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Exosomes and inflammatory biomarkers: potential tools for the study of heterogeneity in asthmatic inflammation

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INTRODUCTION

Asthma, a chronic inflammatory airway disease, has a global prevalence of 5-10%, affecting 339 million people worldwide [1,2]. The inflammatory nature of the disease is related to clinical asthmatic symptoms, such as breathlessness, wheeze, chest tightness, and cough, together with a variable expiratory airflow limitation that also differs over time and in intensity. This expiratory airflow instability is usually reported by peak flow variability, reversibility to fast-acting bronchodilator drugs, or by bronchoconstriction after bronchial challenge [3]. Another clinical aspect of the disease is airway hyperresponsiveness to direct or indirect stimuli, such as exercise, allergen/irritant exposure, weather changes and viral respiratory infections. The resolution of symptoms may occur spontaneously or after treatment [1].

Diagnosis of asthma is based on the history of typical symptoms and an increase in forced expiratory volume in 1 second (FEV1) by 12% and > 200 mL from baseline using bronchodilator reversibility testing or using other tests that report a variable airflow limitation [2-5]. Despite the fact that patients with asthma often present similar clinical symptoms, triggering factors, natural history and underlying molecular biology may vary significantly among patients.

When facing the clinical challenge of diagnosing asthma, it must not be forgotten that, at the centre, there is a specific phenotype and an underlying endotype for each patient, the recognition of which is fundamental for the choice of the most precise therapeutic strategy. Given the heterogeneity of asthma, the use of clustering methods (phenotypes and endotypes) and the emergence of targeted molecular-based therapies have rapidly advanced both the concept of, and the approach to, this disease. Phenotypes are the observable characteristics of patients that often result from the interaction between genetics and the environment; endotypes can be defined as subtypes of disease based on underlying pathobiological processes. Although different phenotypes frequently overlap, a distinct biologic pathway underlies the clinical heterogeneity of this disease [6-8].

The severity of asthma is variable, and the most severe cases are often further complicated by various comorbidities, implying more difficult therapeutic

management. Patients with uncontrolled asthma, between 40% - 54% of adults with asthma in the United States, are frequently affected by poorly controlled comorbidities [9,10]. These patients have significantly reduced health-related quality of life (QoL), worsening of lung function, with up to 10% having severe asthma exacerbations, a worsening of their type 2 comorbidities, and associated healthcare costs, despite the use of high doses of oral corticosteroids (OCS) [9-12].

Asthma and chronic rhinosinusitis with nasal polyps (CRSwNP) are often associated; indeed, patients with CRSwNP are particularly predisposed to asthma, with an incidence of up to 70%, and are mainly characterized by significant eosinophilic infiltration and augmented levels of immunoglobulins E (IgE) in nasal polyp tissue [13,14]. Moreover, the presence of higher levels of eosinophils in the serum and sputum in patients affected by asthma and by CRSwNP may be an expression of a close relationship between the two diseases [15]. Indeed, type 2 inflammatory airway diseases, driven by a similar underlying type 2 pathophysiology, often coexist in the same patient, with a close positive correlation between increased risk of worsening asthma symptoms with an increased incidence of CRSwNP, to which a higher probability of poor asthma control is associated [16].

However, while fraction of exhaled nitric oxide (FeNO) values, total IgE, allergen specific IgE levels, blood and sputum eosinophil counts are useful biomarkers in identifying a type 2 inflammation, they do not always predict patient response to a type 2 biologic therapy and there is no evidence of any disease-modifying effects [17-20]. The Global Initiative for Asthma (GINA) guidelines suggest add-on biological therapies for asthmatic patients not responding to standard care, and to use type 2 biomarkers for better phenotyping of asthma and to guide the therapeutic approach. The criteria that define type 2 airway inflammation, reported in the recent asthma guidelines, are: blood eosinophils $\geqslant$150 cells /µL and/or FeNO$\geqslant$20 ppb and/or sputum eosinophils $\geqslant$2% and/or asthma clinically allergen-driven and/or need for maintenance OCS therapy [3]. Instead, there are currently no recommendations on type 2 inflammatory expression biomarkers to be used in CRSwNP [19].

Therefore, the identification of biomarkers and early targeted treatment is a relevant need for a heterogenous disease such as asthma and in particular for uncontrolled patients [13]. In understanding these clinical and pathophysiological aspects of type 2 inflammation, with the purpose of identifying the various endotypes for more effective treatment for severe asthma, considering that a type 2 inflammation background shows different molecular and cytokine pathways (or the same pattern with activation to various extents), the present work is focused on recognizing prodromal signals, sensitive and disease-specific biomarkers. These could be reflected in proteomic expression. Changes in the protein content of peripheral cells and blood, and alterations in exosomal fraction could represent predictive biomarkers for asthma, helping towards a more precise approach in the management of the condition.

Type 2 inflammation in asthma

Asthma, a heterogenous disease characterized by multiple different pathogenetic subgroups with various cellular and molecular characteristics, is categorized in a consistent subgroup by type 2 inflammation, driven by T helper 2 (Th2) cells and group 2 innate lymphoid cells (ILC2). This subgroup is characterized by the production of cytokines such as interleukin (IL)-4, IL-5 and IL-13, which induce chemokines, such as eotaxin-3, thymus and activation-regulated chemokine (TARC) and vascular cell adhesion molecule-1 (VCAM-1), all involved in trafficking eosinophils to tissues, with consequent clinical symptoms typical of chronic inflammatory airway diseases [21]. Airway inflammation in asthmatic patients mediated by cellular infiltration and by the release of inflammatory mediators is the key point of asthma pathogenesis [19]. Asthmatic bronchial walls present altered wound repair response with secretion of growth factors that induce remodelling during chronic airway inflammation. Remodelling, characterized by smooth muscle hypertrophy, goblet cell hyperplasia, angiogenesis and subepithelial basement membrane thickening, leads to irreversible airway obstruction and hyper-responses [22,23].

Type-2 immune effector leucocytes play a key role in inducing a link between the innate and adaptive Th2 response with the recruitment of T cells and eosinophils.

During allergen sensitization of the airways, there is differentiation of Th2 lymphocytes from naive T cells and the requirement of IL-4 to activate the transcription factor signal transducer and activator of transcription 6 (STAT6) and GATA-binding protein 3 (GATA3) [21]. IL-4 and IL-13 play a significant role in inducing B–cell class switching and IgE production, in releasing proinflammatory mediators, barrier dysfunction and tissue remodelling. IL-13 is also involved in goblet-cell hyperplasia, mucus production, smooth muscle contractility and basement membrane thickening [21,24]. Moreover, IL-13 causes airway obstruction with the production of mucous plugs by binding to mucus-producing cells in the epithelium, altering mucociliary transport, identified by the expression of mucin 5AC (MUC5AC) protein, known as a marker of airway goblet cells and mucus hypersecretion [25]. In asthma patients, IL-13 is also involved in the upregulation of nitric oxide (NO) production [26]. As well as IL-5, also IgE synthesis, IL-25 and IL-33 are involved in airway eosinophilia, and IL-9 augmented levels are highlighted after exposure to allergens with consequent mucus production. This later effect is related to both direct action on airway epithelia and interaction with IL-13 [19]. IL-5 is involved in the differentiation and maturation of IL-5Rα^+ eosinophil progenitors in the bone marrow, in mobilisation and survival, and in the development of other type 2 cells, such as mast cells and basophils [8].

