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Immune modulatory effects of novel monoclonal antibodies target therapies in severe eosinophilic asthma patients

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Summary

New anti IL-5 antibodies, mepolizumab and benralizumab, have recently been approved for severe asthma, sharing the same inclusion criteria. To contribute on biomarkers research leading to the personalized choice, we investigated L-selectin, KL-6 and lymphocyte subsets as bioindicators of airways hyper-responsiveness and remodelling and to phenotype patients according to their answer to the treatment. L-selectin mediates leukocyte rolling of lung endothelium and its expression on T cells is increased in asthma patients. Regulatory T cells (Tregs) suppress inflammation by secreting a wide variety of cytokines that inhibit T cell proliferation.

A cohort of 28 patients affected by severe eosinophilic asthma were treated with anti IL-5 drugs: 20 with Mepolizumab and 8 with Benralizumab. Lymphocytes subsets, Regulatory T cells and CD4+CD62L+ cells were analyzed through flow cytometry, Serum L-selectin quantification was performed by bead-based multiplex analysis, while KL-6 was analyzed through CLEIA.

Clinical, functional and immunological data at baseline (T0), after one month (T1) and 6 months of therapy were collected in a database. All treated patients showed variations in FEV1, FEV1/FVC ratio and peripheral eosinophils for both drugs. Mepolizumab treated patients also showed significant differences between T0 and T1 in $CD8^+$ and NK-T like cells percentages and a significant increase in L-selectin concentrations. Stratifying the cohort of our patients in "early responders and partial responders at T0 they showed significant differences in peripheral eosinophils, sL-selectin, and KL-6, while no differences were found at T0 between "early responders" and "partial responders" patients treated with Benralizumab. In a subgroup of 14 mepolizumab treated patients, immunological data showed an increase in Tregs and CD4+CD62L+ at T1. Soluble L-selectin concentrations were lower at T1. CD45+ and CD62L+ cell features differed significantly in early and partial responders before and after therapy. The FEV1/FVC ratio showed an indirect correlation with L-selectin levels ($r=-0.6$, $p=0.03$), peripheral eosinophilia ($r=-0.7$, $p=0.01$). This real-life study provides new insights for the personalized approach to severe asthma therapy. Although preliminary, the results indicate that besides to peripheral eosinophils, other molecules are useful as biomarkers of early response that can also involving in the pathogenesis of severe asthma. Mepolizumab therapy was found to modulate immune response, restoring immune balance in patients with SEA. L-selectin and Tregs were also proposed as biomarkers of response to mepolizumab treatment.

1. **INTRODUCTION**

1.1 Asthma definition and epidemiology

Asthma is an heterogeneous disease, characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, variable expiratory airflow limitation, chest tightness and cough that vary in intensity and over time (1).

The impact of asthma is felt not only by patients, but also by families, healthcare systems and society. Asthma is a global health problem affecting approximately 300 million people worldwide, of all age groups, with a prevalence from 1% to 21% in adults (2). The World Health Organization estimates that asthma is responsible for about 250,000 deaths/year (3).

Although genetic factors are of great importance in determining a predisposition to developing asthma, environmental factors play an important role in disease susceptibility.

Asthma is common in industrialized nations and factors that have been implicated include urbanization, air pollution and exposure to environmental allergens (4).

In Europe, asthma affects around 30 million people with different prevalence according to geographic area:

- 3% of the population in Eastern European countries
- 9% in the United Kingdom and Scandinavian countries (5).

Asthma occurs mainly in males during childhood, with a male/female ratio of 2/1 until puberty, when the male/female ratio becomes 1/1 (6).

The prevalence of asthma is higher in women after puberty and most cases of adult onset diagnosed in individuals over the age of 40 occur in women, with gender differences related not only to incidence and prevalence but also the severity of symptoms (7,8).

The prevalence of asthma is increased in very young and very old people due to airway reactivity and lower levels of lung functions (9).

2/3 of all asthma cases are diagnosed before 18 years old, however, half of all children diagnosed with asthma have a decrease or disappearance of symptoms in early adulthood (10).

The cost of asthma include:

A. direct cost:
    - drugs,
    - medical examinations
    - access to the emergency room
    - hospitalization
B. indirect costs:
    - loss of working days

- limitations in daily activities
- premature mortality that represent more than 50% of the total cost for asthma.

The high costs of asthma are also attributable to uncorrect use of diagnostic resources, failure to control the disease and the development of exacerbations (11,12).

1.2 Etiology of asthma and risks factors.

Based on clinical criteria, asthma can be differentiated into:

- extrinsic (atopic-allergic)
- intrinsic
- occupational
- mixed

The separation into these different groups was initially based on clinical evaluation, but is now also supported by immunological studies that have found basic differences and common features between these manifestations of asthma.

Asthma is defined as:

- **Extrinsic:** when an exogenous cause can be identified. Extrinsic asthma usually begins in childhood (13).
  - Atopic asthma: In most cases, this cause is represented by an allergen that acts through a mechanism mediated by class E immunoglobulins (IgE), responsible for allergic syndromes, with allergic familiarity, positive skin allergic tests and coexistence of other allergic manifestations such as rhinitis, conjunctivitis or atopic dermatitis.
  - Non-atopic subjects: develop asthma following exposure to substances present in the workplace, pharmacology or food. A particular case is represented by occupational asthma, determined by a sensitizing agent present in the environment (14).
- **Intrinsic:** when an exogenous cause cannot be identified. In most cases they are negatively for allergic familiarity and skin allergy tests. Intrinsic asthma usually begins later, often in combination with a viral infection of the upper airways. Sometimes, it can be associated with nasal polyposis, aspirin sensitivity and steroid dependence (17).

The risk factors for asthma are divided into:

- **Individual:** which act as predisposing factors

1. **Genetic predisposition**

   Different genes are involved in the pathological mechanisms of asthma: Polymorphisms in the gene encoding platelet activating factor hydrolase (PAFH), an intrinsically neutralizing agent of platelet activating factor may play a role in asthma susceptibility and asthma severity (15). The mutation of genes in loco 17q21, is found in most cases of severe asthma and in asthma with severe exacerbations. It resulted implicated in gene transcription, cellular apoptosis and degranulation of eosinophils (16). Other gene mutations result in increased susceptibility to asthma, favoring the activation of inflammatory cells, smooth muscle contraction, cell differentiation, epithelial polarity and fibrosis (17). These variants have been identified thanks to association genome-wide studies (GWAS) and show alterations in the cellular barrier / function and in the innate or adaptive immune response that cause asthma (18,19).

2. **Atopy**

   the genetically determined ability to develop IgE-mediated immune reactions, is an important risk factor for the onset of asthma, in particular for asthma with early onset (before age 10) and for occupational asthma (16). The diagnosis is made on the basis of clinical-anamnestic criteria, on the positivity of the skin prick tests and on the serological tests (19).

3. **Obesity:** Obesity is closely related to the development of asthma due to:
   - Mechanical impairment: reduction of lung volumes, compression of the small airways.
   - Biological changes, linked to a chronic systemic inflammatory state.

     A study by Cottrell et al. described an association between asthma, obesity and abnormal lipid and glucose metabolism (20). Increase weight in early childhood is associated with an increased risk of developing asthma (21). In adults, the increase in BMI predicts the severity of asthma, also given the reduced control of inflammation with corticosteroid therapy (22). Obesity also affects the asthma phenotype, which manifests itself in these subjects with a non-eosinophilic inflammatory pattern (23,24)

4. **Gender:** Asthma manifests itself with different onset, pathogenesis and clinical characteristics in the two sexes (25). The prevalence of asthma is higher in paediatric males; on the other hand, most diagnoses in adulthood involve the female sex (26).

Women have more pronounced atopy, bronchial hyper-reactivity and more severe clinical course of asthma, taking into account a greater number of hospital admissions, hospitalization time, number of re-hospitalizations (27,28).

- **Environmental:** which favor the onset of the disease in predisposed subjects.
    1. **Allergens:** These are protein substances present in some volatile and easy to inhale agents: pollen, dust, pet hair, mites and insects. They act through an IgE-mediated mechanism following ingestion, inhalation, injection (29).
    2. **Smoking:** Is a major risk factor for the development of asthma. Smoking-induced asthma is associated with poor disease control, an impaired response to corticosteroid therapy, an accelerated decline in lung function and an increased rate of healthcare utilization (30).
    3. **Pollution and Work place:** Urban pollution, especially particulate matter is associated with an increased risk of asthma exacerbations (31).

       The work environment is a potential risk factor for the development and exacerbation of occupational asthma (32).

        - High molecular weight allergens: flours, enzymes, latex, foods, animal derivatives.
        - Low molecular weight: isocyanates, metals, various chemical compounds.

       About 10-15% of asthma cases in adults are due to occupational factors. The most involved work activities are related to agriculture, painting, cleaning work and the production of plastic materials (33).

1.3 Pathogenesis

The pathogenesis of asthma is due to interactions between predisposing genetic factors and environmental factors.

It involves the following components:

1.3.1 Airway inflammation

Allergic inflammation is mediated by Th2 lymphocytes. The aeroallergens within the airways interact with the dendritic cells located at the intraepithelial level (34). Dendritic cells process the

allergen by facilitating the formation of peptide fragments, subsequently exposed, through the HLA molecule of the major histocompatibility complex II, on cell surface (35). Dendritic cells migrate to the T dependent regions of the thoracic regional lymph nodes, where they interact with naive T lymphocytes, inducing their subsequent differentiation into Th2 (36,37). The formation of Th2 is favored by IL4, released by mast cells, eosinophils and basophils and by TSLP (thymic stromal lymphopoietin), a cytokine of innate immunity secreted by bronchial epithelial cells and mast cells (38). Th2, release cytokines such as IL-3, IL-4, IL-5, IL-9 and IL-13 (39). IL-4 and IL-13 induce the production of IgE trough a phenomenon called "phenotypic switching" by B lymphocytes (40). The IgE produced interact with mast cells via high affinity receptors FcεRI, resulting in the release of preformed mediators (histamine) and neoformates (cystenyl-leukotrienes, prostaglandin D2) and the activation of genes encoding cytokines, chemokines and growth factors (IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, GM-CSF) (41,42). In particular, the cytokine IL-5 induces the differentiation and maturation, activation and chemotaxis of eosinophils, in synergy with two chemokines: eotaxin and RANTES (43,44). IL-9, secreted by Th9 deriving from Th2, induces the differentiation and activation of mast cells (Fig.1) (45).

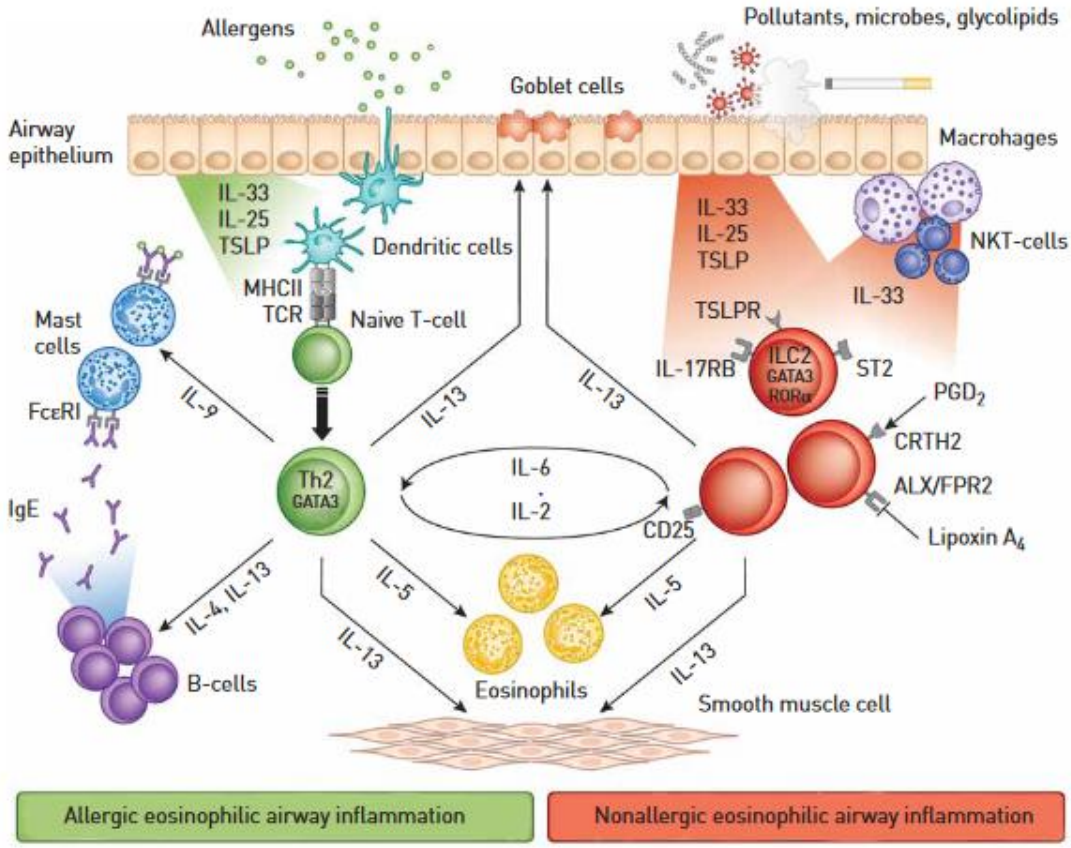


Fig.1 *Two different pathways lead to eosinophilic airway inflammation in asthma. In allergic asthma, dendriticcells present allergens to CD4+T-cells, inducing T-helper (Th)2 cells, which*

produce interleukin (IL)-4, IL-5 and IL-13,and leading to IgE switching in B-cells, airway eosinophilia and mucous hypersecretion. In nonallergic eosinophilicasthma, air pollutants, microbes and glycolipids induce the release of epithelium-derived cytokines, including IL-33,IL-25 and thymic stromal lymphopoietin (TSLP), which activate innate lymphoid cells (ILCs) in anantigen-independent manner via their respective receptors (IL-17 receptor B (IL-17RB), ST2 and TSLP receptor (TSLPR). Activated ILC2s produce high amounts of IL-5 and IL-13, leading to eosinophilia, mucus hypersecretion andairway hyperreactivity. CRTH2: chemoattractant receptor homologous molecule expressed on Th2 cells; ALX/FPR2:receptor for lipoxin A4;FcεRI: high-affinity receptor for IgE; GATA3: GATA-binding protein 3; PG: prostaglandin;ROR: retinoic acid receptor-related orphan receptor; NK: natural killer; MHC: major histocompatibility complex; TCR:T-cell receptor. Reproduced from (46): Management of the patient with eosinophilic asthma: a new era begins. Jantina C. de Groot, Anneke ten Brinke and Elisabeth H.D. Bel.

