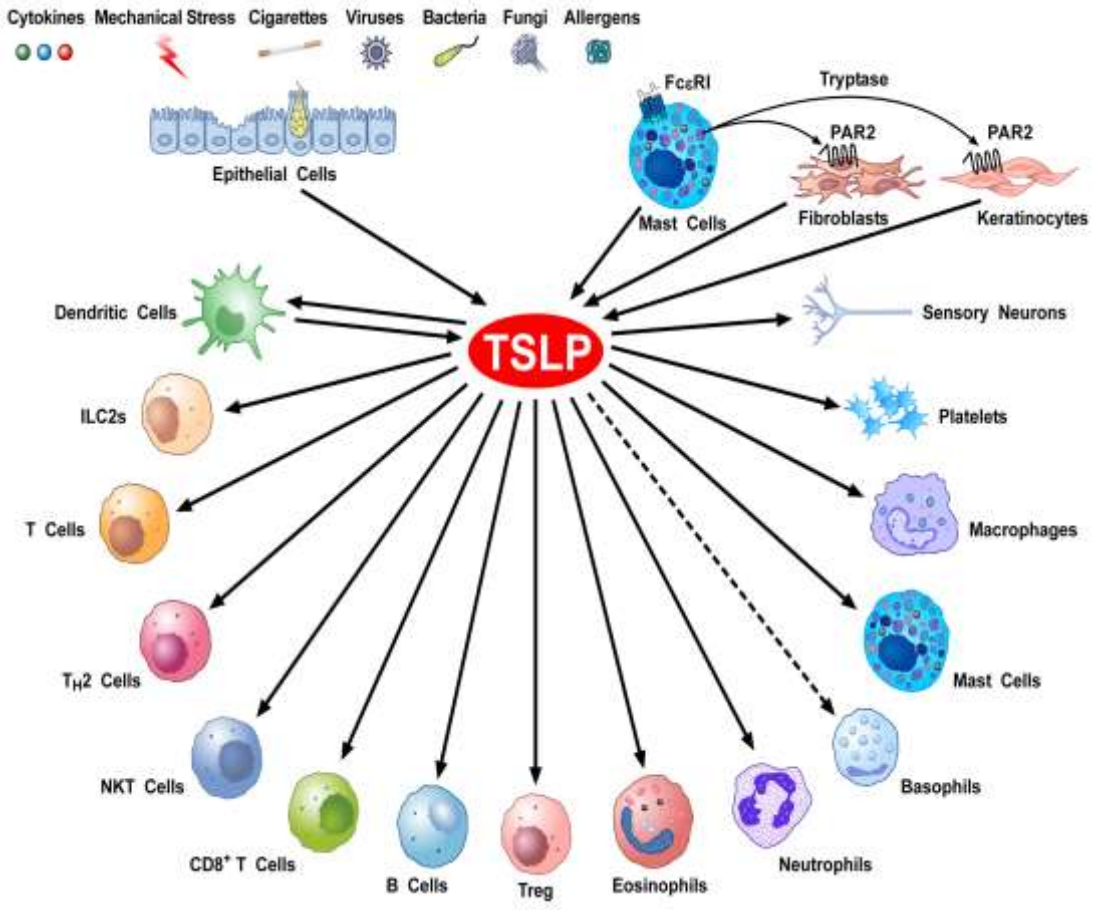


**Figure 1.**

Schematic representation of cellular targets of thymic stromal lymphopoietin (TSLP). Several triggers can activate lung epithelial cells to release TSLP. This cytokine can also be produced by activated mast cells and dendritic cells (DCs). Tryptase, released by mast cell activates the protease-activated receptor 2 (PAR2) receptor on fibroblasts and keratinocytes to release TSLP. TSLP activates DCs, ILC2, $CD4^+$ T and Th2 cells, NKT cells, $CD8^+$ T cells and B cells, Treg, eosinophils, neutrophils, murine, but not human basophils, mast cells, macrophages, platelets, and sensory neurons [from (Varricchi, Pecoraro et al. 2018)].