Other mechanisms are at the base of airway inflammation in specific subsets of patients, such as Th17 cells, related to corticosteroid (CS) insensitivity, which are another subgroup of cells involved in asthma inflammation [27]. An emerging role in understanding the pathogenetic mechanisms of asthma is played by the bronchial epithelium whereby an intrinsic defect characterized by incomplete formation of tight junctions causes the penetration of inhaled allergens. The penetration of allergens facilitates inflammation in submucosal cells and tissues, a process that is also induced by other environmental stimuli such as respiratory viruses with consequent asthmatic exacerbations. The injury of the airway epithelium induced by recognition receptors such as toll-like receptors (TLRs) or by the cytotoxic epithelial injury is mediated by thymic stromal lymphopoietin (TSLP), IL-33 and IL-25 that connect innate with adaptive immunity promoting

Th2 inflammation [19]. In particular, viral or bacterial infections play a role in asthma inflammation inducing activation on innate immunity, such as macrophages and natural killer (NK) cells. Moreover, Th17 cells play a key role in the defence against infections, recruiting neutrophils into the bronchial mucosa in severe forms of asthma [28]. Chronic injury of the airway epithelium causes not only an increased permeability of inhaled allergens but also a reactivation of the epithelial-mesenchymal trophic unit (EMTU) [19,29]. As reported in literature, IL-4 and IL-13 play a key role in epithelial barrier dysfunction in upper and lower airways, contributing to the disruption of epithelial junctions, and to increased epithelial permeability, both in asthma and in CRSwNP [30].

Phenotypes and related biomarkers of asthma inflammation

In the light of the heterogeneity of asthma, the use of a clustering approach and the introduction of targeted molecular-based therapies has led to the need for a more precise phenotyping of the disease. The identification of various phenotypes and of type 2 (T2) biomarkers is playing a key role in the diagnosis and treatment of the disease [19].

Traditionally, asthma was differentiated into extrinsic (or atopic) or intrinsic (non-atopic) types [19, 20]. The Th2 molecular phenotype presents in about 50% of asthmatic patients, from mild to severe asthma, which includes allergic asthma, patients with exercise-induced asthma and eosinophilic asthma. Allergic asthma is clinically characterized by the identification of allergic sensitization and a correlation between allergen exposure and symptoms of asthma. Allergic eosinophilic asthma is considered the most common phenotype of asthma with an underlying Th2-type inflammatory process [31-33].

Truyen E. et al. reported that patients with allergic asthma had a higher percentage of eosinophils, of mRNA for IL-4, IL-5 and IL-13 in induced sputum compared to a non-allergic group. Moreover, if compared to controls, only IL-5 and IL-13 messengers were increased in allergic patients. The authors also reported a positive correlation between the augmented levels of mRNAs for IL-4, IL-5 and IL-13 and the percentage of eosinophils in the airways. IL-9, another cytokine with a key role in allergic asthma, is responsible of the expansion of

mononuclear cells [34]. Indeed, as reported by Shimara et al., increased levels of mRNA and protein for IL-9, mainly produced by cluster of differentiation (CD) 3+ lymphocytes, are present in bronchial biopsies of mild atopic asthmatics, and increased levels of mRNA for IL-9 correlated significantly with bronchial obstruction and responsiveness of airway to methacholine [35]. Furthermore, in allergic asthma, in particular eosinophilic asthma, eotaxin-1, eotaxin-2 and eotaxin-3 are involved in the stimulation and migration of eosinophils. In particular, eotaxin-3 may play a relevant role in persistent allergen-induced bronchial eosinophilia [36]. However, other metabolic pathways, involving ILC2, regulate eosinophilic asthma. In literature, it has been reported that ILC2, high levels of which are present in the peripheral blood of asthma patients, is involved in the pathogenesis of non-atopic eosinophilic asthma [37-39].

In asthma, a T2 response in the bronchial mucosa begins with the release of TSLP, IL-25 and IL-33 (alarmins) in response to epithelial tissue damage, pathogen recognition or following exposure to allergens. These alarmins directly activate ILC2s, leading to the production of Th2 cytokines. IL-33 is mainly expressed by mast cells, ILC2s, eosinophils and regulatory T cells (Tregs), while IL-25 is expressed in lung epithelial cells after exposure to allergens and during helminth infection [40,41]. Prèfontaine et al. reported a progressive increase in mRNA for IL-33 in bronchial biopsies of asthmatic patients, and greater expression of epithelial immunoreactivity for IL-33 in patients affected by severe asthma compared to patients affected by mild asthma [42].

The other broad asthma phenotype includes patients that do not present typical evidence of biomarkers associated with Th2 inflammation. This “non Th2” associated asthma, poorly CS responsive, includes obesity/smoking associated asthma, neutrophilic asthma, and paucigranulocytic asthma [43]. Neutrophilic asthma is associated with higher levels of severity and with Th1-type cells, in particular cells that produce interferon-γ (type I immunity) and Th17 cells that produce IL-17, IL-21 and IL-22 (type 3 immunity). In severe asthma, in contrast to mild asthma, there is a higher level of IL-8 and neutrophilic myeloperoxidase (MPO) and a higher number of neutrophils. Neutrophilic asthma is also reported

in patients affected by severe refractory asthma, in which bacterial colonization of the airways can contribute to the neutrophilic phenotype of asthma [44-47].

Asthma and associated comorbid nasal polyps

Various comorbidities, often associated to asthma, such as rhinosinusitis, nasal polyps, gastroesophageal reflux disease, obstructive sleep apnoea and hormonal disorders, may have a common pathophysiological mechanism with asthma and, moreover, may influence asthma control and response to treatment [48]. However, despite the fact that these diseases frequently coexist, they are often managed separately with consequent poor asthma control [19].

Chronic rhinosinusitis, with a prevalence of 12% in the general population, can be divided into two phenotypes: chronic rhinosinusitis with nasal polyps (CRSwNP) and chronic rhinosinusitis without nasal polyps (CRSnNP) [49].

As reported above, patients with CRSwNP that have a Th2-predominant type of inflammation, present high levels of IL-5 and eosinophilic inflammation, a phenotype that is often also seen in severe asthma. [50,16] CRSwNP affects around 40 - 60% of severe eosinophilic asthma. The association between asthma and CRSwNP, with T2 inflammation being the cornerstone of both diseases, is not just a simple coexistence; a coexistence which, however, can be managed with the use of the same monoclonal antibodies. From a clinical point of view, patients affected by chronic rhinosinusitis, characterized by inflammation of sinonasal mucosa, have nasal obstruction, pressure or face pain, loss of smell and poor drainage lasting > 12 weeks [19]. In literature, an increased risk of asthma symptoms and of poor asthma control in the presence of CRSwNP is reported [51,52]. Similarly, in the presence of a comorbidity with a T2 inflammatory profile the severity of CRSwNP increases, with significantly greater severity in patients with concomitant asthma than in those with CRSwNP alone [53]. Moreover, as reported by Coumou H. et al. in patients with adult-onset asthma, the presence of comorbid nasal polyps was significantly related with a decline in post-bronchodilator forced expiratory volume in 1 s (FEV1) per year [54]. In literature, it has also been reported that uncontrolled asthma and CRSwNP increase disease burden, in particular related to the short- and long-term effects of

the use of OCS (e.g. mood and sleep disturbance, osteoporosis, type 2 diabetes, obesity, glaucoma, cataracts), lead to a reduction of QoL and have a negative impact on mental and physical health [55-57].