- **neutrophilic vs eosinophilic asthma**

In addition to these cell groups, in viral exacerbations of the disease, in asthmatic smokers and severe asthma, neutrophils and Th2 lymphocytes are associated with Th17 and Th1/IL-12 dependent lymphocytes (47,48). IL-12 dependent Th1 lymphocytes, following the release of TNF alpha by monocytes / macrophages, recruit inflammatory cells and induce structural changes in the airways, as occurs in some severe asthma phenotypes (49).

In the mild and moderate forms, instead, there is a Th2 dependent flogistic response with bronchial eosinophilic infiltration. In both phenotypes, the activation of Th2 and Th17 is associated with a deficit/dysfunction of Treg lymphocytes (50).

Based on the pathogenetic action of Th2 in allergic phenotypes, we speak of eosinophilic asthma as Th2 high profile, while asthma with a predominantly neutrophilic inflammatory pattern is defined as having low Th2 characterization (Th2 low) (51). For this reason, they are defined as type 2 and non-type 2 respectively. In the case of non-allergic eosinophilic asthma, IL-5 is not by Th2 lymphocytes but derive from innate lymphoid cells type 2 (ICL2) (52).

Type 2, dependent on the activation of Th1 and Th17, includes asthma associated with obesity, neutrophilic asthma associated with cigarettes and paucigranulocytic asthma mediated by dysfunction of the bronchial smooth muscle (Fig.2) (53).

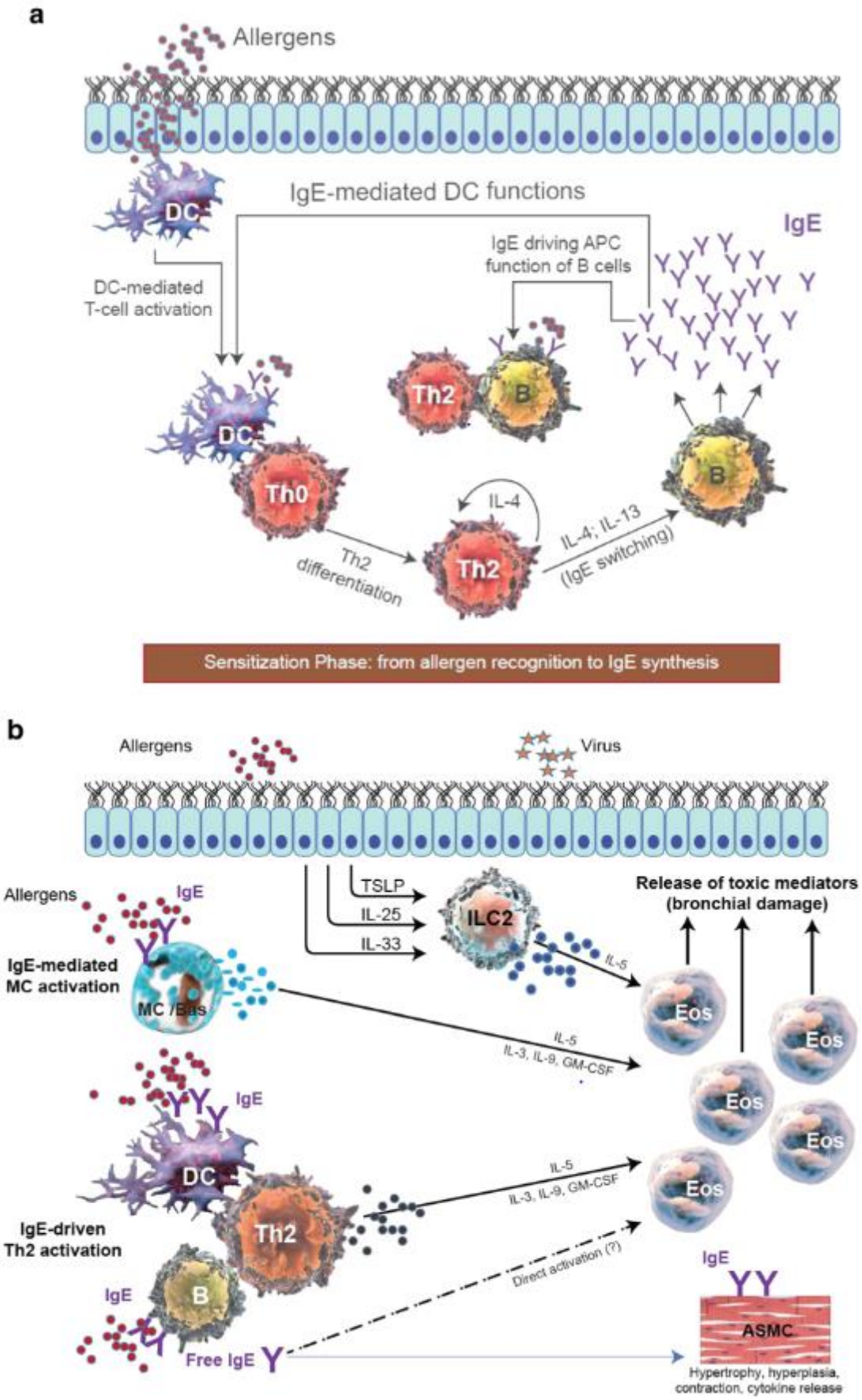


Fig.2 *Cellular components and pathways involved in (a): acute (sensitisation), and (b): chronic (cellular damage) phases of allergic asthma pathogenesis. Reproduced from (54): "Matucci A,*

Vultaggio A, Maggi E, Kasujee I. Is IgE or eosinophils the key player in allergic asthma pathogenesis? Are we asking the right question? Respir Res. 2018 Jun 8;19(1):113. doi: 10.1186/s12931-018-0813-0. PMID: 29879991; PMCID: PMC5992661"

1.3.2 Structural remodelling of the airways

In case of allergic and non-allergic phenotype, structural remodelling involves both proximal and distal airways (55). Structural modifications involve epithelial cells, endothelial cells and mesenchymal cells (smooth muscle fibro cells, myofibroblasts, fibroblasts) (56).

At the epithelial level, there is a numerical increase in mucus-secreting goblet cells following the action of IL-33 and EGF (57). Eosinophils release the major basic protein and the eosinophilic cationic protein (ECP), which have a damaging action at the endothelial level. Eosinophils also release TGF-β. TGF-β together with damaged epithelial cells and activated mesenchymal cells, determines the proliferation of fibroblasts and myofibroblasts with deposition of extracellular matrix proteins and consequent sub epithelial fibrosis (58). The histological finding is given by the thickening of the reticular layer of the basement membrane. The bronchial smooth muscle undergoes hypertrophy and hyperplasia. This phenomenon directly correlates with the duration and severity of the disease (59). Finally, there are neo-angiogenic phenomena induced by VEGF released both by the endothelium and by inflammatory cells (fig.3) (60).

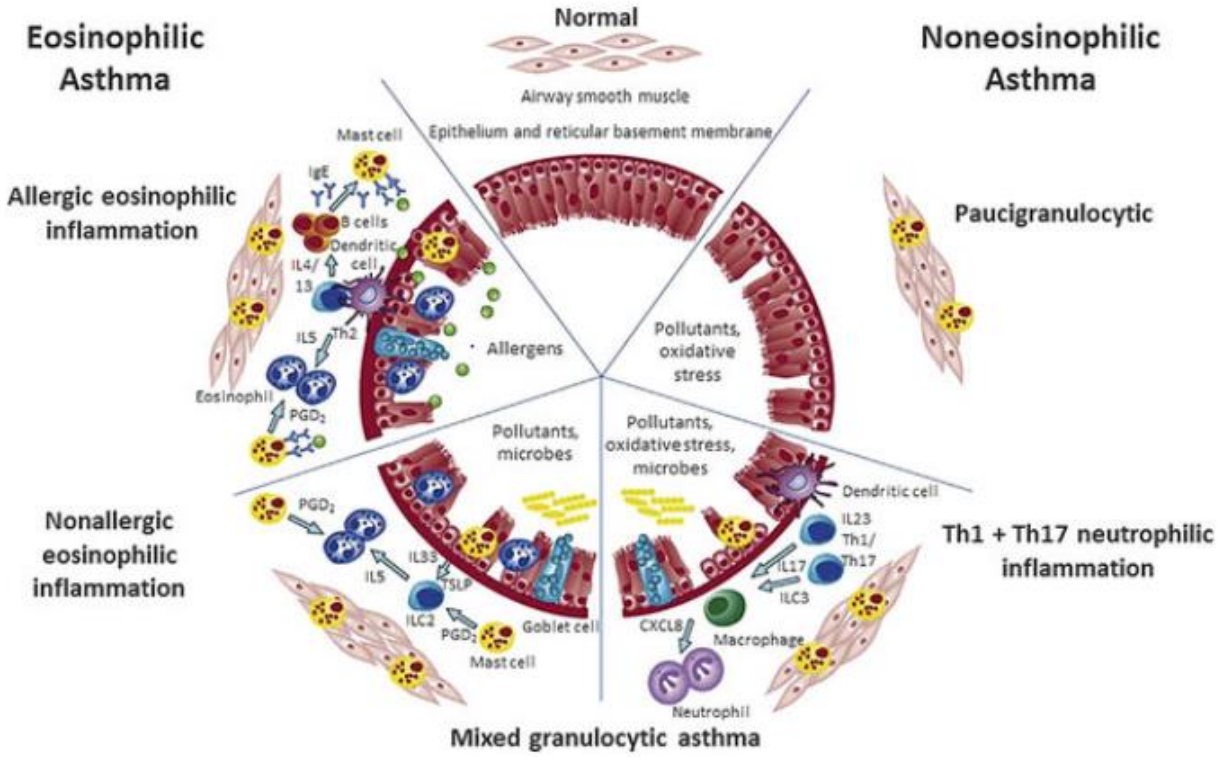


Fig.3 *Mechanisms and characteristic pathological features of asthma immunopathology. Features are divided into eosinophilic (allergic and non-allergic), non-eosinophilic (neutrophilic type 1 and type 17 and paucigranulocytic), and mixed granulocytic inflammation. IL, interleukin; TH, T helper; PDG2, prostaglandin D2; TSLP, thymic stromal lymphopoietin; ILC2, type 2 innate lymphoid cells; CXCL8, C-X-C motif chemokine ligand 8; ILC2, type 3 innate lymphoid cells. Reproduced from (61): Papi A et al. Lancet 2018; 391:783e800. Biological treatments for severe asthma: A major advance in asthma care Feb 2019.*

1.4 Severe asthma

It is a pathology with a prevalence of around 5-10% of asthma pictures. It represents an important health problem, both for the high costs of managing the disease and for the expense linked to the failure to control the disease (62). The therapeutic aim of patients with severe asthma is to identify a personalized treatment, adhering to the phenotypic and endotypic characteristics of the disease (63). According to ERS/ATS 2014 document (64), asthma is defined "severe" if in the previous year was treated with:

- High doses of CSI + a symptomatic drug (LABA, anti-leukotrienes or theophylline)
- Oral corticosteroids for a period > 6 months / year.

Patient diagnosed with Severe asthma, requires a level of therapy to keep the disease under control or, despite treatment, cannot achieve good symptom control (65).
The main findings that define the control of asthma sympoms are self-administration questionnaires such as the Asthma Control Test (ACT) and Asthma Control Questionnaire (ACQ). ACT score <20 and ACQ> 1.5 indicates poor asthma control (66,67).

In severe asthma, from an pathological point of view, there is an increase in bronchial smooth muscle and the reticular basement membrane. In 50% of asthmatic patients, inflammation is triggered by activation of Th2 lymphocytes and specific cytokines, such as IL-4, IL-5, IL-13 and mediated by eosinophils, mast cells, basophils and B lymphocytes that produce IgE (68,69). Phenotypes with an eosinophilic inflammatory pattern are flanked variants of asthma linked to neutrophilic activation and the action of IL-17, probable causes of a lack of response to treatment with corticosteroids (70–72).

1.5 Therapy

The treatment of asthma is articulated according to a progressive "step" mechanism. The rationale of the asthma therapeutic protocol is based on the progressive increase in the level of therapy from "Step 1" to "Step 5", with the possibility to choose, based on the characteristics of the patient

(73,74). The possibility of achieving asthma control through a "step-up" approach with the main therapeutic options was demonstrated by the GOAL study in which control was achieved, with different levels of therapy, in almost 80% of patients (in relation to the initial severity of asthma)(75).

Significant changes in the management of mild asthma were introduced in the 2019 GINA report (76). These recommendations represent a clear departure from the decades of clinical practice involving the use of SABAs and the possible additional use of ICS in patients with mild asthma. Given the low frequency of symptoms in mild asthma, patient adherence to their medications, particularly ICS, was usually unsatisfactory. Such patients often rely solely on SABAs to relieve symptoms, which can lead to abuse and which can have serious consequences such as poor symptom control, exacerbation and even death (77,78). The new treatment, indicated in the 2019 document, provides at Step 1 level the administration of ICS-formoterol (off-label) at a low dose when necessary (79). This combination excludes daily ICSs which, in fact, are no longer listed in step 1 under the "other controller option". Instead, the recommendations suggest an ICS inhalation whenever a "as needed" SABA inhaler is used (fig.4) (2).