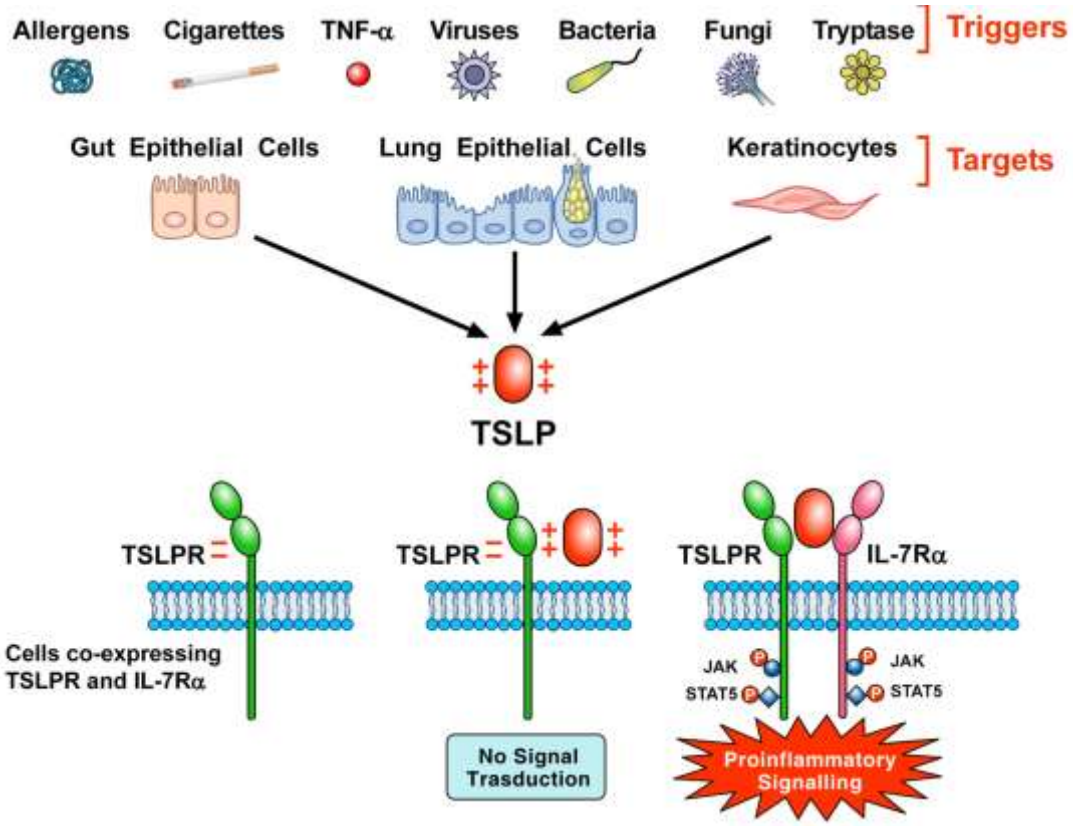


**Figure 2.**

Schematic representation of the production of thymic stromal lymphopoietin (TSLP) and its signaling complex *via* a cooperative stepwise mechanism on the surface of cellular targets. A plethora of triggers (allergens, cigarette smoke extracts, cytokines, viral, bacterial and fungal products, and tryptase) can activate lung and gut epithelial cells and keratinocytes to release TSLP. The latter, which is positively charged, binds to thymic stromal lymphopoietin receptor (TSLPR), which is negatively charged, with high affinity and fast kinetics. Then, IL-7Rα associates with performed TSLPR:TSLP binary complex to form the ternary TSLPR-TSLP-IL-7Rα complex. This receptor complex on cells co-expressing TSLPR and IL-7Rα phosphorylates JAK and STAT5 to initiate proinflammatory signaling in several immune cells [from (Varricchi, Pecoraro et al. 2018)].

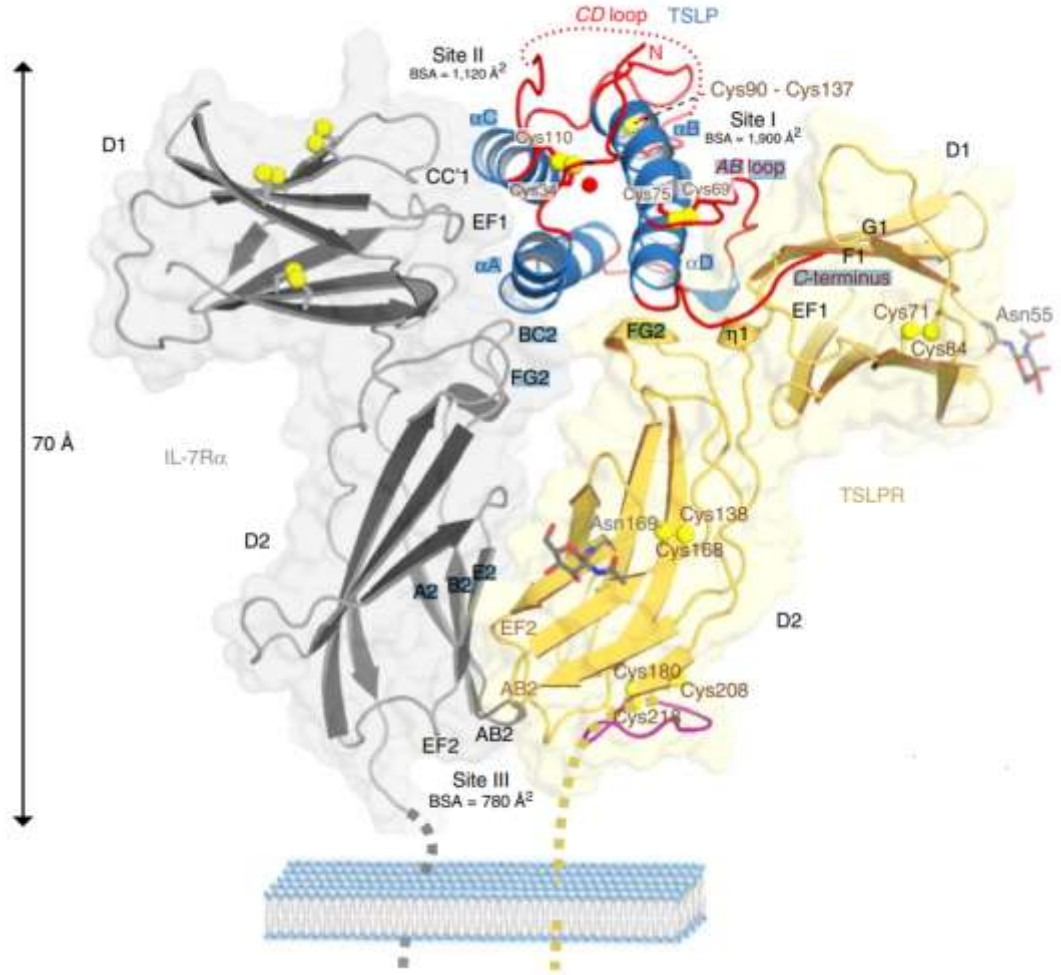


**Figure 3.**

View of the determined X-ray structure for the TSLP:TSLPR:IL-7Rα complex and TSLP. TSLP (blue) is shown in cartoon representation with its four helices labelled as Aa, αB, αC, and αD. TSLP loop regions are highlighted in red with the disordered CD loop region represented as a dashed line. The extracellular regions of TSLPR (yellow on the right) and IL-7Rα (grey on the left), each comprising two domains, D1 and D2, are shown as cartoons overlaid onto transparent surface representations. Disulfide bridges are shown as yellow spheres. The water molecule in the core of TSLP is shown as a red sphere [from (Verstraete, Peelman et al. 2017)].