Regarding asthma in nasal polyps, there is, in addition, an epithelial barrier dysfunction since the expression of tight junction and cell adhesion proteins is significantly thwarted, compared to healthy individuals, as a direct result of IL-5 on airway epithelial cells expressing the IL-5 receptor (IL-5R). Alteration of the epithelium facilitates alteration of the local microbiome, causing persistent inflammation [58]. Indeed patients affected by CRSwNP present with a more frequent colonization by bacteria such as *Staphylococcus aureus* and are also more prone to viral infections, which further activate the adaptive and immune response systems, favouring T2 inflammation with a consequent increase of T2 biomarkers after exposure to viruses such as eosinophils, IL-4, IL-5 and IL-13 in nasal fluids and bronchoalveolar lavage (BAL) [59,60]. This immunological response to viral infections and the production of T2 cytokines leads to reduced production of type I interferons that normally have anti-viral effects, highlighting the presence of increased viral replication in asthmatic patients compared to healthy subjects [60,61].

There is also a distinct phenotype characterized by the coexistence of asthma and nasal polyps and aspirin sensitivity. This phenotype known as aspirin-exacerbated respiratory disease (AERD) is defined as CRSwNP, difficult-to-control asthma and adverse respiratory reactions to medications such as aspirin or non-steroid anti-inflammatory drugs (NSAIDs) which inhibit cyclooxygenase-1 (COX-1). In the pathophysiology of this phenotype, eosinophils, and in particular mast cells, have a key role in releasing inflammatory mediators, such as prostaglandin D2 (PGD2), prostaglandin F2α (PGF2α) and cysteinyl leukotrienes such as LTE4 [58,62]. PGD2, an inflammatory mediator which can bind to the chemoattractant receptor homologous molecule (CRTH2) found on the surface of Th2 cells, ILC2, eosinophils and basophils, activates and recruits them to the airways. Additionally, it can bind to the prostaglandin D2 receptor 1 (DP1) receptor, inducing chemotaxis of pro-inflammatory cells and causing nasal oedema by inducing vasodilation [62].

PGF2α, an eicosanoid produced by eosinophils, mast cells and epithelial cells of the respiratory airways, acts as an agonist for the CRTH2 receptor and has a significant role in inducing bronchoconstriction, worsening asthma symptoms. LTE4, another eicosanoid produced mainly from eosinophils and mast cells, can upregulate the production of PGD2 and PGF2α and levels are drastically reduced by anti-IL-5 or anti-IL-5R biologics. This aspect is very important considering that IL-5 is the most crucial cytokine in the pathogenesis of T2-high inflammation and, in patients with AERD, there is an augmented expression of IL-5R on the surface of eosinophils and mast cells, facilitating the T2 signalling process. Moreover, as IL-5 has recently been implicated in the process of weakening the epithelial barrier between airway epithelial cells, blocking IL-5 signalling is currently the most dominant weapon against T2-high inflammation [58, 63, 64].

Asthma control and severe asthma

Severe asthma is characterized by persistent symptoms and/or frequent exacerbations, despite treatment with high dosage therapies or continuous or frequent use of CS. In particular, according to ERS guidelines, when the diagnosis of asthma is confirmed, and comorbidities addressed, severe asthma is classified as asthma that needs high doses of inhaled corticosteroids (ICS) plus a second controller and/or systemic corticosteroid to prevent the disease from becoming "uncontrolled" or remaining "uncontrolled" despite this treatment. This has an impact on patient QoL, their use of healthcare services and associated costs [65].

In the 1990s, the increased use of ICS had a positive impact on asthma, determining a sharp decrease in asthma mortality [66,67]. However, during the last decade, asthma mortality rates have plateaued, and many patients are experiencing uncontrolled asthma, with consequent impaired QoL and chronic respiratory symptoms, which cause sleep disturbance, excessive daytime sleepiness and decreased work productivity [4,5,68]. There are many reasons at the basis of this lack of improvement in achieving asthma control. Asthma is a chronic inflammatory airway disease needing regular long-term anti-inflammatory treatment for symptom control and prevention of acute attacks and/or lung function decline. ICS are the mainstay of asthma treatment, but many patients do not present good adherence to regular treatment with consequent under-

treatment of the chronic inflammation [69]. Another possible reason is the heterogeneity of asthma; some patients need different interventions, based on a personalised approach according to the various phenotypes [70]. Poor inhaler adherence and technique, lack of self-management support, exposure to triggers, unavoidable environmental factors, limited accessibility to diagnostic facilities and medication are some of the other factors that can contribute to poor asthma control [71-73]. Poor asthma control has been described in patients affected by different GINA-based asthma severity levels, with a consequent impact on both direct (health care services, medications) and indirect (sick leave from work, disability, other) costs [66,74].

However, although most asthmatic patients achieve good asthma control with the use of ICS and bronchodilators, it is also important to analyse what happens in patients that not have a good response to this therapy. Indeed, it is also important to analyse the role of ICS, which, through the glucocorticoid receptor (GR), an intracellular receptor and transcription factor belonging to the nuclear receptor family, is based on the local suppression of Th2 cell-mediated inflammation, such as IL-4/IL-5/IL-13 cytokines, epithelium-derived cytokines, chemotactic chemokines and adhesion molecules. Various mechanisms can cause a reduced response to ICS in patients affected by moderate/severe asthma. For example, it may be caused by the overexpression of cytokines, such as IL-1, TNFα, IL-4, IL-13, or NO, which reduces GR nuclear translocation and function. Moreover, as many patients affected by severe asthma are refractory to ICS therapy poor symptom control is frequent. Indeed, given that numerous uncontrolled patients have severe asthma, they need to be treated with specific biological therapies targeting the IL-5, IgE and IL-4/IL-13 pathways [65, 75].

Biological anti-severe T2-high asthma

The key role of Th2 cells in modulating airway inflammation has aroused much interest in the therapeutic potential role of “anti-Th2 approaches”. The available biological asthma therapies are based on monoclonal antibodies against key Th2 mediators, which treat the disease by targeting these pathways [76, 77].

Omalizumab, a humanized IgG1 monoclonal antibody that specifically binds to free IgE and prevents it from binding to the high-affinity IgE receptor (FcεRI) on

basophils and mast cells, induces FcεRI downregulation in basophils and mast cells, rendering those cells much less sensitive to stimulation by allergens and consequent degranulation. This antibody was approved for the first time in 2003 by the US Food and Drug Administration (FDA) for the treatment of moderate-severe allergic asthma from the age of 12. It is administered by subcutaneous injection every 2–4 weeks and received an extension of its indication from the age of 6 in 2009 for European countries and, later, also in the US for patients that have a sensitivity to perennial aeroallergens [78,79].

It is indicated for adults and paediatric patients affected by moderate-severe persistent allergic asthma, with a skin or in vitro positive perennial aeroallergen, with frequent respiratory symptoms, also at night, and repeated asthma exacerbations, despite optimized treatment with maximal doses of ICS + long-acting ß(2)-agonist (LABA) [80]. Many studies have reported that the use of omalizumab induces a reduction in asthma exacerbations, hospitalizations, relevant improvements in symptom control, QoL, intake of OCS and a significant and persistent increase in FEV1, lasting 5,7, and even 9 years [19, 77, 81-83]. The action in reducing exacerbations related to viral infections is related to the enhancement of the antiviral response mediated by IFNα [19]. A French real-life study also reported that effectiveness and long-term safety of omalizumab persists even 24 months after the suspension of the biological treatment [84]. Moreover, there is a related long-term, very good safety and tolerability profile to this real-life therapeutic effectiveness of omalizumab [85].

In the light of the key role of IL-5 in mediating eosinophilic inflammation, there are FDA-approved biologic drugs that target IL-5 signalling, reduce blood eosinophil counts and sputum eosinophils, attenuating T2-high inflammation [19].