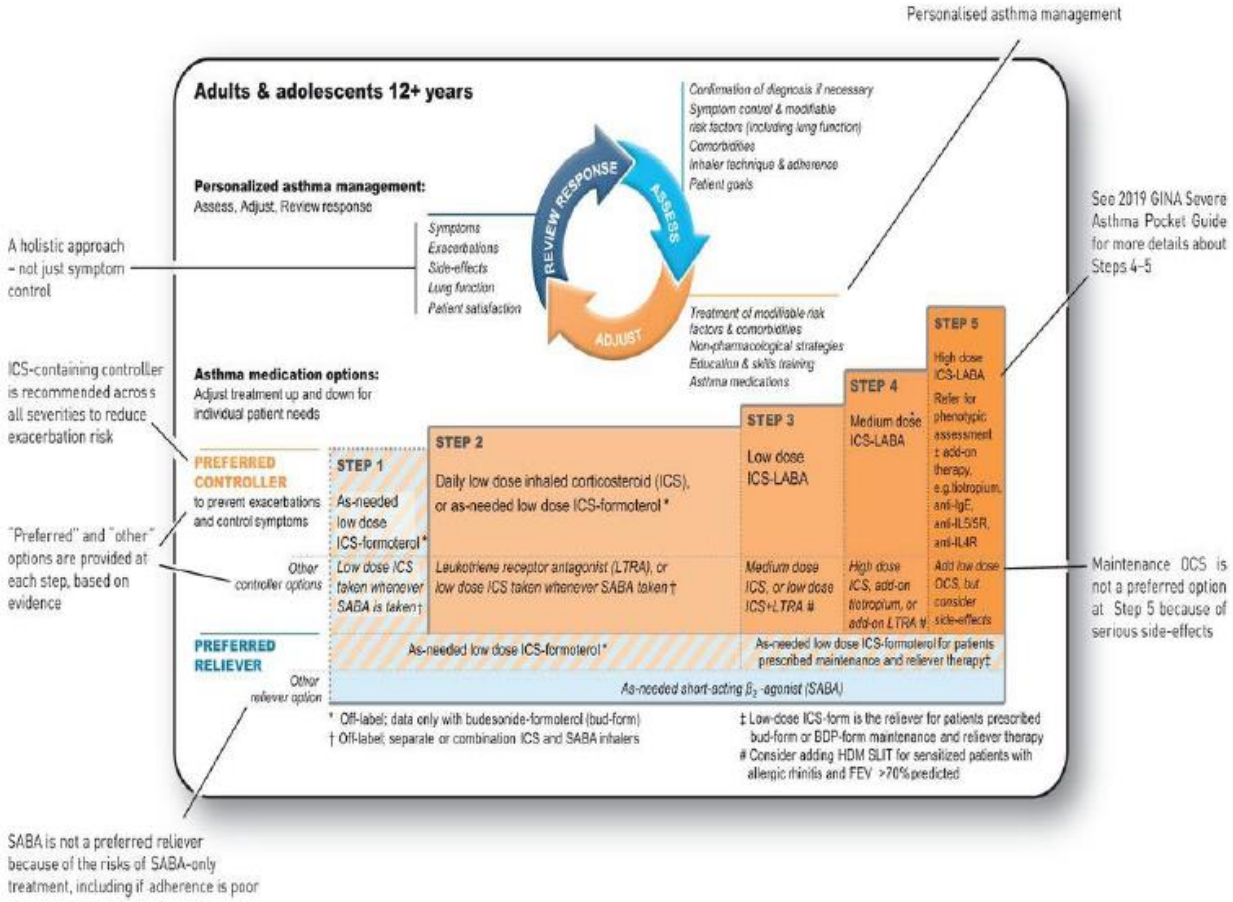


Fig.4 *The 2019 Global Initiative for Asthma (GINA) treatment strategy figure for adults and adolescents, annotated to highlight key features. ICS: inhaled corticosteroids; SABA: short-acting β2-agonists; LTRA: leukotriene receptor antagonists; LABA: long-acting β2-agonists; OCS: oral corticosteroids; BDP: beclometasone dipropionate; HDM: house dust mite; SLIT: sublingual immunotherapy; FEV1: forced expiratory volume in 1 s; IL: interleukin. Modified with permission of the Global Initiative for Asthma (www.ginasthma.org). Reproduced from: (79) GINA 2019: a fundamental change in asthma management.*

1.6 New anti IL-5 monoclonal antibodies

Mepolizumab and benralizumab have recently been approved to reduce exacerbations and to improve lung function in patients with severe and uncontrolled asthma with eosinophilic inflammation (80–83). These drugs have different mechanisms of action: mepolizumab blocks the activity of circulating cytokine IL-5 and benralizumab interferes with the IL-5 receptor alpha (IL-5Rα) of eosinophils (84). Although the role of the two drugs in remodelling or modulating the hyper-responsiveness of airways in severe asthma patients were not fully elucidated, they are very effective in reducing eosinophils in peripheral blood, airways, and bone marrow (55,85,86).

1.6.1 Mepolizumab

Mepolizumab is a humanized anti-IL-5 IgG monoclonal antibody that selectively inhibits eosinophilic inflammation, reducing the number of eosinophils in both sputum and blood, leading to a reduction in exacerbations and the need for corticosteroid treatment (87). In the DREAM study, researchers defined key targeting characteristics of patients who respond to mepolizumab (88). In the MENSA study, serum eosinophils, the number of previous exacerbations and the dose of ICS were used to establish the eligibility of patients and to compare them with placebo patients. It has been found that the use of anti-IL-5 therapy drastically reduces the frequency of exacerbations and moreover, the same patients reduced the need of OCS (89). Finally, the MUSCA study also demonstrated an important and significant improvement in health-related QoL and pre-bronchodilator FEV 1 values, which was maintained until the end of the study period (90) .

1.6.2 Benralizumab

Benralizumab is a humanized IgG1 monoclonal antibody. It binds to the interleukin 5 receptor α which is expressed on eosinophils and basophils. It induces apoptosis of these cells through cell-

mediated cytotoxicity (CD8+ T lymphocytes) following reduction of eosinophilic inflammation (91). The SIROCCO, CALIMA and ZONDA studies evaluated the effect of Benralizumab on asthma exacerbations and the need to reduce OCS (89-91). As reported in the CALIMA study for both patients with serum eosinophilia > 300 cells/ml and the other group with serum eosinophilia <300 cell/ml, exacerbations were lower than in placebo group (95).

1.7 Regulatory T cells in severe persistent asthma in the era of monoclonal antibodies target therapies

1.7.1 Classification

Tregs markers including:

- CD4
- CD25 (IL-2R)
- CD127(IL-7R)
- FOX (forkhead box) P3-transcription factor

Immune tolerance and prevention of inflammatory diseases are supported by Tregs (tab.1). Development of several autoimmune and inflammatory diseases, including type-1 diabetes, rheumatoid arthritis and multiple sclerosis (96) is linked with the alteration of Tregs.

Recently, the classification of Tregs is based on the expression of neuropilin-1, (96–99) as:

- natural (nTregs): that are thymus-derived and express neuropilin-1+
- inducible (iTregs):
    a) iTreg: $CD25^{+}FOXP3^{+}$
    b) Th3: $CD25^{low}FOXP3^{+}$, releasing IL-10 and TGF-β. According to literature data, TGF-β could be involved in generating ex-vivo Th3 from their precursors CD4+CD25- (100).
    c) Tr1: $CD25^{low}FOXP3^{-}$ secreting IL-10
- FOXP3 Features:

FOXP3 is the transcription factor normally expressed in Tregs cells, able to inhibits Th2 cell inflammatorion induced by allergic exposure (101). The development and progression of asthma can be supported by FOXP3 alterations, genetic polymorphisms or epigenetic mechanisms (97,99). Indeed, the differentiation into Tregs is stimulated by the interaction IL-2/IL-2R.

- Rare population of Treg cells:

    - Inducible CD8+ Tregs
    - γδ Tregs
    - NK-Tregs (CD4+Vα14+)

The main mechanisms of action involved in these kind of Tregs are:

- Secretion of inhibitory cytokines IL-10 and TGF-β
- Granzyme-perforin axes for induction of apoptosis in T effector cells.
- Dendritic cell functions inhibition.

Other functional markers that have been validated are:

- CD39: act through hydrolysis of adenosine triphosphate (ATP),
- CTLA-4 (cytotoxic T lymphocyte-associated antigen-4): the inhibitor checkpoint act through the antigen presenting cell (APC) and human leukocyte antigen-D related (HLA-DR) systems (103–106).
- Helios: as a novel marker to distinguish phenotypically and functionally Tregs subpopulation (107,108).

PRINCIPAL FUNCTIONS OF REGULATORY T CELLS	
• Immunological anergy • Suppress and regulate immune homeostasis • Produce lytic enzymes (perforins, granzymes)	• Secrete proinflammatory suppressive mediators (IL-10, TGF- β, IL-35) • Regulate infection tolerance • Regulate CD4 and CD8 cell activation and proliferation inhibition

Table 1 *The main functions of regulatory T cells. Reproduc by (86): Bergantini L, Cameli P, d'Alessandro M, Vietri L, Perruzza M, Pieroni M, Lanzarone N, Refini RM, Fossi A, Bargagli E. Regulatory T Cells in Severe Persistent Asthma in the Era of Monoclonal Antibodies Target Therapies. Inflammation. 2020 Apr;43(2):393-400. doi: 10.1007/s10753-019-01157-0. PMID: 31853715.*

1.7.2 Severe asthma and atopy

Although the functions of Tregs have been widely investigated, few studies have evaluated their role in severe asthma. Tregs play a crucial role in the equilibrium of immune system and in the regulation of Th2 responses. Exacerbation in severe uncontrolled asthma can be cause to Tregs dysfunction, and the release of IL-10 and TGF-β mechanisms during acute phase (109). IgE and

other effector cytokines can be regulated by IL-10 and TGF-β. This cytokine is able to preserve pulmonary homeostasis through equilibrium of Th2/Th1 responses and the regulation of IgE production (109). When Tregs are affected by antigens and viral infections they suppress excessive Th2 response, enabling immune-inflammatory disorders (such as atopy and asthma) to evolve. During asthma progression, Treg depletion and dysfunction play a key role in hyperresponsiveness and bronchial tissue remodelling (110–112). However, the precise role of Tregs in the etiopathogenesis of severe asthma is not resolved. Severe persistent asthma has been linked to a defect in regulatory responses, although their role it is not clear (34,113–116) (fig. 5). Several mediators has been studied in the pathogenesis of this asthma phenotype:

- inducible nitric oxide synthase (iNOS)
- periostin
- lipoxin A4: low levels of this molecule in severe asthma are reported to decrease activation of NK cells, regulating the apoptosis of eosinophils and neutrophils.
- Eotaxin: involved in eosinophil and Th2 recruitment, allows persistence of eosinophilia and steroid resistance (114).

1.7.3 Steroid resistance in severe asthma

Low affinity between ligands and their receptors (type 1) or a low number of cells expressing glucocorticoid receptors (type 2), cooperate for steroid resistance in severe asthma (117). Persistent airway eosinophilic inflammation and subsequent irreversible remodeling, involve both, innate and adaptive immunity with the cooperation of Tregs. The persistence of neutrophils, eosinophils and lymphocytes is connected with progressive thickening of the airways and changed composition of the airway walls and extracellular matrix. IgE, IgG and Tregs alteration induce peripheral airway inflammatory thickening (118). Recent paper demonstrated that atopy is linked to the onset of severe asthma with pulmonary function test impairment and respiratory failure, high level of serum IgE, Th2 responses and Tregs alteration (119,120). Obesity contributes to severe asthma through the persistence of non Th2 airway inflammation. In obesity patients, vitamin and microbiome facilitating severe asthma development during all phases of inflammatory pathways involved (116,121). In a recent manuscript, a cohort of children was considered, showing that lower expression of regulatory T cells is linked to steroid resistance and it is associated with the worsening of atopy. For these reasons, Tregs have been demonstrated to have a protective modulatory role in severe asthma (122).

1.7.4 Monoclonal antibody treatments for severe asthma

Several factors (IgE, IL-4, IL-13 and IL-5) are involved in severe asthmatic inflammation; in particular they are involved in mast cell activation and degranulation, airway eosinophilia and overproduction of mucus. High blood and sputum eosinophil count, serum IgE, flow exhaled nitric oxide (FeNO), serum periostin and all atopy mediators are associated with severe asthma (120). Novel monoclonal antibody treatments have been developed and approved by the FDA for severe asthma. These drugs target the predominant inflammatory endotype and they have modified the management and prognosis of severe asthmatic patients (113,123,124). Among these drugs, Omalizumab is the only one commercially available in Italy (since more than 10 years). Very recently, Mepolizumab and Benralizumab have been proposed as option therapies for severe eosinophilic asthma.

Omalizumab: anti-IgE

The first drug approved for severe asthma patients was omalizumab, a recombinant anti-IgE humanized monoclonal antibody, demonstrated to induce a significant "steroid-sparing" effect and to improve asthma symptoms and reduce the exacerbation rate (113). Few literature data reported the effect of omalizumab on modulatory functions of Tregs in severe asthma patients. A recent study hypothesized the contribution of omalizumab (combined with oral immunotherapy) in restoring allergen-specific Tregs functions in food allergy (125). The authors reported a desensitization by these drug-combination through depletion of allergen-reactive T cells and implementation of allergen-specific Tregs functions in these patients (125).

Mepolizumab, benralizumab and reslizumab: anti-IL-5

The role of Tregs modulatory functions in severe asthma patients treated with novel monoclonal antibodies therapies are not fully investigated. In particular, mepolizumab is a humanized monoclonal antibodies target circulating IL-5, while benralizumab and reslizumab target IL-5 receptor. These drugs are recommended for severe asthma patients that report eosinophil count ≥300 cells/μl (126). Unfortunately, the effects of these drugs on Tregs expression have not been elucidated. However, some authors reported Tregs dysfunctions in severe hypereosinophilic asthma patients associated with a strong Th2 local response (127).

Lebrikrizumab and Dupilumab: anti IL-4/IL-13

Among cytokines involved in the pathogenesis of asthma, IL-13 and -4 play a crucial role. The first is an anti-inflammatory cytokine contributing in the maintenance of peripheral tolerance and its function is coordinated by Tregs (128). Lebrikrizumab, a humanized monoclonal antibody target IL-13 improves lung function parameters in severe asthma adults patients, blood eosinophil counts

and IgE concentrations (129). Dupilumab is a human monoclonal antibody targeting receptor α chain of IL-4 (IL-4Rα) and it stops downstream signaling *via* IL-4 and IL-13 receptors. This drug can reduce asthma exacerbations, steroid requirements and pulmonary function decline according to the reduction of Th2-related inflammation in severe asthmatics (130). Tregs maintain B cell homeostasis through the regulation of IL-4 and IL-13 released by Th2 and type 2 innate lymphoid cells (ILC2) (131). Unfortunately, the modulation of Tregs by anti-IL4 and -IL13 therapies are not still reported.

Tezepelumab: anti-TSLP

Tezepelumab (AMG 157) targets thymic stromal lymphopoietin (TSLP) blocking the bond with its receptor. This drug could reduce bronchoconstriction and attenuate the early and late phase response in allergic asthma patients (132). AMG 157 could be considered an important upstream regulator of airway-type 2 inflammation due to reduction of blood and sputum eosinophil counts and FeNO before allergen challenge (133). One paper reported an inverse correlation between Tregs expression TSLP in a pediatric cohort of asthma patients (133).

Fevipiprant: Anti-PGD2

Fevipiprant is a monoclonal antibody therapy targeting prostaglandin D2 (PGD2) able to be in contrast with lipid inflammatory mediator that is overreleased in Th2 cells, eosinophils, basophils and macrophages of asthmatic patients by cyclooxygenase and PGD2 synthase (134). Some authors reported the secretion of soluble factors (IDO (idoleamine 2, 3-dioxygenase), TGF-β, and PGE2 (prostaglandin E2)) are inducted by Tregs adipose-derived stem cells in a murine model. Moreover, the same authors reported the contribution of Tregs in the downregulation of Th2 cytokines, facilitating upregulation of the Th1 immune response. An expansion of Tregs in this model was associated with an improvement pulmonary function and airway inflammation through interaction with adipose-derived stem cells (135) (fig. 6).