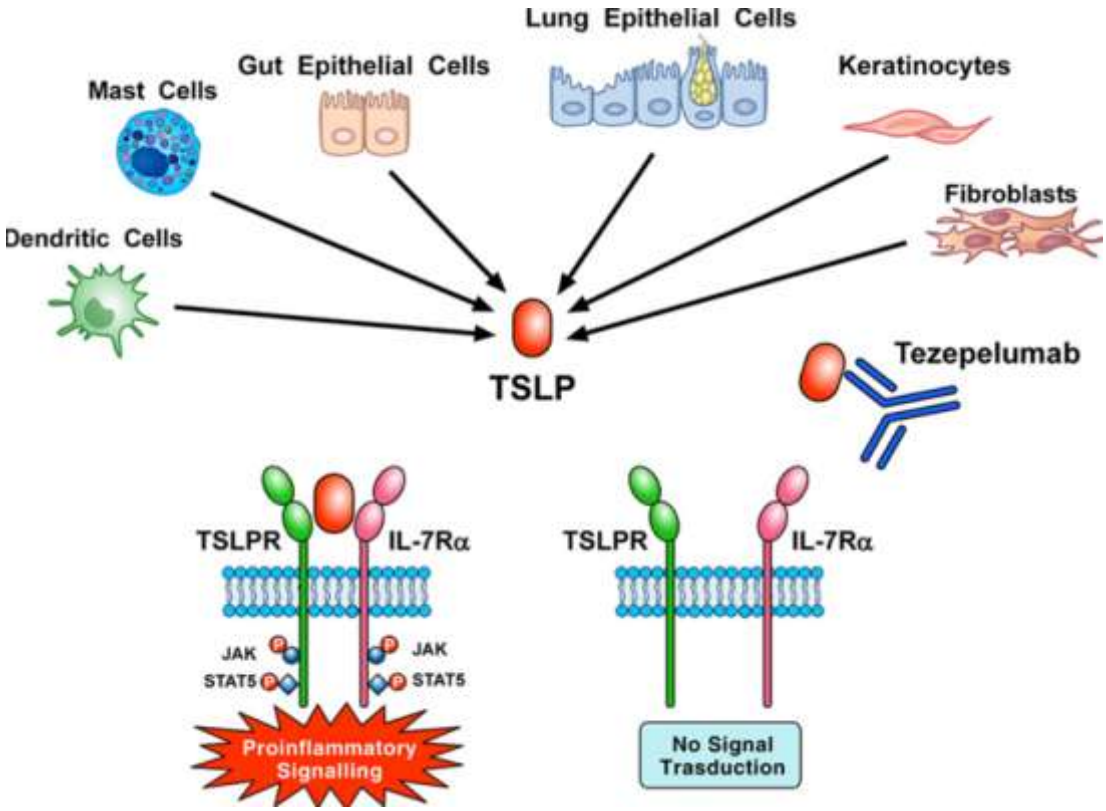


**Figure 4.**

Thymic stromal lymphopoietin (TSLP) produced mainly by lung and gut epithelial cells and keratinocytes but also by dendritic cells (DCs), mast cells, and fibroblasts initiates signaling by establishing a ternary complex with thymic stromal lymphopoietin receptor (TSLPR) and IL-7Rα. Tezepelumab, a human monoclonal antibody (mAb) anti-TSLP, binds with high affinity to TSLP and blocks the formation of TSLPR:TSLP: IL-7Rα ternary complex on effector cells. In particular, the variable heavy ($V_H$) chain of Tezepelumab binds to TSLP, while the variable light ($V_L$) chain does not interact with TSLP. Tezepelumab inhibits *in vitro* human DC maturation and chemokine production induced by TSLP and reduced exacerbations in patients with severe uncontrolled asthma [from (Varricchi, Pecoraro et al. 2018)].