Mepolizumab is a humanized IgG1 monoclonal antibody, administered by monthly subcutaneous injection and approved as an add-on treatment for patients 6 years and older affected by severe eosinophilic asthma, which recognizes and blocks IL-5 and prevents its binding to the IL-5 receptor alpha subunit (IL5Rα or CD125) on the surface of eosinophils [86,87]. The efficacy of mepolizumab in reducing disease exacerbations, and blood and sputum eosinophils was firstly reported by Nair et al. and by Haldar et al. and later confirmed by the phase IIb/III

DREAM (Dose Ranging Efficacy And safety with Mepolizumab) trial [88-90]. Moreover, MENSA (MEpolizumab as adjunctive therapy in patients with Severe Asthma), SIRIUS (SteroId ReductIon with mepolizUmab Study) and phase IIIb MUSCA trials, reported that, in patients affected by severe eosinophilic asthma, mepolizumab reduced asthma exacerbations, improved QoL, symptom control, and respiratory function, and reduced the need to take 50% OCS, reporting good drug safety and tolerability [91-93].

The efficacy of mepolizumab is reported both in non-allergic and allergic patients with severe eosinophilic asthma, with the possibility of efficacious use in the case of switching from omalizumab due to inadequate asthma control provided by anti-IgE therapy [94-96].

Its efficacy is also reported in severe nasal polyps, inducing an improvement of subjective symptoms, and endoscopic nasal polyp score, and decreasing the need for surgical polypectomy [97].

Reslizumab is a recombinant humanized IgG4k monoclonal antibody, which, similarly to mepolizumab, blocks IL-5 and interferes with its functions. However, it is not administered subcutaneously but intravenously (approved at a dosage of 3 mg/kg every 4 weeks) [98,99]. The clinical and functional effects have been evaluated in many randomized trials, which reported that reslizumab induced a reduction in both sputum and blood eosinophil counts, incremented respiratory function with positive effects not only on FEV1, but also on the small airways resulting in significant enhancements of mid-expiratory flow at 25–75% of forced vital capacity (FEF25-75) [100,101]. In literature, it has also been reported that severe asthma patients with a blood eosinophil count of 400 cells/mL and 12% FEV1 reversibility show a good response to reslizumab and, moreover, this biological drug induces a significant reduction of 50% - 59% of the annual exacerbation rate when compared with a placebo. It has also been responsible for a 70% - 50% decrease in the daily dose of OCS [102-103]. Reslizumab, similarly to omalizumab and mepolizumab, has a good, satisfactory profile of safety and tolerability [104].

Benralizumab, a humanized afucosylated anti-eosinophilic IgG1 monoclonal antibody, approved for patients 12 and older and administered once every 4–8

weeks by subcutaneous injection, is instead directed against IL-5Rα/CD125, inducing antibody-mediated eosinophil depletion and a rapid eosinophil reduction in sputum, bone marrow and blood [105,106].

The IL-5 receptor is specifically expressed on the surface of eosinophils and basophils [107,108]. Randomized clinical trials, in particular phase III SIROCCO and CALIMA studies reported that the use of benralizumab is related to a reduction of the annual rate of severe eosinophilic asthma exacerbations, an improvement of asthma symptom control and increased FEV1 [109,110]. An analysis of these trials reported the efficacy of benralizumab as an adjunctive biological therapy in both atopic and non-atopic patients with severe eosinophilic asthma [111]. A positive impact on lung function, reduction of OCS consumption, asthma symptom control, nasal polyps, long-term safety and tolerability have also been highlighted for benralizumab [112-118].

The inhibition of IL-4 and IL-13 play a crucial role in reducing/suppressing inflammatory response, and, in particular, the cytokine and chemokine cascade induced by their activity. This correlates to the action of another biological drug, dupilumab, a fully humanized molecule, which is able to bind the α subunit of the IL-4 receptor, shared by the IL-4 type I receptor complex (IL-4 α/gc) and the IL-4/IL-13 type II receptor complex (IL-4α /IL-13Rα), with high affinity and specificity, inhibiting the signal of both cytokines [119].

Some studies have reported that asthmatic patients with a more marked type 2 inflammation, in particular with high values of eosinophilia and FeNO, are those which better respond to dupilumab treatment [120]. Dupilumab studies reported a positive impact of the biological drug dupilumab on asthma exacerbations, on lung function, and on a reduction of OCS by 70% [120-123]. Moreover, Corren et al., in a post hoc analysis of the LIBERTY ASTHMA QUEST study, highlighted that the above reported beneficial effects of dupilumab are found in both allergic and non-allergic asthmatic patients [123].

Dupilumab displays a more than satisfactory profile of safety and tolerability, even if in some patients it may induce conjunctivitis or a marked blood eosinophilia, which usually presents a spontaneous resolution [124]. Moreover,

dupilumab has beneficial therapeutic effects also in relevant asthma comorbidities such as atopic dermatitis and nasal polyps [76, 125-127].

Extracellular vesicles

For many years it was thought that intercellular communication was regulated through direct contact between cells or via release of soluble molecules that send the signal by binding to a suitable receptor on the target cell, and/or via uptake into that cell. A revolution in this aspect was reported with the discovery of small secreted vesicular structures, containing complex cargo both in their lumen and on the lipid membrane that surrounds them [128].

Extracellular vesicles (EVs) can be considered as a novel means for intercellular communication and can be divided into exosomes (30-150 nm), microvesicles (100-500 nm) and apoptotic bodies (500-1000 nm), according to differences in size, cellular origin and functions. Firstly, when discovered in 1967 by Wolf, exosomes were thought to be a system of discarding plasma membrane (PM) proteins in maturing reticulocytes [129,130]. Exosomes, which present a cup-like morphology when observed under the transmission electron microscopy and with a buoyant density of 1.10–1.14 g/mL, are lipid bilayer-enclosed nanoparticles released by cells. In particular, they are the smallest EVs derived from the release of intraluminal vesicles (ILV) following the fusion of multivesicular bodies (MVBs) with the PM. Cells first generate early endosomes by endocytosis [128,131-133]. MVBs, late endocytic compartments that contain many ILVs, originate from the maturation of the first endosomes. Fusion of MVBs with the plasma membrane leads to the release of ILVs into the extracellular space as exosomes [132]. Depending on their origin, exosomes can modulate various immune-regulatory processes [133,134].

Exosomes are rich in cholesterol, sphingomyelin, ceramide and phosphatidylserine. They are formed by a lipid bilayer membrane enclosing a small organelle-free cytosol containing a heterogeneous array of macromolecules, defined as luminal cargo, and specific markers such as members of the tetraspanin family (e.g. CD9, CD63, CD81), heat shock proteins (e.g. HSP70) or proteins related to MVB biogenesis (e.g. ALIX, TSG101). Exosomes can play a significant

role in cell-to-cell communication and carry some components of the parent cell, such as protein, metabolites, receptors and nucleic acids, including DNA, RNA, and miRNAs. Some exosome proteins are specific to originated cells, while others are invariable, independently of cell origin [135].

EVs have been isolated from many biological fluids, such as blood, saliva, malignant ascites, amniotic fluid, and urine [136-137].

The interaction of exosomes with target cells can be carried out following various pathways. There is the possibility of a direct interaction of exosome lipids and/or trans-membrane proteins with receptors on plasma membrane of the target cell, inducing intracellular signalling cascades. Moreover, there is the possibility of fusion events of exosome membrane with PM, delivering luminal cargo directly into the cytosol or phagocytosis and macropinocytosis of exosomes, with subsequent fusion with other endosomal structures. Furthermore, there may be other endocytic internalization processes that include both clathrin-dependent and -independent pathways [138].