Daclizumab: anti-IL-2 receptors

Daclizumab, a humanized monoclonal antibody, binds IL-2 receptor (IL-2R) α-chain with strong efficacy in mild and severe asthmatics (136). Tregs usually express IL-2R and some authors reported a crucial role of IL-2 in induction and expansion of Tregs (137). In particular, low dose of IL-2R administration in severe asthma could induce an expansion of Tregs (138).

1.7.5 Th17 severe asthma endotype

A subset population of effector T cells, Th17, have proinflammatory properties and they are implicated in allergic/autoimmune disease. TGF-β stimulate the differentiation of naïve T cells into Th17 effector cells. The production of IL-6 and IL-21 inhibit Tregs differentiation to the benefit of Th17 cells (139–141). High concentrations of IL-17A were reported in BAL fluid, sputum and peripheral blood from severe asthma patients associated with increased disease severity (142, 143).

IL-17A, IL-17F and IL-22 are involved in mucous cell production in AEC and they are associated with airway smooth muscle proliferation and migration (144). Due to the expression of receptors for IL-17 and IL-22 by these cells, IL-17A or IL-17Rα may be potential targets in patients with severe asthma (145). Secukinumab and brodalumab are monoclonal antibodies targeting IL-17A, however few data are available about the efficacy in severe asthma patients and the role of Treg in this field (146) (fig. 6).

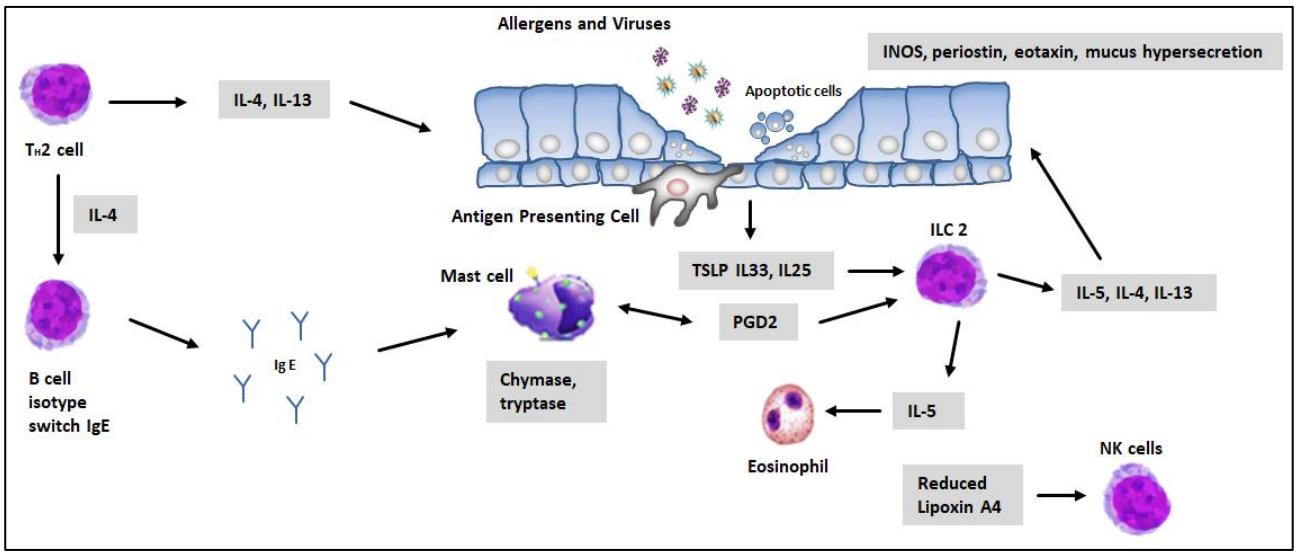


Fig. 5. *The role of T helper-2 (Th2) cells in pathogenesis of asthma. Abbreviations: IgE, immunoglobulin E; IL-, interleukin; iNOS, inducible nitric oxide synthase; TNFα, tumor necrosis factor-alpha; TGF-β, transforming growth factor.*

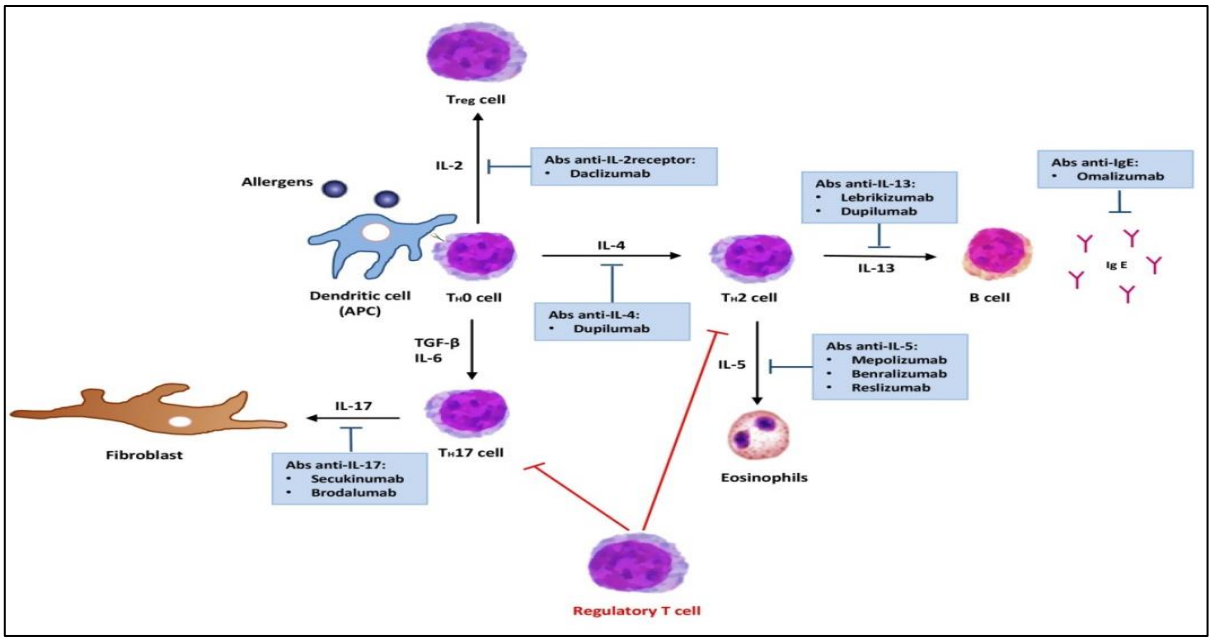


Fig. 6 *The role of regulatory T cells in suppression of T effector cells and monoclonal antibodies therapies. Abbreviations: Treg, regulatory t cells; Th-, T helper; IgE, immunoglobulin E; IL-, interleukin; TGF-β, transforming growth factor.*

1.8 Adhesion molecules and KL-6

In animal model of asthma it has been found that L-Selectin (a molecule mediating leukocyte rolling and promoting their migration into airways) plays a crucial role in the development and control of airway hyper-responsiveness (147). Actually, the L-selectin expression by T cells is greatly increased in asthma patients (148,149).

In severe asthma the airway remodeling usually consists in abnormal changes of cells with thickness of the walls and scarred. A target of remodeling and diseases activity widely investigated among fibrotic lung diseases is KL-6 (150). Serum KL-6 is a mucin-like glycoprotein, over-expressed in alveolar epithelial cells, reflecting alveolar damage and regeneration of type II pneumocytes (151,152).

As a rule, mepolizumab and benralizumab show a good safety profile, favourable clinical efficacy, and allow alternative treatment options for hyper-eosinophilic patients (153–155). However, there are no reliable predictive markers to select patients for mepolizumab vs benralizumab (sharing the same prescription criteria) and to detect the response of each patient to treatments with these drugs (156–158).

2. Aim of the study

Despite the clinical control of treatments and management of comorbidities, severe asthma can remain uncontrolled with increasing risks of drug-related adverse effects. Although pathogenic mechanisms of this disease are not still elucidated, recent studies suggest the involvement of specific immunological pathways and novel monoclonal antibodies, mepolizumab and benralizumab, have recently been approved to reduce exacerbations improving lung function parameters in patients with severe and uncontrolled asthma with eosinophilic inflammation.

The two drugs have different mechanisms of action: mepolizumab blocks the activity of circulating cytokine IL-5, benralizumab interferes with the IL-5 receptor alpha (IL-5Rα) of eosinophils**.** Although the role of the two drugs in remodelling or modulating the hyper-responsiveness of airways in severe asthma patients are not fully investigated, they are very effective in reducing eosinophils in peripheral blood, airways, and bone marrow.

Migration of leukocytes from the circulation into the airways depends on adhesion molecule activity and interactions during inflammatory responses. Among these molecules, L-selectin (also called CD62L), can mediate leukocyte rolling in arterioles and venules of inflamed lung endothelium and its expression on T cells is greatly increased in asthma patients. Tregs are a subpopulation of T cells of the tolerance system able to suppress inflammation by secreting a wide variety of cytokines (such as IL-10 and TGF-β) inhibiting T cell proliferation and IgE production. Tregs decrease in asthma patients may favor persistent severe inflammation.

In severe asthma, the airway remodeling usually consists in abnormal changes of cells with thickness of the walls and scarred. Serum KL-6 reflect alveolar damage and regeneration of type II pneumocytes and no data is available on this protein in severe asthma patients.

As a rule, mepolizumab and benralizumab show a good safety profile, favourable clinical efficacy, and allow alternative treatment options for hyper-eosinophilic patients. However, there are no reliable predictive markers to select patients for mepolizumab vs benralizumab (sharing the same prescription criteria) and to detect the response of each patient to anti-IL5 treatments with these drugs. Aim of the present study was to contribute to phenotype patients according to anti-IL5 treatment responses and to investigate L-selectin and KL-6 as bioindicators of airways hyper-responsiveness and remodelling. Together, we also investigated lymphocyte subpopulations in patients with severe eosinophilic asthma before and after anti-IL5 treatment.

3 MATERIALS AND METHODS

3.1 Study Design and Population

A cohort of 28 patients (age 54.4 ± 13.4 years) affected by severe eosinophilic persistent asthma refractory to conventional therapies were treated with anti-IL-5 biological therapy: 20 patients (72%) with mepolizumab (100 mg s.c. every 4 weeks) and 8 patients (28%) with benralizumab (100 mg s.c. every 8 weeks). Ten healthy controls (6 male, 51.4±13.4 years) were also enrolled in the study. Cohort study was reported in Fig.7. They had no history of asthma or allergy and were not on any medication. They were monitored for 12 months and did not develop any disease. All patients and controls were Caucasians and gave their written informed consent to participate in the study, which was approved by our Local Ethics Committee OSS-REOS (12908).
The diagnosis of severe eosinophilic asthma was performed according to international guidelines. These patients were monitored at Siena University Regional Referral Centre for Rare Lung Diseases from January 2018 to March 2020.

Patients were followed up for at least 6 months and stratified as:

- (a) “partial responders” (those with a reduction in exacerbation ≥30% and <70% requiring maintenance with oral corticosteroids [mOCS] or a ≥50% reduction in prednisolone dose);

- (b) “early responders” (patients without exacerbation and off mOCS at 6 months of follow-up)

All patients maintained the treatments unchanged during follow-up as they tolerated the therapy without any major side effects. Clinical, functional, and immunological data at baseline (T0), after 1 month (T1), and 6 months of therapy (T6) were collected in a database. Patients with 6 months follow-up included 14 Mepolizumab patients.

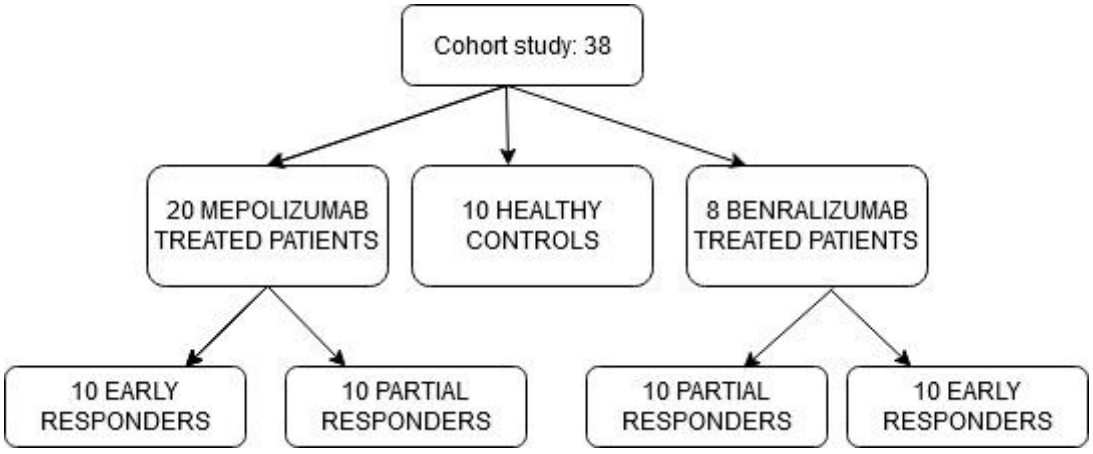


Fig. 7 Cohort study

3.2 Pulmonary Function Tests

The following lung function measurements were recorded according to ATS/ERS standard parameters using a Jaeger Body Plethysmograph with corrections for temperature and barometric pressure: forced expiratory volume in the first second (FEV1) and FEV1/FVC ratio. Both were expressed as percentages of predicted values.

3.3 Preparation and Storage of PBMCs

Cytofluorimetric analysis was performed at Siena University, Respiratory Diseases and Lung Transplant Unit, from January 2019 to December 2019. Lymphocyte subset percentages in PBMCs from SEA patients and controls were determined by flow cytometric analysis from 28 patients at T0 and T1 and for 14 patients treated with mepolizumab at T6. The peripheral blood samples were collected after 8-hour fasting in a tube containing EDTA anticoagulants (BD Vacutainer® EDTA Tubes, BD biosciences, CA, USA) and processed within 8 hours. Briefly, a layer of blood was added to 15 mL Ficoll Histopaque®-1077 (Sigma-Aldrich, INC) in a conical 50 mL tube and centrifuged for 30 minutes at 1693*g* in a swinging-bucket rotor without brake. The mononuclear cell layer was transferred to a new conical 50 mL tube (Corning® 50 mL centrifuge tubes, Sigma-Aldrich , INC), adding 15 ml RPMI 1640 medium (Gibco® - Thermo Fisher Scientific, Inc.), and centrifuged at 423*g* for 10 minutes. Supernatant was carefully removed and the cells stored in liquid nitrogen until the experiments.