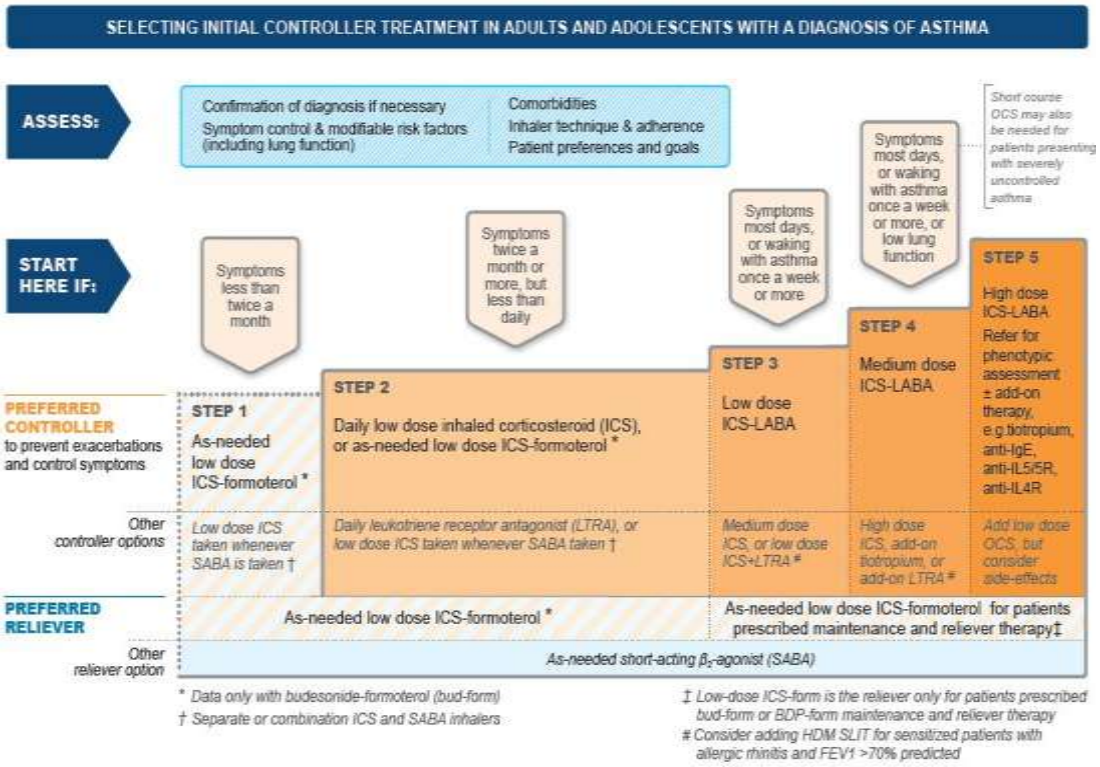


**Figure 5.**

Bronchial asthma is a chronic low-grade inflammation disorder of airways. Asthma is a genetic and phenotypically heterogeneous disorder. Therefore, treatment of patients with different forms of asthma (mild, moderate, and severe) requires a multistep procedure. This figure schematically summarizes the GINA guidelines concerning Controlled and Reliever drugs for the treatment of different forms of asthma [from www.gina.org (2021)].

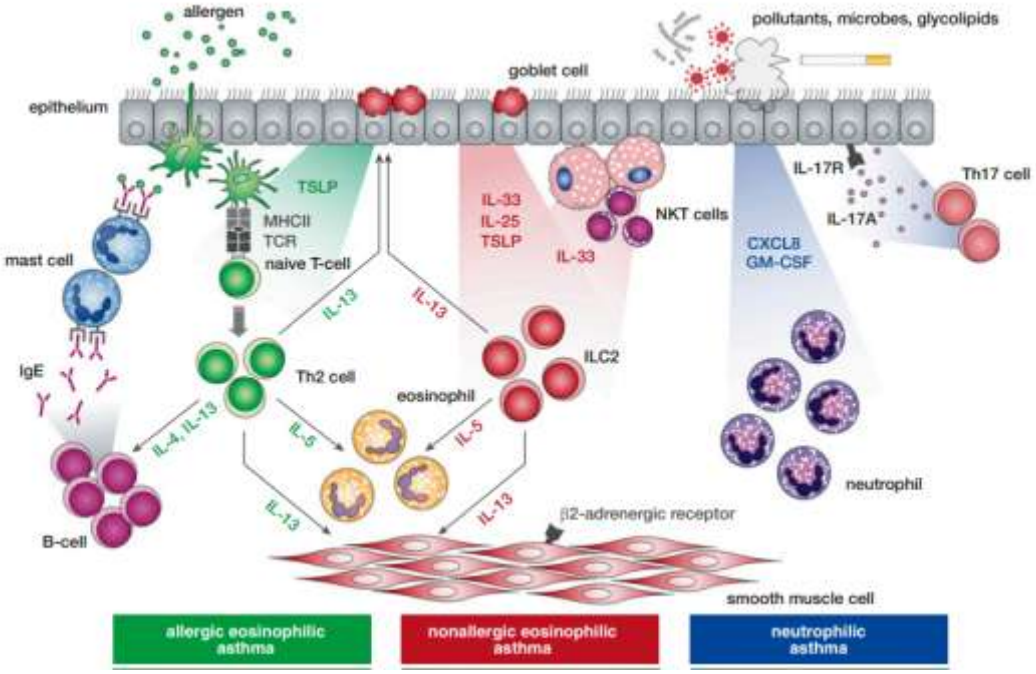


**Figure 6.**

Simplified scheme of three different types of chronic airway inflammation in patients with asthma. In allergic eosinophilic asthma (green box), Th2 lymphocytes and mast cells drive eosinophilic airway inflammation in an allergen-specific, IgE-dependent manner. In non-allergic eosinophilic asthma (red box), innate lymphoid cells type 2 (ILC2) and presumably natural killer T cells (NKT cells) might contribute to airway eosinophilia *via* the production of IL-5. The mechanisms underlying neutrophilic asthma (blue box) remain to be fully elucidated, but IL-17 and CXCL8 pathways have been associated with airway neutrophilia [reprinted with permission of the American Thoracic Society. Copyright© 2021 American Thoracic Society. From (Brusselle and Bracke 2014)].