Exosomes may be isolated from almost every cell, not only from eukaryotic but also from prokaryotic cells, participating in the regulation of central normal biological processes such as immune response, pregnancy, tissue repair, and blood coagulation. Exosomes are also involved in pathobiological mechanisms related to the most frequent types of diseases affecting the population, such as neurodegenerative disorders, tumorigenesis and infectious diseases [139-143]. Additionally, they are stable both in vivo (in systemic circulation) and in vitro (they can be preserved frozen for a long period of time without losing their biological properties) making them an extremely useful source of information and biomarkers [144].

Recent evidence has shown that exosomes can promote inflammation and immune activation in chronic respiratory pathologies, even if their impact on respiratory diseases is not completely clear and is still under investigation [145-146]. Analysis of EVs from patients affected by respiratory pathologies may help to improve the diagnosis, prognosis, and response to therapy. EVs, in fact, might be a target for personalized medicine. As already reported above, EVs, which can be detected in various biological fluids (sputum supernatant, mucus, epithelial lining

fluid, pulmonary circulation, nasal and bronchoalveolar lavage fluid), represent a useful tool for both investigating the pathophysiology of respiratory disease and for biomarker discovery [136-140,147].

The most commonly used methods for exosome isolation, for various purposes and applications, are density-based, size-based, polymer-based precipitation, immunoaffinity, and micro-fluidic techniques. The methodologies currently in use for exosomes isolation and characterization are indicated in Figure 1 [148].

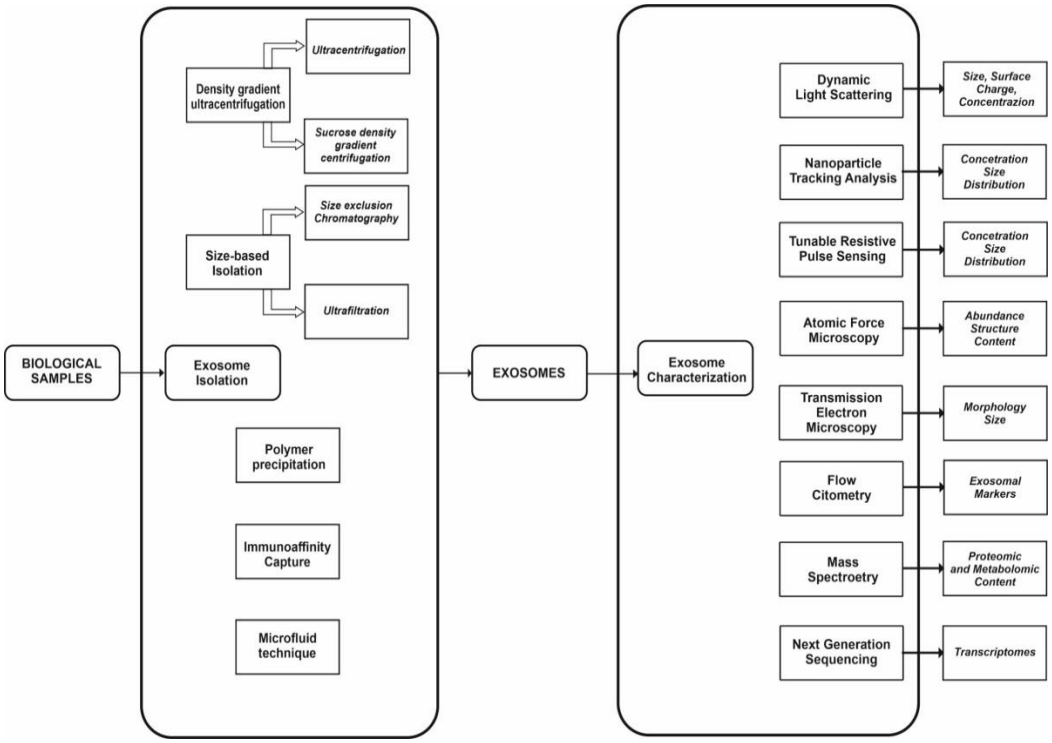

Figure 1 - Summary of methodologies currently in use for exosome isolation and characterization in chronic respiratory diseases.

Exosomes in physiopathology of asthma

In the lung, and in particular in asthma patients, exosomes, which are released from the key cells implicated in disease such as mast cells, eosinophils, dendritic cells (DCs), T-cells and bronchial epithelial cells, play a crucial role in lung biology and function through cell-cell communication [34-35]. Moreover, exosomes can induce the activation, or repression, of other asthma-associated cells and enhance allergic responses [149]. Analysing the different cellular origins of exosomes in the lungs, DC-derived exosomes have costimulatory molecules on

their surfaces that can activate allergen-specific T2 cells [32,37]; eosinophil-derived exosomes, whose number is increasing in asthmatic patients, play a significant role in the modulation of asthma [144, 150-151]. Eosinophil-derived exosomes contain eosinophilic proteins, such as eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil cationic protein (ECP), and may perform a crucial role in promoting asthmatic inflammation like their cell of origin. Indeed, isolated from asthmatic patients, they may have both autocrine and paracrine functions, inducing an increased production of cytokines, reactive oxygen species (ROS), and NO from target eosinophils, along with eosinophil migration by upregulating the expression of adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and integrin α2 that is relevant in asthma pathogenesis [152-153].

Other cellular players in the inflammatory response in allergy and asthma include lymphocytes. B-lymphocytes play various roles, e.g. the production of antigen specific IgE following T2 cell activation, the release of T2 cytokines, the triggering of an asthmatic reaction by acting as an antigen-presenting cell (APC) without the involvement of IgE and T lymphocytes [154-155]. They are also involved in the differentiation of naïve Th0-lymphocytes into T1-or T2-lymphocytes by releasing IFN-γ or IL-4, respectively [156]. Moreover, B-cells or regulatory B (Breg) cells that produce IL-10 play a key role against inflammation in hyperresponsiveness airways and against allergic inflammation, recruiting natural Treg (CD4+ CD25+ FoxP3+) cells to the lung [157]. B-cell-derived exosomes, which mirror their parent cells, carry major histocompatibility complex (MHC) classes I and II and integrins β1 and β2, along with the costimulatory molecules CD40, CD80, and CD86, and can present antigenic peptides to T-cells, induce T-cell responses, contain HSP70 which is relevant in DC maturation and can also modulate the proliferation and production of T2 cytokines [158-159].

T-lymphocytes can also release exosomes [160]. Cytotoxic CD8+ T-cells release granules containing cytolysis mediators, but the potential function of T-cell-derived exosomes is not clear [58, 161].

Exosomes released by activated CD4+ T-cells, which contain proteins such as lysosomal-associated membrane protein 1 (LAMP-1), lymphocyte function

associated antigen-1 (LFA-1), CD4+ T-cell markers such as CD4, TCR, CD25, and Fas ligand, play a key role in suppressing cytotoxic responses and antitumor immunity through CD8+ T-lymphocytes. Activated CD3+ T-cells also release exosomes that together with IL-2 induce the proliferation of autologous resting CD3+ T-cells and a specific cytokine profile [162].

Mast cells are also involved in allergic reactions and T2 responses, releasing bioactive mediators such as histamine, prostaglandins, and leukotrienes (LTs), and through the production of proinflammatory cytokines such as TNF-α and IL-13 which are involved in the innate and adaptive immune responses in asthma. Mast cells constitutively release exosomes which have downstream effects on other immune cell types. Mast cell-derived exosomes stimulate the activation of B- and T-lymphocytes, the production of cytokines such as IL-2, IL-12, and IFN-γ and the production of IgE by B-cells in the absence of T-cells through their CD40 surface ligand. Moreover, mast cell-derived exosomes carrying FcεRI that can bind to free IgE cause a reduction of serum levels of IgE and of mast cell activation, being thus a potential novel anti-IgE factor for patients affected by severe asthma [163-166].