3.4 T cells immunophenotyping

Blood samples were processed by flow cytometry using a panel of monoclonal antibodies (BD Multitest™ 6-color TBNK, San Jose, CA, USA), including FITC-labeled CD3, PE-labeled CD16 and CD56, PerCP-Cy5.5-labeled CD45, PE-Cy7-labeled CD4, APC-labeled CD19 and APC-Cy7-labeled CD8, in all patients at T0 and T1 and in 14 mepolizumab treated patients at T6. Briefly, a total of 10^6 cells were stained with 20 µl of antibody cocktail for 30 minutes. Washing was performed with 1 ml RPMI 1640, followed by centrifuging for 10 minutes at 423*g*. Supernatant was discarded and the cell pellet resuspended in 400 µl RPMI medium. At least 100,000 events were read by flow cytometer for each sample. Data was analysed using DIVA software (BD-Biosciences San Jose, CA, USA). Lymphocytes were distinguished on the basis of forward (FSC) versus side (SSC) scatters and additional gating was applied using SSC versus CD45 to distinguish lymphocytes from cell debris. Specific panels were subsequently assessed to identify T lymphocytes, B lymphocytes and NK cells. T lymphocyte subpopulations were gated in order to distinguish $CD3^+CD4^+$ (T-helper), $CD3^+CD8^+$ (T-cytotoxic) and $CD3^+$ $CD16/56^+$ (NKT) cells.

3.5 Analysis of Serum Soluble L-Selectin

Serum soluble L-selectin quantification was performed by bead-based multiplex LEGENDplex™ analysis (LEGENDplex™ Adhesion Molecule Panel (Biolegend)), in all patients at T0 and T1 according to the manufacturer's instructions. Briefly, the plate were Pre-wet by adding 100 µL of LEGENDplexTM 1X Wash Buffer to each well. To remove the excess volume, the vacuum was applied until wells are drained. In each well, 25 µL od assay buffer, 25 µL of sample/standard and 25 µL of mixed beads were added with a final volume of 75 µL in each well. The plate were placed on a plate shaker at 818*g* for 2 hours at room temperature followed by vaccum application. 200 µL of 1X Wash Buffer to each well were added and remove by vacuum filtration. After, 25 µL of Detection Antibodies to each well were added and the plate was placed on a plate shaker at approximately for 1 hour at room temperature. 25 µL of SA-PE to each well directly was added and shake on a plate shaker at approximate 818*g* for 30 minutes at room temperature. Vaccum were applied for the excess of fluids, and 150 µL of 1X Wash Buffer to each well were added. The samples were read on a flow cytometer. Reactions were run in duplicate. Analysis was performed with BD FACSCantoII flow cytometer (BD-Biosciences San Jose, CA, USA). Data was processed by Legendplex V8.0 software (Biolegend) and concentrations were expressed in ng/ml.

3.6 Analysis of Krebs von Den Lungen-6

Krebs von den Lungen-6 was measured in serum by KL-6 reagent assay (Fujirebio Europe, UK) as previously reported (160). The principle of the assay is agglutination of sialylated carbohydrate

antigen in samples with KL-6 mAb by antigen-antibody reaction. The change in absorbance reflects KL-6 concentrations. The detection limit of the assay is 13.47 U/mL consistent with the guidelines in the CLSI Protocol EP17-A (161). KL-6 concentrations in samples were expressed in IU/mL.

3.7 Regulatory T cell lymphocytes detection by flow cytometry

The analysis of regulatory T cells was performed at T0 and T1 in 14 mepolizumb treatment and in 10 healthy controls. Multicolor immunofluorescent staining followed by flow cytometric analysis were used to determine the phenotype of multiple peripheral T cell subsets. Three major T cell subsets were characterized: naïve, regulatory and effector T cells. Blood samples were processed by flow cytometry using a panel of monoclonal antibodies (BD Human Regulatory T Cell Cocktail San Jose, CA, USA), including FITC anti-Human CD4 (clone SK3), PE-Cy7 anti-Human CD25 (clone 2A3) and Alexa Fluor® 647 anti-Human CD127 (clone HIL-7R-M21). A panel containing FITC anti-Human CD4 and PE anti-Human CD62L (CD4/CD62L BD Simultest™ BD Biosciences, San Jose, CA, USA) was also analysed to identify CD62L regulatory subpopulations of T lymphocytes among CD4+ cells. Processing of samples was assessed according to the manufacturer's instructions. At least 1,000,000 events were read by flow cytometer for each sample. Data was again analysed using DIVA software. Lymphocytes were distinguished on the basis of forward (FSC) versus side (SSC) scatters and additional gating was applied using SSC versus CD4. Specific dot-plot was subsequently assessed to identify $CD4^{+}CD25^{bright}CD127^{-/low}$ Tregs, $CD4^{+}CD62L^{+}$ Tregs, $CD4^{+}CD25^{bright}CD127^{+}$ Th effector cells and $CD4^{+}CD25^{-}CD127^{+}$ naïve T cells.

3.8 Statistical Analysis

The Chi-squared test was used for categorical variables as appropriate. All data was expressed as mean±standard deviation (M±SD) or median and interquartile range (IQR). T cell subset percentages were compared in healthy controls and asthma patients at T0 and in asthma patients at T0 and T1 (before and after administration of mepolizumab). The Mann-Whitney U test and Wilcoxon test were used to differentiate 2 different groups. The Spearman test was used to look for correlations between variables. A p value less than 0.05 was considered statistically significant. Statistical analysis and graphic representation were performed by GraphPad Prism 8.0 software. Unsupervised Principal Component Analysis was used to visualize experimental groups in a two-dimensional plane on the basis of the % of lymphocytes subtypes. In order to visualize the percentages of each lymphocytes subsets on serum samples of the three conditions (controls, T0 and T1), it was performed a heatmap analysis. The above-mentioned analysis and the related figures were obtained by ClustVis (http://biit.cs.ut.ee/clustvis/).

4. RESULTS

4.1 Demographic data

There were no statistical differences in age, gender distribution and smoking habits among HC, mepolizumab and benralizumab treated patients. (Tab. 2).

	Healthy Controls (n=10)	Mepolizumab treated patients (n=20)	Benralizumab treated patients (n=8)	P values
Age	51,4 ±13,45	56.3 ±11.8	50.5±16.1	ns
Gender (F:M)	4/6	13/7	4/4	ns
Smoking Habits (Former-current /never)	6/4	12/8	6/2	ns

Tab.2 Demographic characteristics of the population.

4.2 Functional parameters, peripheral eosinophils, KL-6 and sL-selectin at baseline and after one month of therapies

Table 3 reported functional parameters, peripheral eosinophilia, KL-6 and sL-selectin concentrations at time 0 and time 1 in benralizumab and mepolizumab populations. FEV1 (in ml and %), FEV1/FVC ratio significantly increased after one month of mepolizumab or benralizumab therapy associated with a significant decrease of peripheral eosinophilia (in cells/mm^3 and %).

	Mepolizumab group			Benralizumab group		
	T0	**T1**	**P values**	**T0**	**T1**	**P values**
Functional parameters:						
• **FEV1(%)**	84.4±26.6	87.1±21.5	0.04	81.3±25	89.7±15	0.04
• **FEV1(ml)**	2558±88	2820±960	0.0037	2607±1067	2747.1±847	0.005
• **TIFFENAU index**	69.4±12.4	72.8±10.26	0.04	66±8.4	70.5±9	0.04
Peripheral eosinophilia (cell/mm3/%)	915±617 / 11.4±6.5	140±83.5 / 1.6±1.2	<0.0001	718±49 /8.2±4.6	0 / 0	<0.0001
sL-selectin (ng/ml)	1442±1003	630±413	0.0012	1173±683	1360.3±1289	ns
KL-6 (U/ml)	332.7±124	310.7±107.1	ns	334.6±76.8	329.1±88.3	ns

Tab.3 Functional parameters, peripheral eosinophilia, KL-6 and sL-selectin concentrations at time 0 and time 1 in benralizumab and mepolizumab populations.

4.3 Lymphocyte immunophenotyping in HC, mepolizumab and benralizumab groups

Tab. 4 reported lymphocyte immunophenotyping in HC, mepolizumab and benralizumab groups. Interestingly, HC showed statistical increased percentages of CD45+cells than mepolizumab group and benralizumab group (p=0,004 and p=0,03, respectively). The same behaviour emerged for CD19+ Cells that resulted increased in HC than mepolizumab group and benralizumab patients (p=0,005 and p=0,002, respectively). On the contrary, NKT-like cells showed a statistically significant decrease in healthy controls than both patient groups (p=0,003 and p=0,003, respectively). Mepolizumab treated patients also showed significant decrease of $CD8^+$ and NK-T like cells–percentages between T0 and T1 and a concomitant significant increase in L-selectin concentrations (Fig. 8).

	HC (N=10)	Mepolizumab group (N=20)			Benralizumab group (N=8)		
		T0	**T1**	**P values**	**T0**	**T1**	**P values**
Lymphocytes immunophenotyping:							
CD45	35,50±8,8	19.1±5.4	17.9±7.4	ns	24.5±11.8	28.5±1.3	ns
CD3	77,19±6,1	81.8±8.5	79.5±7.7	ns	81.6±5	76±6.7	ns
CD4	46,13±7,7	47.9±12	53.5±8.4	ns	58.5±3.4	52±10	ns
CD8	24,41±6,6	31.7±14	24±8	0.03	20.5±8.6	22±4.8	ns
CD19	13,60±4,6	7.8±5.2	8.3±5.7	ns	8.5±3.1	10.7±2	ns
NK	10,58±4,6	9.9±5.8	13±16	ns	10.7±6.9	11.1±3.4	ns
NKT-like	6,88±2,51	12.21±8.7	8.4±8.3	0.02	15.6±23	15.1±6	ns

Tab. 4 Lymphocyte immunophenotyping in HC, mepolizumab and benralizumab groups

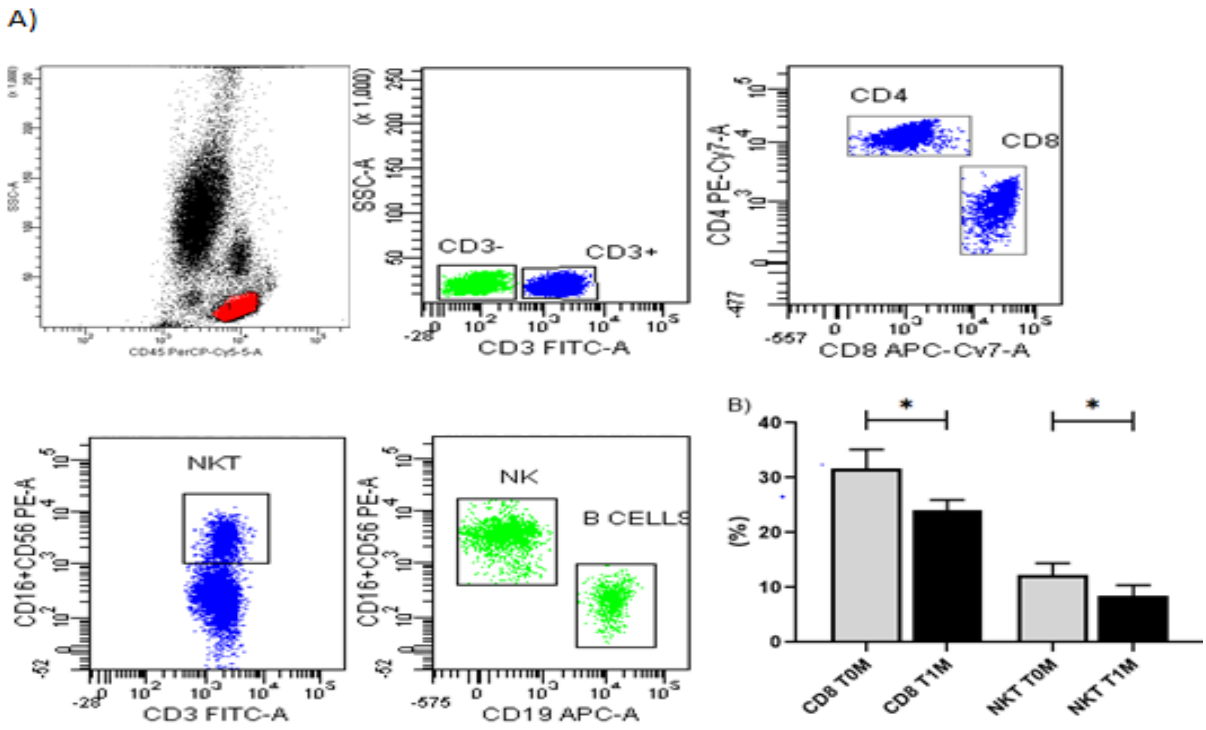


Fig.8 A) Gate strategy of CD4+, CD8+ and NKT-LIKE cells. B) Different percentages of CD8 and NKT cells in mepolizumab gropu at T0 and T1.

4.4 Functional parameters, peripheral blood eosinophils, KL-6 and lymphocytes subsets in mepolizumab group

After 6 months of mepolizumab treatment (T6), pulmonary function tests, immunological data, KL-6 concentrations and peripheral eosinophilia were available for 14 patients. The results showed significant decrease of peripheral eosinophils and significant increase of FEV1/FVC ratio and FEV1(%). The peripheral percentages of NKT-like cells resulted significantly decreased after 6 months of mepolizumab treatment (tab.5).

	Mepolizumab group				
	T0 (n=20)	**T1 (n=20)**	**T6 (n=14)**	**P Values (T0-T1)**	**P Values (T0-T6)**
Functional parameters:					
• **FEV1(%)**	84.4±26.6	87.1±21.5	90.3±24.4	0.04	0.037
• **FEV1(ml)**	2558.86±886	2820±960.7	2861±961	0.0037	0.0026
• **TIFFENAU index**	69.4±12.4	72.8±10.26	74.9±11	0.04	0.032
Peripheral eosinophilia (cell/mm3 / %)	915±617 / 11.4±6.5	140±83.5 / 1.6±1.2	74±61/1±0.8	<0.0001	<0.0001
KL-6 (U/ml)	332.7±124	310.7±107.1	334±136	ns	ns
Lymphocytes immunophenotyping:					
CD45	19.1±5.4	17.9±7.4	18.9±13	ns	ns
CD3	81.8±8.5	79.5±7.7	77±11	ns	ns
CD4	47.9±12	53.5±8.4	51±13	ns	ns
CD8	31.7±14	24±8	21±6	0.03	0.021
CD19	7.8±5.2	8.3±5.7	9±8.7	ns	ns
NK	9.9±5.8	13±16	12±8	ns	ns
NKT-like	12.21±8.7	8.4±8.3	7±4.5	0.02	0.024

Tab. 5 Pulmonary function tests, immunological data, KL-6 concentrations and peripheral eosinophilia at T0, T1 and T6.