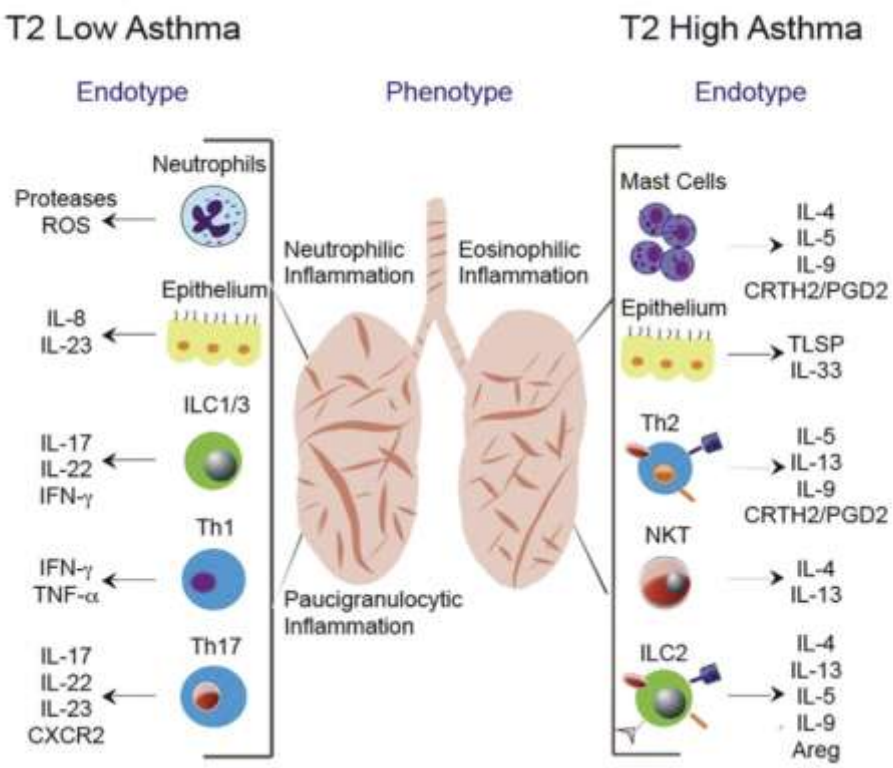


**Figure 7.**

Different inflammatory and immune patterns in the airways contribute to the development of two phenotypes/endotypes of asthma (T2-low and T2-high). In a T2-low pattern, a predominance of neutrophils or paucigranulocytic inflammation has been described. Patients with this phenotype are less likely to respond to glucocorticoids. In neutrophilic-predominant disease, patients might respond to antibodies that block IL-17 or CXCL8. Innate lymphoid cells type 1 (ILC1) and type 3 (ILC3) are more predominant in T2-low disease. These cells produce IL-22, IL-17, and IFN-γ. A T2-high inflammation is suggestive of eosinophilic phenotypes and is more likely to respond to glucocorticoids. A multitude of inflammatory cells and cytokines is involved in this phenotype. Targeting specific type 2 cytokines has proven to be an effective strategy, including mAbs anti-IgE, anti-IL-5/IL-5Rα, and anti-IL-4Rα [from (Tabatabaian, Ledford et al. 2017)].

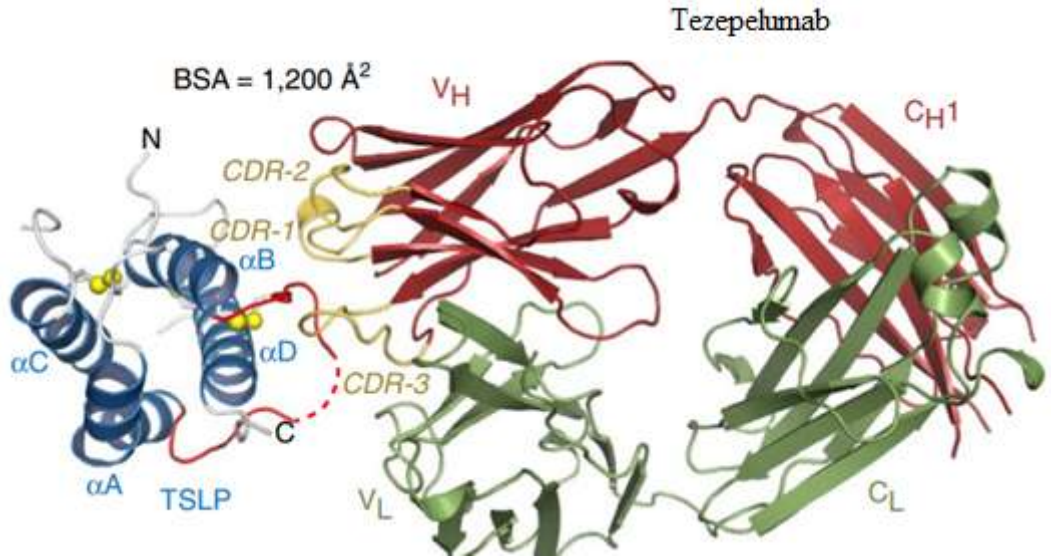


**Figure 8.**

Cartoon representation of the determined X-ray structure for Fab fragment of Tezepelumab in complex with TSLP. TSLP helices (αA, αB, αC, and αD) are colored in blue. The red dashed line represents to two disordered residues in the TSLP loop. N- and C-termini of TSLP are indicated. The $V_H$ and $C_H1$ fragments of Tezepelumab are colored in red, whereas the $V_L$ and $C_L$ fragments of Tezepelumab are in green. This cartoon shows that the $V_H$ fragment of Tezepelumab selectively binds to TSLP [modified from (Verstraete, Peelman et al. 2017)].