Basophiles, granular cells that comprise 0.5–1% of circulating white blood cells but whose levels are augmented in the presence of inflammatory or chemotactic stimuli, can induce the proliferation and survival of naïve B-cells and guide their differentiation into antibody-producing cells. These functions can be modulated via direct cell-to-cell contact as well as through soluble mediators and exosomes, though there is limited data regarding exosome production by basophils [167,168].

Exosomes derived from DCs, specialized cells which act as APC, process and present antigens to T-cells and also have the capacity to phagocytose dead cells and bacteria, are similar to their cellular origin, presenting MHC classes I and II molecules on their surface, presenting allergens and triggering the induction of T2 responses [169,170]. On their surface, DC-derived exosomes contain HLA-DR, MHC, and CD86, which expresses the potential of these exosomes to induce T-cell proliferation and differentiation, and CD54, which instead enables exosomes to interact with T-lymphocytes via LFA-1 [82]. The recruitment and migration of

granulocytes and leukocytes to the site of inflammation is mediated by these exosomes, through metabolites of arachidonic acid such as 5-keto eicosatetraenoic acid (5-KETE) and LTB4 that are produced following transfer of exosome derived enzymes. These proinflammatory lipid metabolites play a relevant role in triggering asthma pathogenesis [171].

Moreover, exosomes released from structural lung cells also mediate the immune response in asthma. In literature, an increased production of exosomes by lung cells and their protein content in a mouse model of asthma is reported [172]. IL-13 has a role in increasing the secretion of exosomes by lung epithelial cells and these exosomes enhance the proliferation and differentiation of macrophages. Inhibition of exosome production by GW4869, an inhibitor of exosome biogenesis/release, reduces the induction of asthmatic characteristics in this model [173].

In literature, comparison and analysis of exosomal miRNAs in patients with severe asthma and in healthy patients highlighted dysregulated miRNAs that were involved in pathways related to airway integrity and related with some clinical data such as eosinophil count or FEV1 [174]. The identification of BAL exosomes from asthmatic patients shows that the expression of the epithelial marker mucin 1 on their surface allows us to understand how they are derived from bronchial epithelial cells [175]. BAL exosomal miRNAs from asthmatics are involved in IL-13 pathways, which in turn promote exosome production by airway epithelial cells and these exosomes are subsequently involved in the proliferation of undifferentiated lung macrophages [173,176].

AIM OF THE PROJECT

The present project is aimed at characterizing inflammation molecular profiles in patients with severe asthma, by identifying specific features in terms of proteomic expression in different clinical phenotypes. Using the available tools and emerging isolation and characterization technologies, the molecular approach that we used is based on exosomes, which can be considered as part of an intercellular cross-talking like a “shuttle system” for biological communication, and that may be used not only as a potential biomarker to characterize endotypes in several different inflammatory conditions in severe asthma but also in diagnosis, prognosis and therapeutics [177]. To further characterize the circulating EV profile in patients affected by severe asthma, we used an innovative flow cytometry multiplex bead-based platform, which evaluates the expression of a comprehensive panel of 37 membrane antigens expressed on the EV surface. Moreover, considering that the inflammatory process underlying asthma is coordinated by a cytokine network, we added an analysis of the cytokines expressed in the enrolled patients, identifying possible inflammatory and molecular differences, to evaluate if there is a relationship between the molecular and inflammatory profile with clinical phenotypes, identifying new possible predictive biomarkers of disease.

MATERIALS AND METHODS

Study population

We enrolled 22 asthmatic patients; in particular, 13 affected by severe asthma and 9 affected by non-severe asthma + CRwNP. The patients were enrolled at the Asthma Centre and Allergy Unit, Verona University Hospital, Verona, Italy. The diagnosis of nasal polyps had already been confirmed at the Verona operating Unit, prior to enrolment in the study and was confirmed by endoscopy or CT scan.

Focusing on patients with severe asthma, the inclusion criteria that we developed were: 1) confirmed diagnosis of severe T2-high asthma according to the European Respiratory Society/American Thoracic Society (ERS/ATS) definition; 2) age ≥18 years old; 3) eligibility to biological treatment, according to the prescription requirements established by the European Regulatory Agency. Exclusion criteria were: 1) ongoing biologic treatment, with at least an interval of 6 months since the previous biological treatment in the case of switch to a new biologic; 2) ongoing systemic steroid treatment > 10 mg prednisone or equivalent.

To explore the relevance of nasal polyps within the inflammatory pattern, patients affected by non-severe asthma + polyps were also enrolled.

The following patient characteristics were collected from each patient at baseline, or at the moment of enrolment in the study: age, gender, smoking, body mass index (BMI), naïve vs switch (i.e. if, on the date of enrolment, they had started biological therapy for the first time or had changed by that date from another biologic therapy), type of biological therapy to begin, eosinophil count. Moreover, for the group of patients affected by severe asthma we also collected: total serum IgE, FeNO, asthma control test (ACT), comorbidities, spirometry and in the case of presence of polyps we also collected visual analogue scale (VAS) and sinonasal outcome test 22 (SNOT-22). For asthma control using the ACT questionnaire, we considered patients with an ACT score ≥ 20 as well-controlled. These parameters have been and will be collected at time of inclusion, and thereafter at 3, 6 and 12 months from enrolment in the study and start of biological therapy.

The study was conducted in accordance with the ethical standards established in the Declaration of Helsinki. All patients provide their written informed consent.

The study was approved by the Ethics Committee of our institution, Azienda Ospedaliera Universitaria Integrata, Verona, Italy, register number 2987CESC.

Sample collection

Serum samples, approximately 10 ml, were obtained from samples of peripheral blood and placed in vacuette tubes containing separating gel. Vacuette tubes containing patient samples were centrifuged at 3000 rpm for 10 min at 4°C and the serum was aliquoted and stored at -80°C until used for subsequent analysis. Where possible also nasal lavage fluid (NLF) samples were obtained for patients.

NLF collection was obtained according to an established method with minor adjustments: briefly, patients were placed in a sitting position, made extend their neck gently backward to 30° from a horizontal position and fluid (physiologic solution, generally 0.9% NaCl, pre-warmed to 37°C) was instilled and not lost anteriorly due to gravity. To limit posterior loss, patients were asked to close their soft palate, hold their breath for the time of nasal lavage retention, and then keep their mouth open a little. The fluid was left within the nasal cavity for 10 seconds, then the patient expelled the fluid from the nostrils by gently exhaling into a collecting funnel that drained into a container on ice. Five mL of volume was instilled per nostril per patient and the NLF obtained volume was measured (by a graded container) to determine the retrieved volume (a recovery of around 80% is usual). NLF was filtered to remove mucin and then centrifuged at 4°C at 1000g for 20 minutes to sediment the cell pellet. Before centrifugation a small aliquot was taken and stored at -80°C. On the contrary, after centrifugation the supernatant was removed and stored in aliquots (0.5-1.0 mL), in appropriate polypropylene tubes and frozen at -80°C until assay. The cell pellet was re-suspended in 1 mL PBS containing 0.1% wt/vol human serum albumin, and 100 µL aliquots taken to make microscope slides for differential cell counts or specific cell immune staining [178].