4.5 Treg subsets in HC and mepolizumab group

Fewer Tregs were observed in mepolizumab patients than HC although the difference was not statistically significant (Fig. 9).

CD62L+ cell percentages were lower in the mepolizumab group than in HC (p=0.01) (fig.10), whereas higher CD4+CD62L+ cell percentage was observed in asthmatic patients than controls (p=0.004). No other significant differences were observed between mepolizumab group and HC. At the same time, significant increase in Tregs and CD4+CD62L+ percentages (p=0.0001 and p=0.02) (fig. 9, fig. 10) were reported at T0 than T1 in mepolizumab group (Tab. 6).

	Healthy Controls	**T0**	**T1**	**P values**
Regulatory T Cells (%)	6,10±1,47	4,99±1,88	9,53±4,70	0,02
Effector T cells (%)	9,01±2,83	11,84±8,88	10,21±8,17	ns
Naive T cells (%)	77,93±7,10	68,79±12,30	71,64±22,52	0,18
CD4+CD62L+ cells (%)	1,61±1,24	8,66±6,86	17,15±7,02	<0,0001
CD4-CD62L+ cells (%)	4,14±2,71	1,79±1,78	1,78±2,95	0,01

Tab.6 *Tregs subsets of healthy controls, baseline (T0) and one months after therapy (T1). Data are expressed as Mean ± Standard Deviation (M. ±S.D.)*

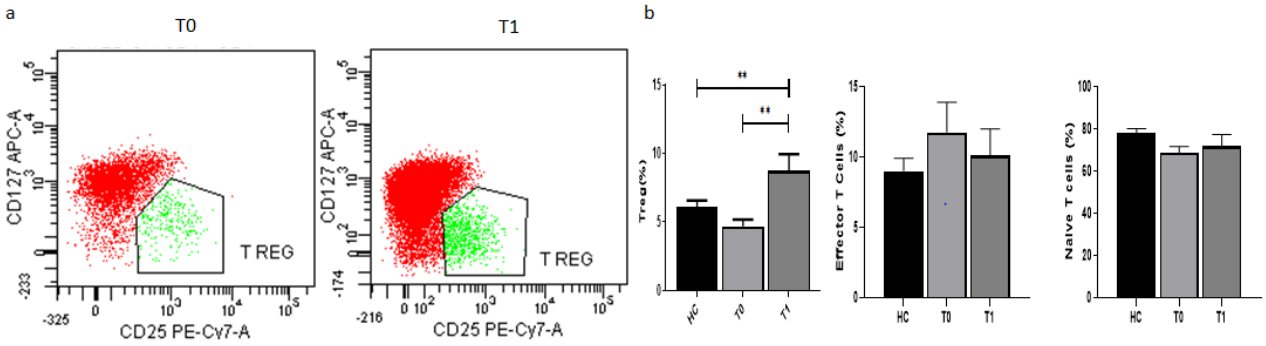


Fig. 9 *Gate strategy of Tregs and the percentages at T0 and T1. B) Different T cells subsets between HC and T0 and T1 groups; *p value<0,01, **p value<0,001 ***p value<0,0001 (not significant if not indicated).*

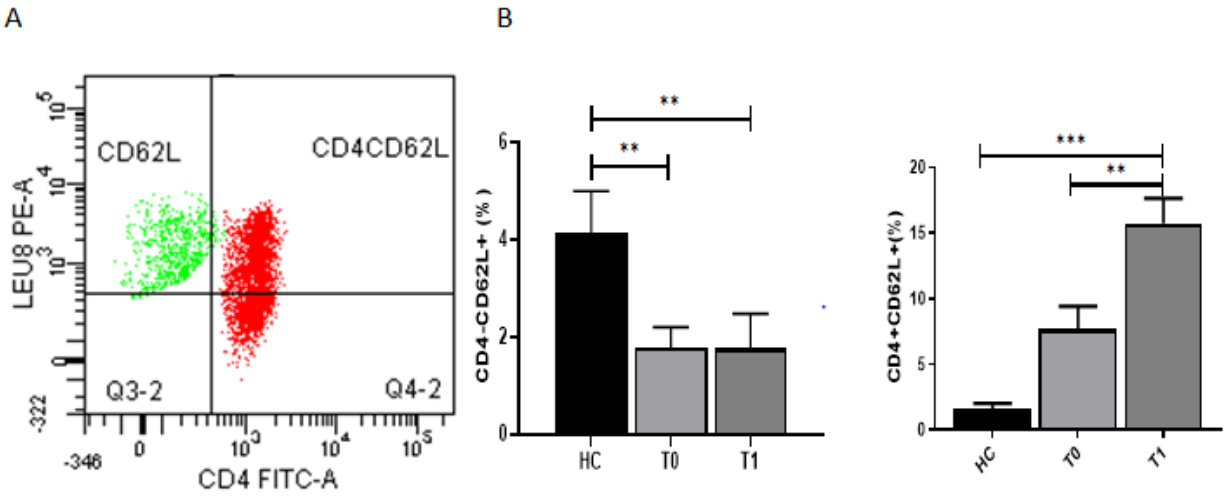


Fig. 10 *A. Gate strategy of CD4CD62L. B. CD4+CD62L+ and CD4-CD62L+ percentages among HC, T0 and T1; *p value<0,01, **p value<0,001 ***p value<0,0001 (not significant if not indicated).*

4.6 "Early Responders" versus "Partial Responders" in Mepolizumab and Benralizumab Treatment.

Stratifying the cohort of mepolizumab patients in "early responders" (n = 10) and "partial responders" (n = 10), significant differences were observed in peripheral eosinophils, sL-selectin, and KL-6 at time 0 (Fig. 11). At T1, "partial responders" showed significant variations in peripheral eosinophilia, sL-selectin concentrations and percentages of NK cells than T0. While

"early responders" showed significantly different peripheral eosinophilia, sL-selectin concentrations, percentages of NKT-like cells, KL-6 levels, FEV1 percentages, and FEV1/FVC ratio at T1 than T0. Considering a subgroup of 14 mepolizumab treated patients, trends of FEV1(%), FEV1/FVC ratio, Peripheral blood eosinophils and KL-6, were reported in fig.12. Concerning Benralizumab, no differences were found at T0 between 5 "early responders" and 3 "partial responders" patients. At T1, the 2 groups showed statistically significant changes in peripheral eosinophilia than T0 (676 ± 365 cell mm^{-3} vs. 0 cell mm^{-3}; $p < 0.0001$, 8.5 ± 4.1 vs. 0%; $p < 0.0001$ in "partial responders," and 760 ± 679 cell mm^{-3} vs. 0 cell mm^{-3}; $p < 0.0001$, 8 ± 5.6 vs. 0%; $p < 0.0001$ in "early responders").

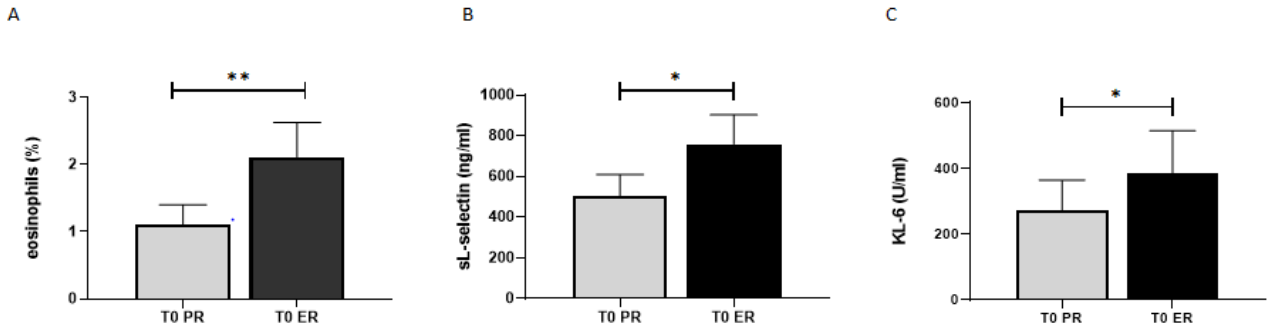
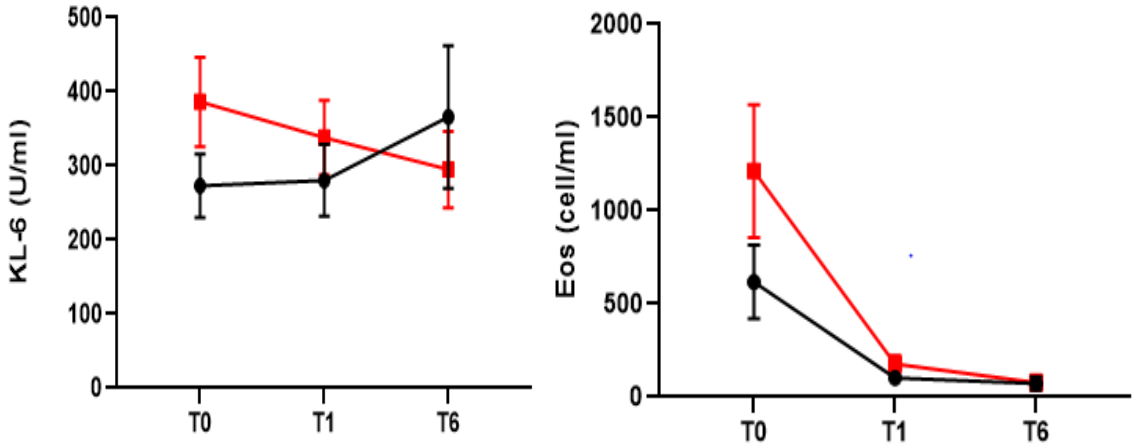


Fig.11 A) The percentages of *eosinophils at T0 mepolizumab group. B) Differences of sL-Selectin (ng/ml) at T0 mepolizumab group. C) Differences of KL-6 (U/ml) at T0 mepolizumab group. Abbreviations: T0PR= baseline values of mepolizumab: Partial Responders subgroup (PR). T0ER= baseline values of mepolizumab: Early Responders subgroup (ER). **p<0,001 *p<0,05*



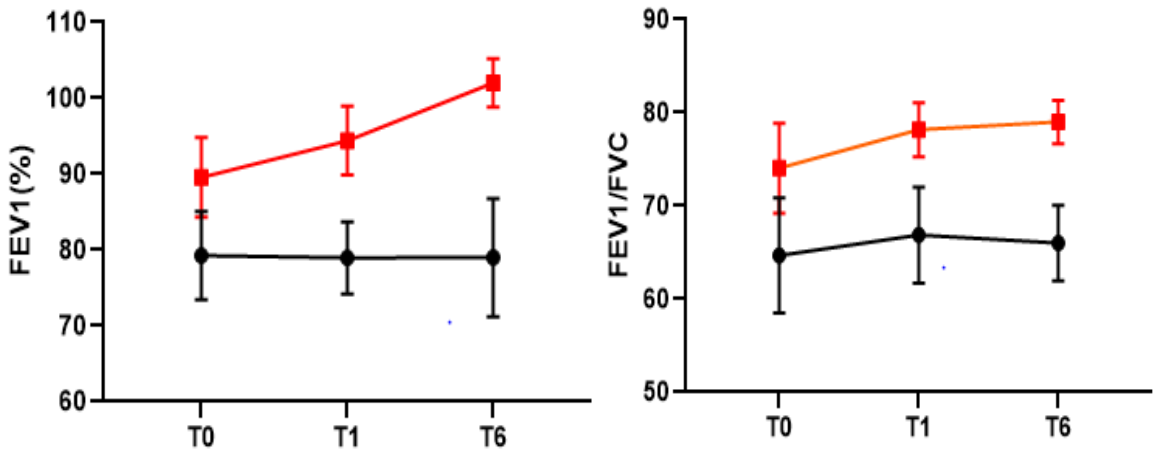


Fig. 12 *Trends of FEV1(%), FEV1/FVC ratio, Peripheral blood eosinophils and KL-6 at T0, T1 and T6 of 14 mepolizumab treated patients. Red line: early responders, Black line: partial responders*

4.7 "Early Responders" versus "Partial Responders" analysis of Treg subsets of 14 Mepolizumab Treated patients.

When the population was stratified according to clinical response to mepolizumab, early clinical responders (50% of patients) and partial responders (n=7, 50%) showed analogous clinical parameters, as reported in tab. 7 After 1 month of therapy the percentages of naïve T cells, CD4+CD62L+, CD62L+ cells and s-Lselectin concentrations differed significantly from T0.

	T0 MEPOLIZUMAB			T1 MEPOLIZUMAB		
	ER T0	**PR T0**	**P Values**	**ER T1**	**PR T1**	**P Values**
Regulatory T Cells (%)	4,76±2,15	5,23±1,69	ns	9,41±5,74	9,64±3,86	ns
Effector T cells (%)	10,94±11,31	12,73±6,43	ns	10,61±9,66	9,80±7,15	ns
Naive T cells (%)	67,07±7,22	70,51±16,39	ns	81,21±11,44	62,07±27,47	0,01
CD4+CD62L+ cells (%)	8,34±9,35	8,99±3,79	ns	20,46±6,69	13,84±6,05	0,01
CD62L+ cells (%)	2,37±2,22	1,21±1,07	ns	3±3,9	0,56±0,41	0,01

L-selectin (ng/ml)	1710±1282,96	1238,84±915,77	ns	743,03±537	485,26±314	0,04

Tab. 7 *Immunological data of baseline (T0) and one months after therapy (T1) after stratifying population in early responders (ER) and partial responders (PR). Data are expressed as Mean ± Standard Deviation (M.±S.D).*

4.8 Predictive Biomarkers in Mepolizumab group.

Patients treated with mepolizumab therapy were stratified according to their clinical responses, and ROC analysis was performed to identify baseline cut-off values of our proposed biomarkers to distinguish "early responders" from "partial responders" (Fig. 13). The results showed better AUCs for "early responders" than for "partial responders": for KL-6, AUC = 0.75, 95% CI: 0.53–0.97, p = 0.004; peripheral eosinophils, AUC = 0.79, 95% CI: 0.57–1, p = 0.04; FEV1/FVC ratio, AUC = 0.76, 95% CI: 0.53–0.99, p = 0.005; and sL-selectin, AUC = 0.73, 95% CI: 0.50–0.95, p = 0.003. In the logistic regression, the early response to mepolizumab therapy was tested as dependent variable, while KL-6, peripheral eosinophils, and L-selectin were tested as independent variables. The combination of these biomarkers allowed the identification of "early responders." The ROC curve analysis of the model revealed an AUC of 0.8778 (95% CI: 0.72–1; negative predictive value (%): 77.78, positive predictive value (%): 80; p = 0.005) (Fig. 13). A cut-off value of KL-6 of 337 IU/mL showed 70% sensitivity and 77.78% specificity; peripheral eosinophilia values <580 (cells/mm3) showed 80% sensitivity and 70% specificity. Finally, sL-selectin concentration higher than a cutoff of 1,051 (ng/mL) showed 70% sensitivity and 80% specificity facilitating the discrimination between "early responders" and "partial responders" patients.