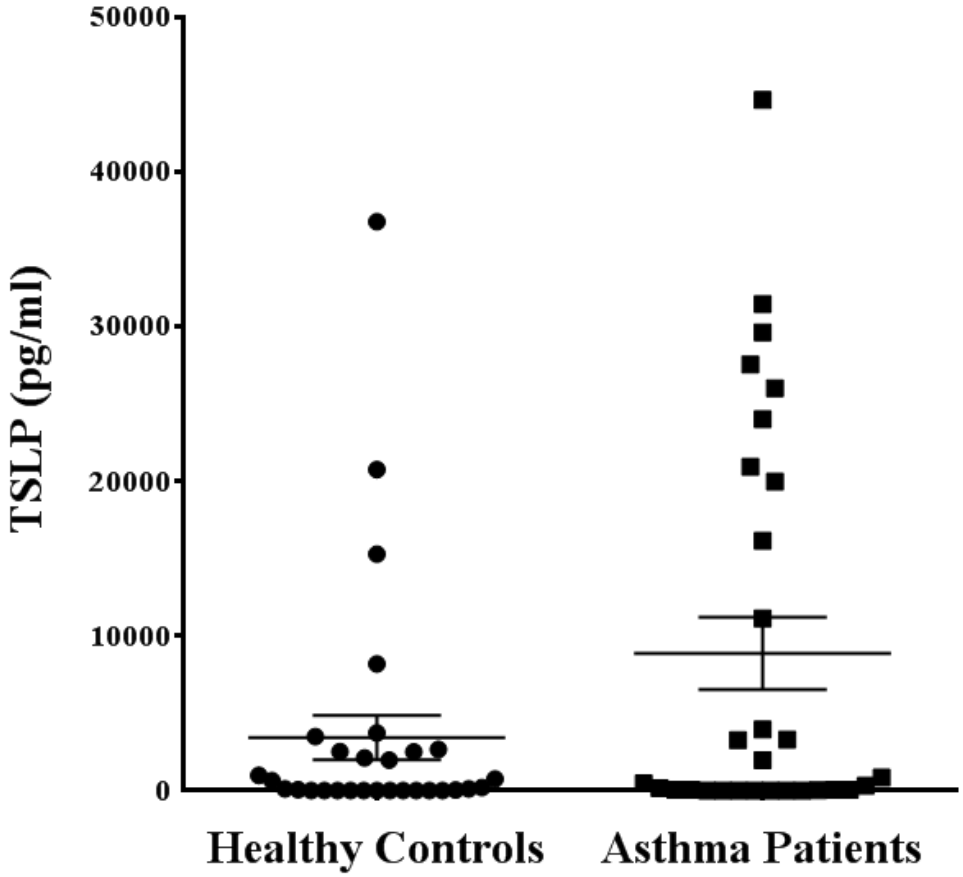


**Figure 9.**

**TSLP concentrations in plasma of healthy controls and patients with asthma.**TSLP in healthy controls (N=30) and in asthma patients (N=30) were determined by ELISA (R&D System). Each point represents a single donor evaluated in duplicate. Data are shown as the mean ± SEM.

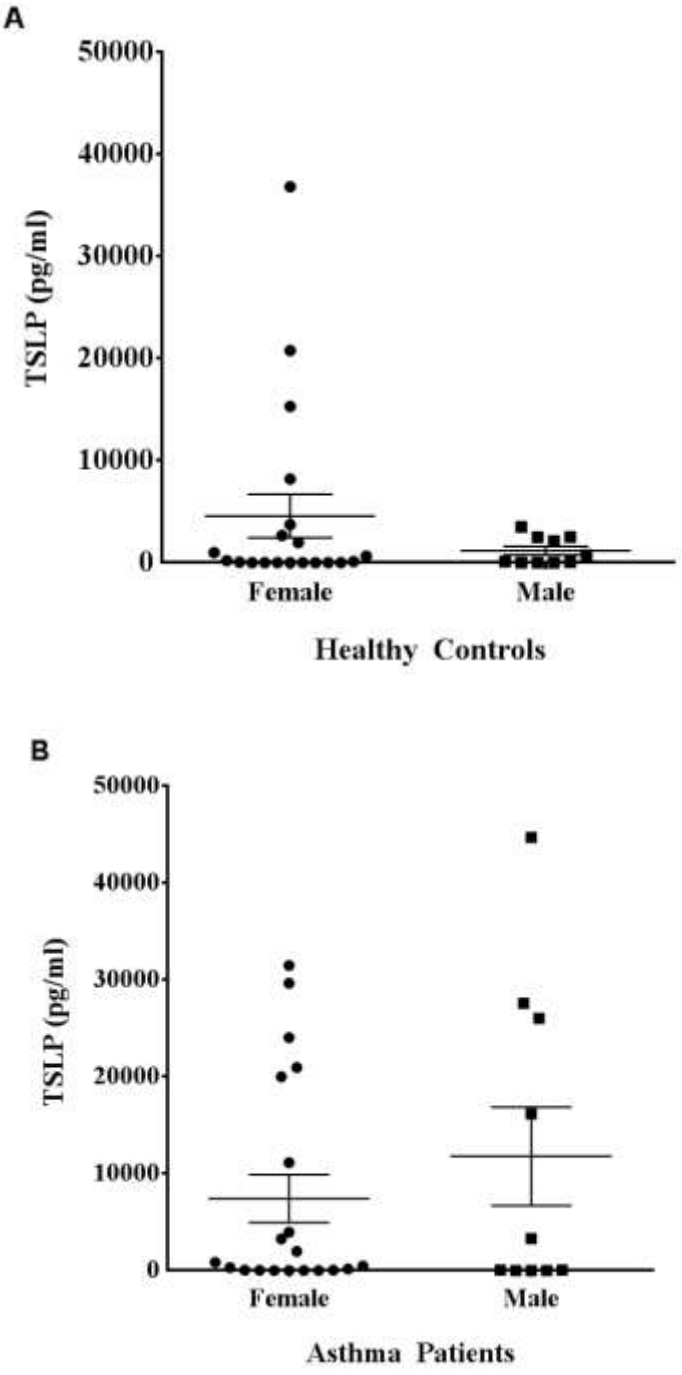


**Figure 10.**

**TSLP concentrations and gender in healthy controls and in patients with asthma.** TSLP in healthy females (N=20) and males (N=10) (A) and in asthma patients females (N=20) and males (N=10) (B) were determined by ELISA (R&D System). Data represent the mean ± SEM.