EV isolation

EVs were isolated from serum samples. One mL of serum was diluted with equal volume of PBS to decrease viscosity. The samples were then centrifuged at 2000xg for 30 min at 4°C and supernatants were transferred into 2 mL ultracentrifuge tubes (Beckman Coulter, Brea, CA, USA) and ultra-centrifuged in a Beckman Optima XPN-80 (Beckman Coulter) at 16500xg for 50 min at 4°C. The supernatants were then transferred into new ultracentrifuge tubes and ultra-centrifuged at 120000xg for 60 min at 4°C. Pellets obtained were resuspended in PBS and ultra-centrifuged at 120000xg for 60 min at 4°C. After these ultracentrifugation steps, supernatants were removed, and the pellets were resuspended in 100 uL cold PBS and stored at -80°C.

EV characterization

Nanoparticle Tracking Analysis (NTA)

Particle size distribution in serum EV samples was determined using a NanoSight NS300 system (Malvern Technologies, Malvern, UK). In this system, light scattering and Brownian motion are used to determine particle size and distribution of small particles suspended in solution (20-2000nm). Particle movement is observed through a microscope and size is calculated using the Stokes-Einstein equation. EVs samples were thawed, mixed thoroughly and diluted 1:250 with PBS solution. Instrument settings were selected according to the manufacturer's software manual. Samples were analysed under constant flow conditions (flow rate = 20) at 25°C according to manufacturer's recommendations. Three videos of 60s were captured with camera level of 14/15. The data were analysed using instrument software and a detection threshold of 5/6. EV concentration is expressed as number of particles per mL of serum.

Transmission Electron Microscopy (TEM)

EV samples were thawed, mixed thoroughly and diluted 1:1000 with PBS solution. Aliquots of 6 µl of the suspension were absorbed for 1 minute on an ultra-thin carbon coated copper grid (CF200H-Cu-UL, Electron Microscopy Sciences) and excess of suspension was removed by gentle blotting. Suspension adsorbed to grid was placed on 1 drop of UranyLess solution (Electron

Microscopy Sciences) for 1 second. Operation was repeated and the second drop was left in place 30 seconds. Grid was then dried by gentle blotting and air. Sample was then visualized on a Morgagni 268D (FEI Philips) transmission electron microscope, setting the voltage to 80kV at the Centro Piattaforme Tecnologiche of the University of Verona.

EV analysis

Multiplex bead-based flow cytometric analysis

The MACSPlex Exosome Kit has been developed for the simultaneous flow cytometric detection of 37 surface epitopes that are known to be present on different exosomes plus two isotype control beads. EVs are captured by polystyrene beads (MACSPlex Exosome Capture Beads), labelled with different amounts of dyes (phycoerythrin [PE] and fluorescein isothiocyanate [FITC]), to realize 37 bead-subsets discriminable by flow cytometry. Each bead subset is coated with a different antibody against a specific EV (sEV) surface antigen, as indicated in Table 1.

No.	Antibody	Isotype
22	CD3	mIgG2a
23	CD4	mIgG2a
24	CD19	mIgG1
32	CD8	mIgG2a
33	HLA-DRDPDQ	recombinant human IgG1
34	CD56	recombinant human IgG1
35	CD105	recombinant human IgG1
42	CD2	mIgG2b
43	CD1c	mIgG2a
44	CD25	mIgG1
45	CD49e	recombinant human IgG1
46	ROR1	mIgG1κ
52	CD209	mIgG1
53	CD9	mIgG1
54	SSEA-4	recombinant human IgG1
55	HLA-ABC	recombinant human IgG1
56	CD63	mIgG1κ
57	CD40	mIgG1κ
63	CD62P	recombinant human IgG1
64	CD11c	mIgG2b

No.	Antibody	Isotype
65	CD81	recombinant human IgG1
66	MCSP	mIgG1
67	CD146	mIgG1
68	CD41b	recombinant human IgG1
74	CD42a	recombinant human IgG1
75	CD24	mIgG1
76	CD86	mIgG1
77	CD44	mIgG1
78	CD326	mIgG1
79	CD133/1	mIgG1κ
85	CD29	mIgG1κ
86	CD69	mIgG1κ
87	CD142	mIgG1κ
88	CD45	mIgG2a
89	CD31	mIgG1
96	REA Control	recombinant human IgG1
97	CD20	mIgG1
98	CD14	mIgG2a
99	mIgG1	control mIgG1

Table 1 - Overview of the 37 surface markers and 2 isotype controls of different purified sEVs analysed with MACSPlex Exosome Kit.

Triggers for side scatter (SSC) and forward scatter (FSC) were set to confine the measurement on capture beads. FITC and PE voltage were optimized to discriminate the 37 bead subsets; single bead subsets were each gated to measure APC median fluorescence signal intensity.

We used $5x10^8$ EVs in a volume of 120 uL MACSPlex buffer (MPB), incubating it overnight with 15 uL MACSPlex Exosome Capture Beads in the 96-well plate included in the kit on an orbital shaker (800 rpm at 10°C) protected from light. MPB was used as blank control. After incubation, 200 uL of MPB was added to each well and the plate was then centrifuged at 3000 g for 3 minutes at 10°C to discard extra volume; 135 uL of MPB and 15 uL of MACSPlex Exosome Detection reagents (5 uL for each APC-conjugated anti-CD9, anti-CD63, and anti-CD8 antibody) were added and incubated for 1 hour on an orbital shaker (450 rpm at 10°C) protected from light. After a further washing step, samples were analysed, with approx. 10000-15000 single bead events being recorded for each sample. A BD LSRFortessa X-20 Cell Analyzer (BD Bioscience/Falcon, San Josè, CA, USA) was used to acquire the samples and the data were analysed by FlowJo software (Treestor, Ashland, OR, USA).

Cytokine analysis

Cytokine analysis on serum samples were performed for the following cytokines: transforming growth factor (TGF)- β1, IL-4, IL-5, IL-6, IL-10, IL-17A, IL-33, IL-13, eotaxin-3 by using Simple Plex assays run on the ELLA microfluidic immunoassay system (ProteinSimple, San Jose, CA). Samples were diluted at a 1:1 ratio with sample diluent, and 50 µl of the solution was added to each sample inlet on the ELLA cartridge, as per manufacturer's instruction. Wash buffer was added to the appropriate wells on the ELLA cartridge. The results of the analysed samples were reported using Simple Plex Runner v.3.7.2.0 (ProteinSimple) and were available about 90 minutes after start of run.

In some serum samples, IL25 was also assessed by a commercially available ELISA kit, following manufacturer's instructions (Novus Biologicals, Colorado, USA).

In some patients, cytokine analysis, for the same cytokines reported above, was performed in NLF samples, if available, following the same procedures.

Statistical analysis

Data analysis and illustration were assessed with Graph Pad Prism software, version 9.3.1 for Mac (GraphPad Software, San Diego, CA, US). Comparison between different groups of numerical variables was assessed using the Mann-Whitney non-parametric test. Significant comparisons are indicated by asterisks as follows: * $p<0.05$; ** $p>0.01$; ***$p<0.001$.

RESULTS

Characteristics of study population

Twenty-two patients were enrolled, 13 affected by severe asthma (all non-smokers, 5 men and 8 women) and 9 were affected by non-severe asthma + polyps (3 smokers and 6 non-smokers). Of the 13 patients affected by severe asthma, 8 patients were also affected by nasal polyps. In particular, of the 7 patients in switch, 2 were affected only by severe asthma, 5 were also affected by nasal polyps; of the 6 naïve patients, 3 patients were affected only by severe asthma and the other 3 patients were affected also by nasal polyps. Regarding biological therapies, of the patients affected by severe asthma, 2 patients started biological therapy for the first time with anti-IL-5; 1 patient started biological therapy for the first time with anti IgE, instead 2 patients that were previously in therapy with anti-IgE started therapy with anti-IL-5. Regarding patients with severe asthma + polyps, 2 patients started therapy with anti-IL5R and 1 with anti-IL5 therapy; 2 patients were previously in therapy with anti-IL-5 and started anti-IL-4/IL-13 therapy at the time of inclusion; 2 patients were previously in therapy with anti-IL-5 receptor and started anti-IL-4/IL-13 therapy at the time of inclusion; 1 patient was previously in therapy with anti IgE and started anti IL-4/IL-13 therapy at the time of inclusion.