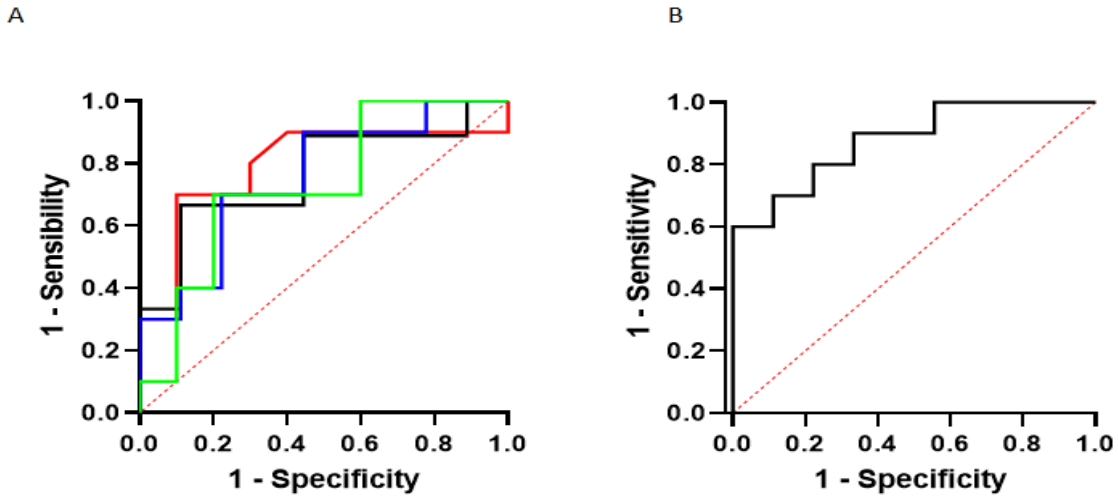
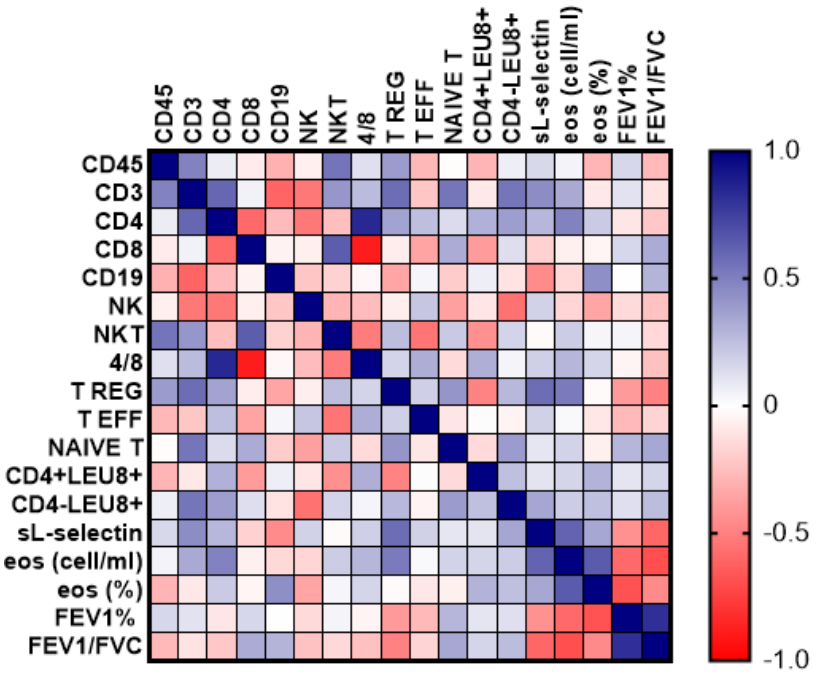


Fig. 13 *A) ROC curve of KL-6 (blue), eosinophils (Red), Tiffenau Index (black) and sL-selectin (Green) in discriminating Early Responders from Partial Responders in Mepolizumab Group. B) ROC curve analysis of logistic regression model: Eosinophils, KL-6 and sL-Selectin*

4.9 Correlation analysis parameters of 14 mepolizumab treated patients at T1

Correlation matrix between variables after one month of therapy was reported in Fig.14a. After one month of therapy, FEV1 (ml) revealed a direct correlation with CD62L+ cells (r=0.6, p=0.04). FEV1/FVC ratio showed an indirect correlation with L-selectin levels (r=-0.6, p=0.03), peripheral eosinophilia (r=-0.7, p=0.01) and CD45+ lymphocyte percentages (r=-0.55, p=0.05). Peripheral eosinophilia also showed a direct correlation with L-selectin levels (r=0.6, p=0.04) (Fig.14b).

a)



b)

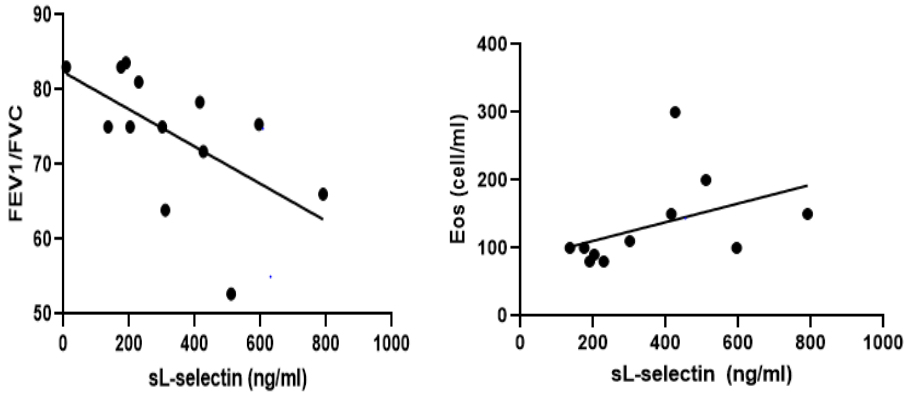


Fig.14 *a) Correlation matrix between variables after one month of therapy. B) Correlation of FEV1 and Eosinophils with sL-selectin.*

4.10 Correlation analysis parameters of 14 mepolizumab treated patients between T0 and T1

Significant correlations were observed between FEV1/FVC ratio and CD45 percentages (r=0.58, p=0.034) at T0 and T1 and between FEV1/FVC ratio and CD62L+ cells at T0 and T1 (r=-0.62, p=0.024). Interestingly, peripheral eosinophilia was indirectly correlated with CD4+CD62L+ levels (r=-0.61, p=0.04).

4.11 PCA and Heat Map analysis of lymphocytes immunophenotyping considering mepolizumab and benralizumab before starting therapy and healthy controls

PCA and Heat map analysis of the three groups (mepolizumab T0, Benralizumab T0 and HC) were reported in Fig. 15. In particular Fig.15a, X and Y axis showed principal component 1 and principal component 2 that explain 50.6% and 17.6% of the total variance, respectively. X and Y axis of the fig. 15b showed principal component 1 and principal component 3 that explain 50.6% and 16.5% of the total variance, respectively. Unsupervised lymphocytes immunophenotyping samples allow to cluster the three conditions separately. In particular, asthmatic patients were spaced from controls relatively to the main component (PC1). Heat Map analysis, in fig. 15c, obtained with the unsupervised data by flow cytometric analysis, showed the percentages of each considered variable. In particular, the clustering of lymphocytes was performed using clustering method and Euclidean distance. Color change from red to blue indicated the different abundance. Heat map showed a homogeneity of lymphocytes in control group.

a) b)

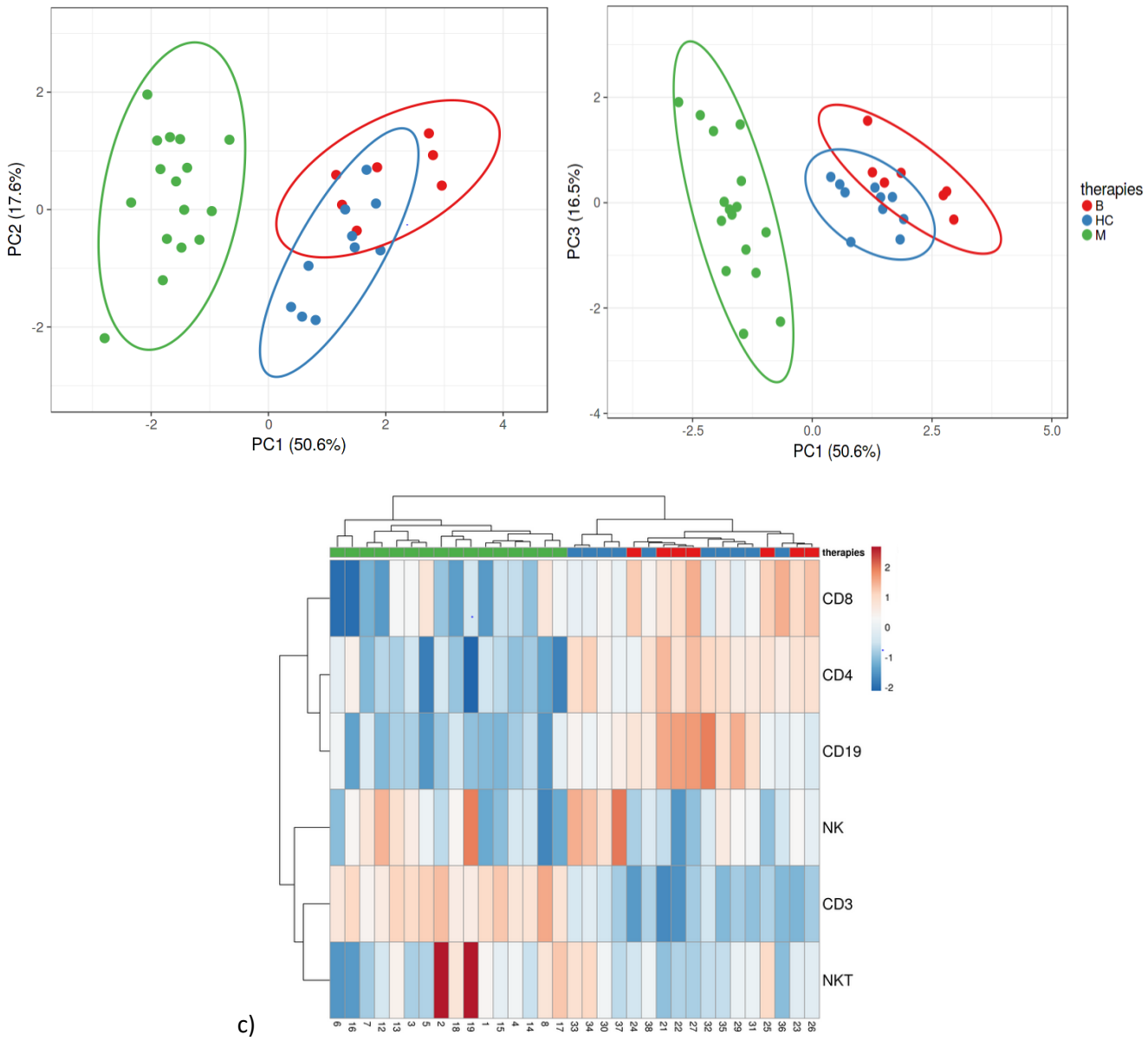


Fig.15 *a) Unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. Prediction ellipses are such that with probability 0.95. B) Unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. X and Y axis show principal component 1 and principal component 3 that explain 50.6% and 16.5% of the total variance, respectively. C) Rows are centered; unit variance scaling is applied to rows. Both rows and columns are clustered using correlation distance and average linkage.*

4.12 PCA and Heat Map analysis of lymphocytes immunophenotyping considering mepolizumab at T0 and T1 and healthy controls

PCA and Heat map analysis of the three groups (mepolizumab T0 and T1 and HC) were reported in Fig. 16. In particular Fig. 16a X and Y axis show principal component 1 and principal component 2

that explain 47.4% and 18.6% of the total variance, respectively. X and Y axis of the fig. b X and Y axis show principal component 1 and principal component 3 that explain 47.4% and 15.4% of the total variance, respectively. Analysis clearly showed as, on the basis of unsupervised lymphocytes immunophenotyping, clusterize completely separately. In particular, asthmatic patients are spaced from controls relatively to the main component (PC1).

a) b)

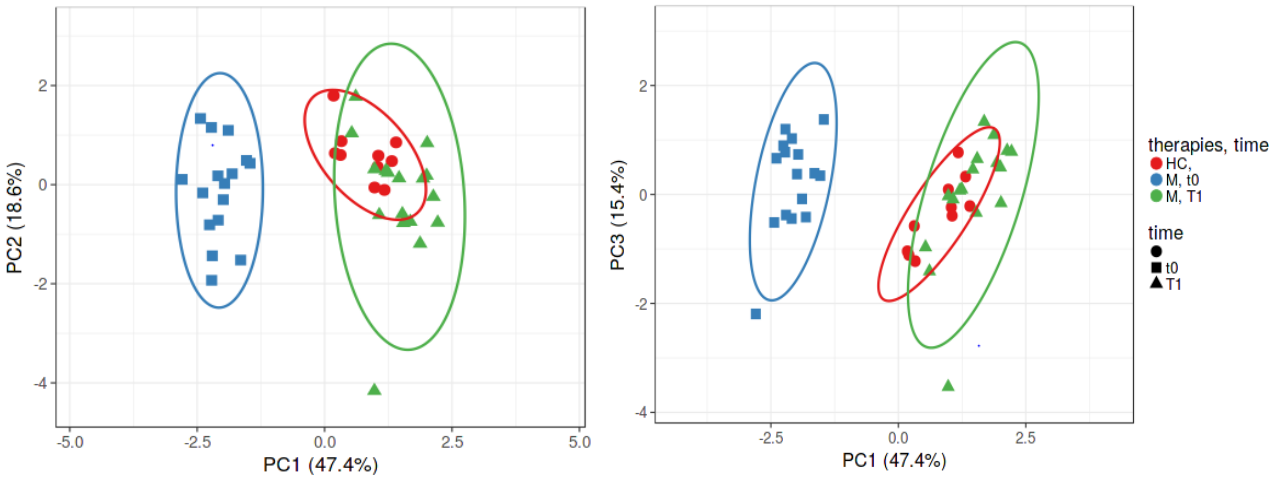


Fig.16 *Unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. Prediction ellipses are such that with probability 0.95. X and Y axis show principal component 1 and principal component 3 that explain 47.4% and 18.6% of the total variance, respectively. B) Unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. X and Y axis show principal component 1 and principal component 3 that explain 47.4% and 15.4% of the total variance, respectively.*