NS: Not significant

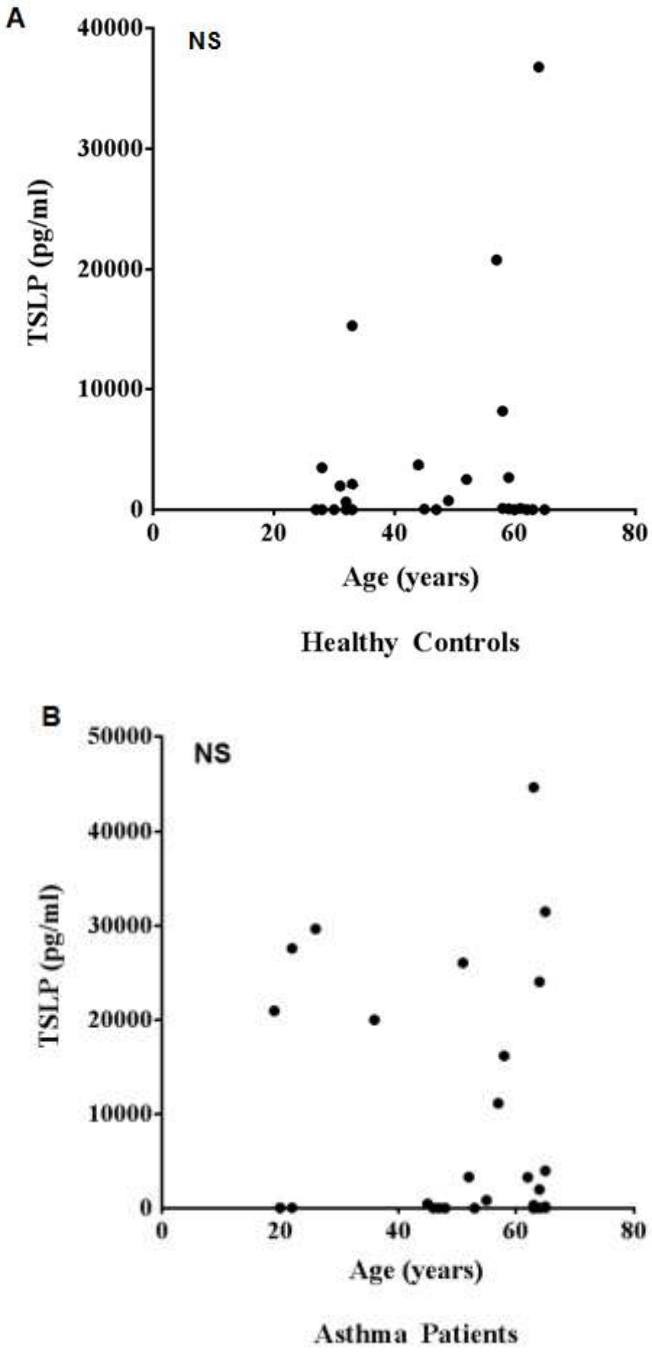


**Figure 11.**

**Relationship between TSLP plasma concentrations and age of healthy controls and patients with asthma.** TSLP were determined by ELISA (R&D System). Correlation between levels of TSLP and age (expressed as years) in healthy controls (N=30) (A) and in asthma patients (N=30) (B) were assessed by linear regression analysis.

NS: Not significant

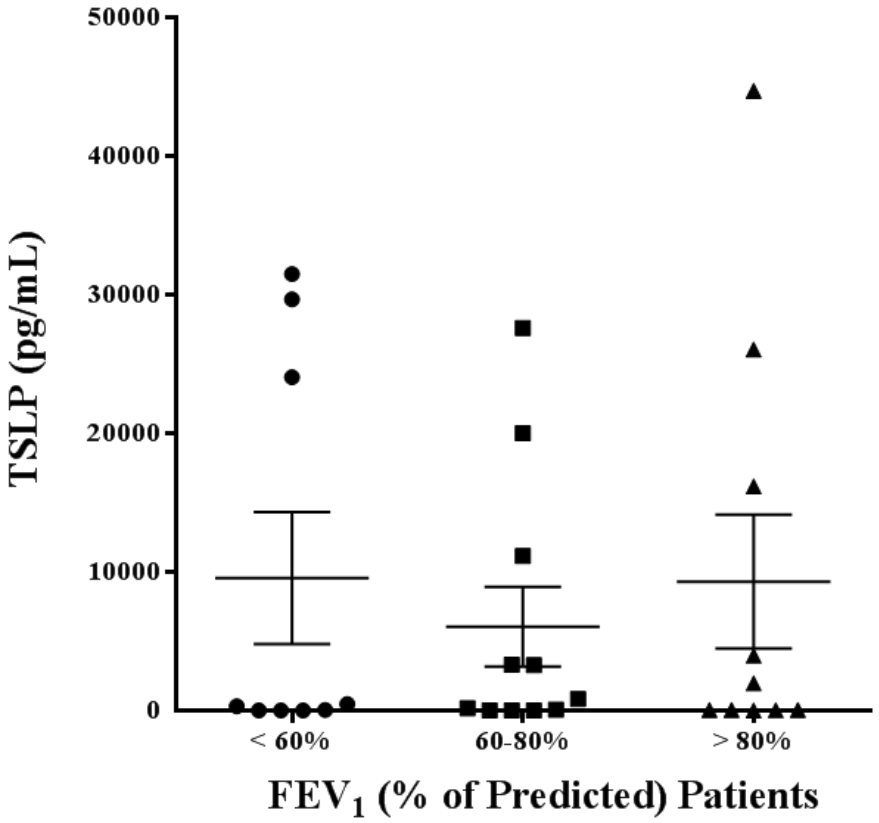


**Figure 12.**

**Effects of $FEV_1$ on TSLP plasma concentrations in patients with asthma.** TSLP concentrations were determined in nine patients with $FEV_1$ > 80% predicted (mild asthma), eleven patients with $FEV_1$ 60-80% predicted (moderate asthma) and ten patients with $FEV_1$< 60% predicted (severe asthma).

Data represent the mean ± SEM.

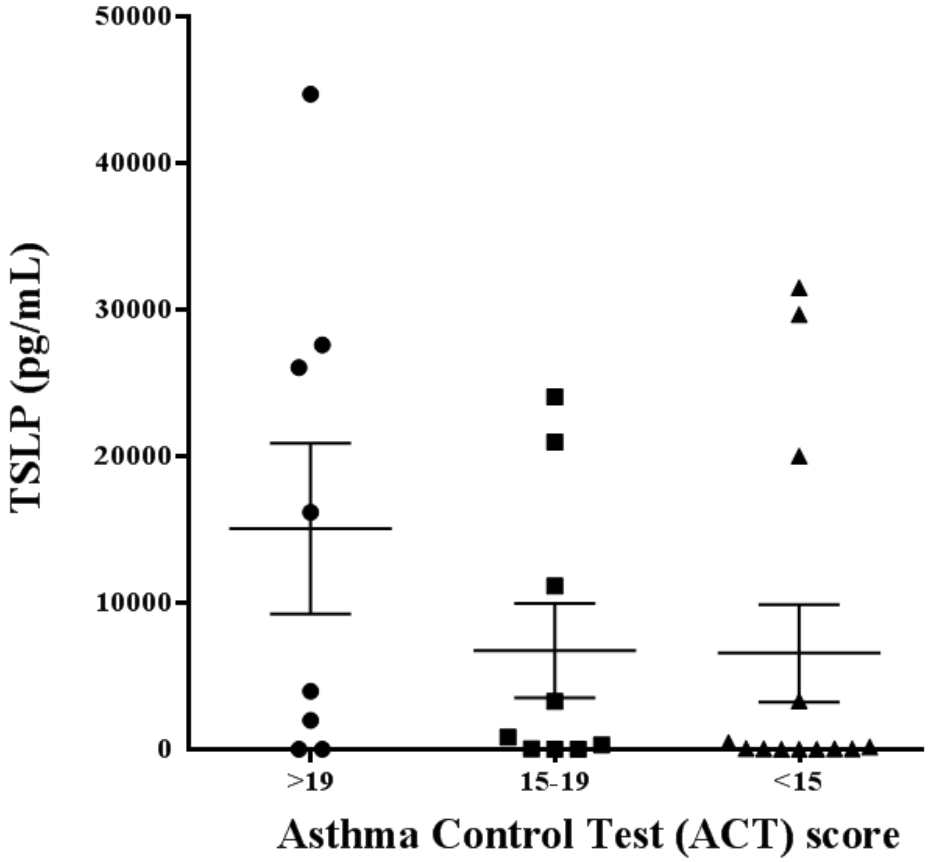


**Figure 13.**

**Effects of Asthma Control Test (ACT) score on TSLP plasma concentrations in patients with asthma.** TSLP concentrations were determined in eight patients with ACT score >19 (well controlled asthma), nine patients with ACT score 15-19 (partially controlled asthma) and thirteen patients ACT score < 15 (poorly controlled asthma).

Data represent the mean ± SEM.

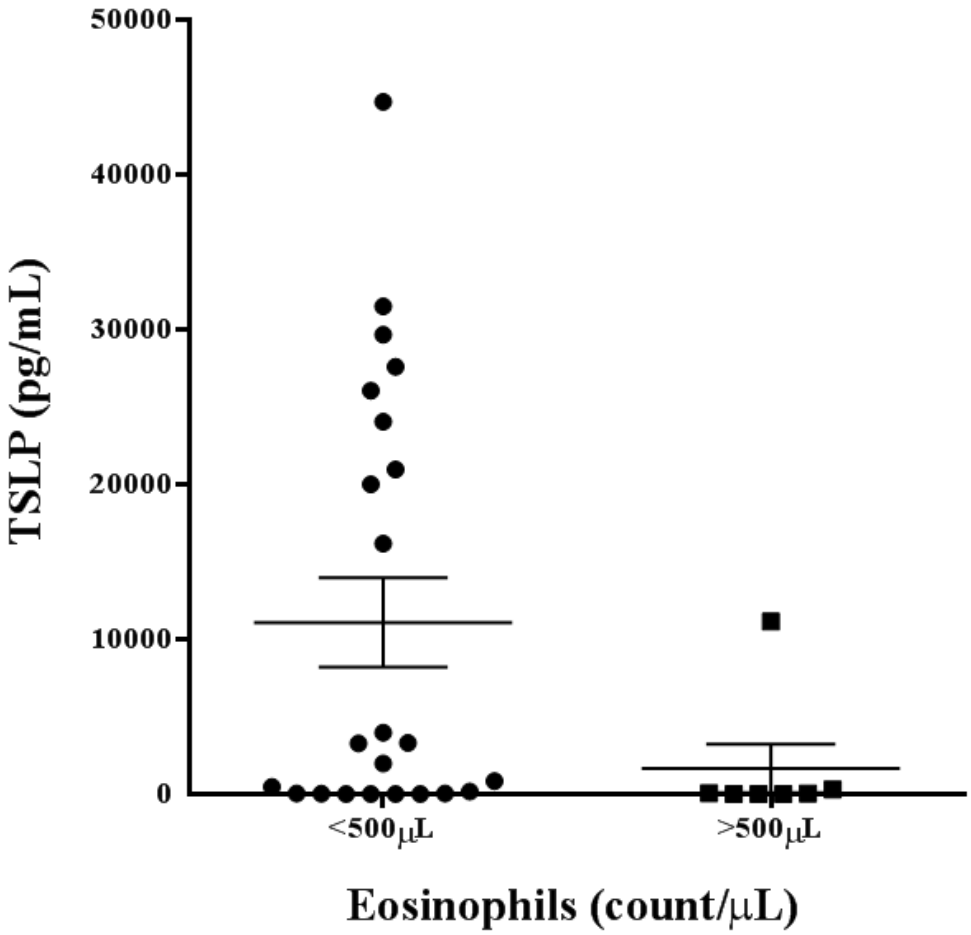


**Figure 14.**

**Effects of eosinophil count on TSLP plasma concentrations in patients with asthma.** TSLP concentrations were determined in twenty-three patients with eosinophil count <500 and seven patients with eosinophil count >500. Data represent the mean ± SEM.

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