4.13 PCA and Heat Map analysis of a subgroup of 14 mepolizumab patients before and after one months of therapy.

PCA and Heat map were reported in Fig. 17a , clearly showed as, on the basis of unsupervised lymphocytes immunophenotyping serum samples of the three conditions, clusterize separately. In particular, Mepolizumab samples are spaced from controls relatively to the main component (PC1). Heat Map analysis, in fig. 17b, obtained with the unsupervised data by flow cytometric analysis, showed the percentages of each considered variables in all serum samples. In particular, the clustering of lymphocytes was performed using clustering method and Euclidean distance. Color change from red to blue indicating respectively greater or lesser abundance. Heat map showed a

homogeneity of lymphocytes in controls group, while T0 and T1 conditions showed an opposite behavior.

a) **b)**

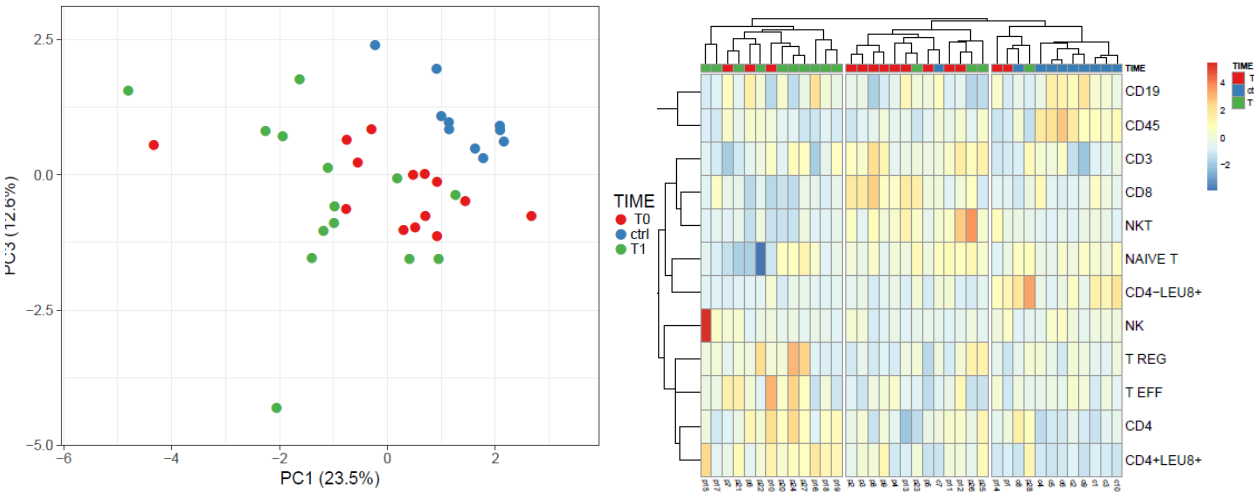


Fig.17a. *Principal Component Analysis (PC) performed with the unsupervised lymphocytes subtypes.* ***b.*** *Heat Map analysis performed with the unsupervised Lymphocytes subtypes. Colors varying from blue for lower expression to red for higher expression. Each cluster has a unique color assigned (bar on the left): blue for control samples, red for T0 samples and green for T1 samples.*

5. Discussion

Patients with severe eosinophilic asthma were enrolled in this study that confirmed the safety and efficacy of the treatment with novel anti IL-5 and anti IL-5Rα monoclonal antibodies mepolizumab and benralizumab. In agreement with the literature data, after the first month of therapy, we recorded the improvement of clinical status and functional parameters with significant increase of FEV1 and FEV1/FVC ratio values and a decrease of peripheral eosinophilia in all patients (88,94,154,155,162). The role of eosinophils in severe asthma and the capability of mepolizumab and benralizumab to reduce blood eosinophil counts are well known (163,164). However, as previously reported, we found that benralizumab treatment leads to a complete depletion of eosinophils in the peripheral blood (31,33,34). Interestingly, patients treated with mepolizumab revealed different values of sL-selectin, CD8+ and NKT-like cells after the first dose.

$CD8^+$T cells are an important source of type 2 cytokines; they are insensitive to corticosteroids and corticosteroid-resistant pathways generally cause asthma exacerbations (167). NKT-like cells have been associated with severe poorly controlled asthma; they play a potential role in airway hyper-reactivity, together with other Th2 cells or independently to adaptive immunity responses (168). Hodge et al. suggested an altered expression of cytotoxic/pro-inflammatory mediators by different lymphocyte subsets such as CD8+ NK and NKT-Like cells in poorly controlled asthma. This immunological pattern of expression is a marker of treatment responsiveness and/or risk of exacerbations (169). In the context of NK and NKT-like cells, Duvall et al. pointed out that NK cells are altered in severe asthma (170). In line with these data, our results reported an altered expression of CD8+ and NKT-like cells after mepolizumab than benralizumab treatment. This difference may be related to the differential molecular target of these two treatment.

The exact role of circulating adhesion molecules, including sL-selectin, in asthma is still controversial. However, we found significant changes in sL-selectin concentrations after the first month of mepolizumab therapy. According to Hamzaoui et al. in severe asthma patients, circulating soluble forms of these molecules may reflect different inflammatory pathways and could be used to monitor the disease activity (171).

The stratification of our patient population showed that higher levels of peripheral eosinophils, sL-selectin and KL-6 concentrations at baseline were associated with early response to mepolizumab treatment. Combination of these three blood biomarkers, significantly improved the identification of "early responders" patients. Comparisons between baseline values and those at T1 showed that peripheral eosinophils and sL-selectin changed significantly in all patients, and in "early responders" there was a significant increase of FEV1 and FEV1/FVC ratio and a decrease of KL-6 and NKT-like cells concentrations. In benralizumab cohort, after one month of therapy, the only

difference between “early and partial responders” was the peripheral eosinophil. Unfortunately, the small size of our cohort did not allow a better evaluation of this aspect.

Although blood eosinophils and severe asthma clinical features are considered the best predictor biomarkers for anti IL-5 and antiIL-5Rα therapies, the detection of blood eosinophils has great limitation and does not support the clinicians in the more suitable drug for each candidate (172).

For the first time this study proposed sL-selectin as marker of hyper-responsiveness and KL-6 as bioindicator of airway remodelling, suggesting a panel of blood biomarkers to discriminate “early responders” from “partial responder” among severe eosinophilic asthma patients. Moreover, Regulatory T cells were also analyzed and found to be higher in asthma patients than controls, further increasing after one month of mepolizumab administration. This interesting finding is in line with the literature suggesting that Tregs contribute to Th2 immune response regulation (96,122,173) and demonstrating that mepolizumab could have a modulatory effect in restoring immune cell homeostasis.

We also analysed the relationship beween L-selectin and regulatory T cells in a subgroup of 14 mepolizumab treated patients. L-selectin levels correlated with blood eosinophil count and FEV1/FVC ratio and were minus 2.5-fold those recorded before mepolizumab treatment. Serum L-selectin emerged in our study as a potential biomarker of severe asthma useful for evaluating treatment responses. L-selectin showed a similar pattern to the gold standard biomarker: blood eosinophilia. L-selectin, compared as a surface marker in PBMC of patients and controls, showed higher CD4+CD62L+ percentages in patients. Cells expressing L-selectin continued to increase during mepolizumab treatment, particularly in clinical responders. The exact role of CD4+CD62L+ cells is unclear. Zhang et al. (174) showed that CD4+CD62L+ T cell-derived Foxp3+ T cells can suppress T effector cell proliferation. Our results therefore contribute to the hypothesis that Th2 inflammation in severe uncontrolled asthma can be associated with a large number of cells with modulatory properties that can help restore equilibrium during inflammatory status. Fiscus et al.(175) demonstrated that L-selectin interferes with selective migration of T lymphocytes and with the development of airway hyper-responsiveness. Tang et al. suggested that eosinophil inflammation and airway hyper responsiveness may be dissociated events in the pathogenesis of asthma regulated by T lymphocytes rather than eosinophils (176). Royce et al. supported the theory that L-selectin is a possible target for asthma therapies, which can have dramatic effects on airway hyper-responsiveness and remodeling (177). Our data supported these findings, especially the evidence that CD45+ T lymphocytes revealed lower expression in asthma patients after one month of therapy than at time 0. At the same time, CD4+CD62L+ cells were highly expressed after one month of therapy in early clinical responders. The indirect correlation between CD4+CD62L+ cells

and blood eosinophil count before and after therapy further support this theory. On the contrary, CD4-CD62L+ cells showed significantly lower percentages in asthma patients than controls.
After mepolizumab therapy a discrepancy between early clinical responders and partial responders was observed: early responders tended to have higher expression of CD62L+ on the surface of cells while partial responders showed a decrease in CD62L+ cell percentages.
CD62L (L-selectin) is highly expressed on naive T cells and down-regulated on activated T cells (178). A further step of our study will be to evaluate the expression of CD62L in many cell types such as B cells, dendritic cells, macrophages, NK cells, neutrophils, eosinophils and Tregs.

6. Conclusion

By stratifying a cohort of 28 severe eosinophilic asthma patients treated with two new anti IL-5 and anti IL-5Rα monoclonal antibodies (mepolizumab and benralizumab) this study provides new insights for the personalized approach to severe asthma therapy. Although preliminary, the results indicate that besides to peripheral eosinophils (nowadays considered the best predictor biomarkers for anti IL-5 and antiIL-5Rα therapies) KL-6 and sL-selectin are useful novel biomarkers of early response to mepolizumab, (interfering with pathogenesis of severe asthma).

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UNIVERSITÀ
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Dipartimento di Scienze della Vita

Siena, Nov. 09, 2020

Evaluation of Dr. Laura Bergantini's Ph.D. thesis:

"IMMUNE MODULATORY EFFECTS OF NOVEL MONOCLONAL ANTIBODIES TARGET THERAPIES IN SEVERE EOSINOPHILIC ASTHMA PATIENTS"

BACKGROUND

Research questions are clearly articulated and sufficient background information are included.

APPROPRIATENESS OF LITERATURE CITED

Dr Berghantini accurately identifies relevant research and literature and clearly summarizes and integrates the information.

METHODOLOGY

Dr Bergantini demonstrates very good understanding and proper application of methodology, she uses state of the art methodologies and applies complementary approaches when necessary. Some minor revisions are suggested

PRESENTATION AND ANALYSIS OF RESULTS

Results are clearly summarized with indication of the candidate's role, discussion is focussed and relevant to issues investigated. Figures/tables are well-structured and self-explanatory. Alternative explanations have been addressed when applicable. Some minor revisions are suggested.

DISCUSSION/IMPLICATIONS

Discussion of results are focused and connected to research questions. Implications for future research are also discussed.

QUALITY OF WRITING

Clear presentation in correct language. Logical progression of thought within the thesis and within each section. Some typos are present.

FINAL CONSIDERATIONS

The thesis is well written, with just some typos and some minor revisions (see below) that can be easily removed in the final version. The thesis provides, for the first time, some new biomarkers that can be used to improve personalized therapies in severe asthma using new anti IL-5 antibodies, mepolizumab and benralizumab. Between them, KL-6 and sL-selectin can be associated with early response to mepolizumab.

The description of the techniques and the figures presented are of good quality. The methodological approach applied guarantee findings and can be considered state-of-the-art in the

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field. The scientific outcome of the thesis is highly significant as demonstrated by the publication of the results in some high-impact international journals and can be helpful for the international scientific community.

MINOR REVISIONS TO BE ADDRESSED UNDER THE SUPERVISION OF THE STUDENT'S TUTOR

Materials and Methods:

Pg 28: It is not clear if the term FULL RESPONDERS is considered as EARLY RESPONDERS as reported in Figure 7 and in the results, discussion and conclusion sections. If yes, please use only Early responders or otherwise specify better the meaning.

Pg 30: When a centrifugation process is reported you must use **RCF (x*g*)** instead of rpm or otherwise you must include the name of the rotor and centrifuge used.

Results

Fig: 12 please include the legend for red and black lines.

Fig. 15 please include letters a, b and c in the pictures.

Fig. 16 please include letters a and b in the pictures and legend.

Fig. 17 please include letters a and b in the pictures.

CONCLUSIONS

As the PhD thesis is of great quality and fulfils all the requirements, I recommend that Dr Laura Bergantini should be allowed to proceed to the oral defence of the thesis and granting the doctoral degree.

Best regards

Prof. Luca Bini

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8th November 2020

Assessment of the PhD thesis with title:

"Immune modulatory effects of novel monoclonal antibodies target therapies in severe eosinophilic asthma patients"

To be discussed by **Laura Bergantini**

The thesis addresses the role of biomarkers in immunophenotyping and identifying biomarkers of the response of severe asthma patients to anti-IL5 biological therapies. Biomarkers studied in this thesis include lymphocyte subsets, serum soluble L-selectin and marker of epithelial cell damage KI6, at baseline and at 1 and 6 month follow up visits in subjects treated with mepolizumab, an anti-IL5 monoclonal antibody, or benralizumab, an anti IL5 receptor antibody.

The author set out to study lymphocyte subsets including Tregs, and serum biomarker responses after treatment with the two anti-IL5 drugs, and also to identify the baseline biomarker profile of early vs partial responders to treatment. The author found clear differences in lymphocyte subsets between patients with severe asthma and healthy controls, with significant increases in CD8+ and NKT cells at baseline. Interestingly, she observed a difference in their response to the two drugs, with a drop in both CD8+ and NKT cells after 1 month of mepolizumab treatment but not with benralizumab.

In addition, the author reports the novel observation that in the mepolizumab-treated group, in addition to baseline levels of eosinophils, serum L-selectin and KL6 were significantly higher in the early responders compared to the partial responders, whereas this difference was not significant in the smaller group treated with benralizumab, and that levels of these biomarkers fell in the early responders but not in the partial responders in the mepolizumab treated group. When combined, these three biomarkers improved identification of early responders to mepolizumab.

The thesis contains a review of the field in the introduction, presents well delineated aims, and explores the issues across a number of experiments. The analysis of the data appears to be appropriate. Limitations of the study include the small number of patients treated with benralizumab, which doesn't allow to reach definitive conclusions about the differences in the predictive biomarker responses between the two treatments. This thesis reports novel data on the immunomodulatory effects of these two drugs, with identification of a set of three biomarkers that can help identify early responders and provides the basis for future confirmation of these very interesting results.

I consider this thesis of high quality and have no hesitation to recommend it to proceed to the thesis defence and granting the doctoral degree.

Sincerely,

Elisabetta Renzoni

Consultant Physician and Honorary Senior Lecturer

Royal Brompton Hospital/Imperial College