Regarding patients with non-severe asthma + polyps, all 9 patients started biological therapy for the first time with anti- IL-4/IL-13.

All the collected patient clinical characteristics and data at baseline regarding patients are reported in Table 2.

Patients	Gender	Age	Diagnosis	Biological Therapy	Smoke	BMI	Comorbidities	FEV1 %	FVC %	FENO	ACT	Total IgE (>76 KU\L)	Eosinophils mm^3	SNOT-22	VAS
1	M	60	Severe eosinophilic asthma	anti - IL5	NO	27	Diabetes; Psoriasis	45	74	25	17	-	600	-	-
2	F	63	Eosinophilic inflammation in severe allergic asthma	anti - IgE → anti – IL-5	NO	29	Allergic Rhinitis	64	81	165.60	14	2.400	170	-	-
3	M	62	Eosinophilic inflammation in severe allergic asthma	anti - IgE → anti – IL-5	NO	24.5	Allergic Rhinitis; Gastroesophageal reflux	25	52	21.97	16	300	300	-	-
4	F	55	Severe allergic asthma	anti – IgE	NO	23.1	Depression	67	79	21.99	9	671.10	100	-	-
5	F	70	Severe eosinophilic asthma	anti – IL-5	NO	31.6	-	77	82	14.47	12	-	650	-	-
6	M	64	Severe eosinophilic asthma	anti - IL5 → anti – IL-4/IL-13	NO	31.5	Nasal polyps; Osas; Gastroesophageal reflux; Hyperuricemia; Hypertension	80	94	24	14	-	300	30	8
7	F	61	Severe eosinophilic asthma	anti – IL-5R	NO	20.8	Polyps; Rheumatoid arthritis	49	89	116	11	-	1580	56	5
8	F	45	Severe eosinophilic asthma	anti - IL5R → anti – IL-4/IL13	NO	25	Nasal polyps	87	104	9	12	98	0	58	10
9	F	56	Severe eosinophilic asthma	anti - IL5R → anti – IL-4/IL-13	NO	26.6	Nasal polyps; Thyroid Disease; Epilepsy	141	62	70.80	12	-	0	56	3
10	F	74	Severe eosinophilic asthma	anti - IL5	NO	26.6	Nasal polyps; Thyroid Disease	67	84	7	13	172	130	-	-
11	M	69	Severe eosinophilic asthma	anti - IL5 → anti – IL-4/IL-13	NO	24.4	Nasal polyps; Allergic Rhinitis; Multiple sclerosis	85	93	16	21	140	980	57	9
12	M	58	Severe allergic asthma	anti - IgE → anti – IL-4/IL-13	NO	23.3	Nasal polyps	122	121	24	25	1012	200	72	10
13	F	67	Severe eosinophilic asthma	anti – IL-5R	NO	23	Nasal polyps; Gastroesophageal reflux; Hypercholesterolemia; Anaemia	76	80	70.67	9	-	2220	66	10
14	F	38	Non-severe asthma + polyps	anti – IL-4/IL-13	YES	22.5	Mild asthma + Aspirin sensitivity	-	-	-	-	-	910	82	10
15	F	49	Non-severe asthma + polyps	anti – IL-4/IL-13	YES	27.8	Mild asthma + Aspirin sensitivity	-	-	-	-	-	520	72	10
16	F	50	Non-severe asthma + polyps	anti – IL-4/IL-13	NO	26.6	Mild asthma + Aspirin sensitivity; Thyroid Disease	-	-	-	-	-	380	69	10
17	F	59	Non-severe asthma + polyps	anti – IL-4/IL-13	YES	22.7	Mild asthma + Aspirin sensitivity	-	-	-	-	-	350	66	10
18	F	37	Non-severe asthma + polyps	anti – IL-4/IL-13	NO	21	Mild asthma + Aspirin sensitivity; Thyroid Disease	-	-	-	-	-	300	53	10
19	M	60	Non-severe asthma + polyps	anti – IL-4/IL-13	NO	21	Mild asthma	-	-	-	-	-	810	23	3
20	M	34	Non-severe asthma + polyps	anti – IL-4/IL-13	NO	21.2	Mild asthma; Thyroid disease	-	-	-	-	-	680	4	0
21	M	50	Non-severe asthma + polyps	anti – IL-4/IL-13	NO	21.1	Mild asthma	-	-	-	-	-	410	76	10
22	F	75	Non-severe asthma + polyps	anti – IL-4/IL-13	NO	22.9	Mild asthma	-	-	-	-	-	780	1	0

Table 2 - Collected patient demographic - clinical characteristics and data of patients enrolled in the study

EV analysis

EVs were extracted by means of ultracentrifugation, from 22 serum samples from patients affected by different respiratory diseases at enrolment, as described above. EVs were then further characterized by NTA and TEM. An image representative of the EVs extracted and visualized by TEM is indicated in Figure 1. Size and shape visualized by TEM analysis confirmed that the isolated EVs are in the range of exosomes (Fig. 2). Each group of patients was analysed by TEM and the results were reproducible. NTA analysis reported concentration of particles and size parameters as illustrated in the following figures.

Summery curves obtained by NTA analysis of representative samples of the 2 main groups of pathologies analysed are shown in Figure 3.

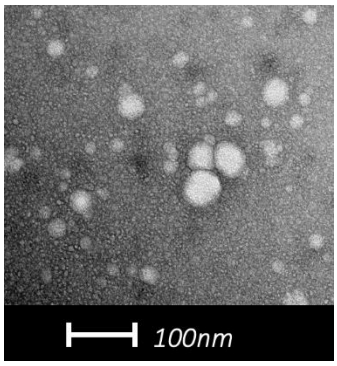

Figure 2 - Picture of EVs acquired by TEM. Size is indicated in white.

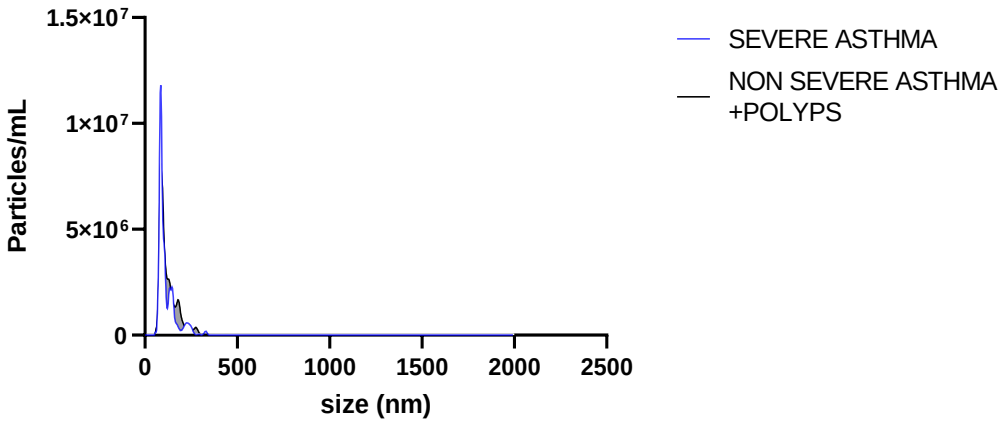

Figure 3 - NTA analysis of the 2 main groups of pathologies investigated. Concentration and size of particles are indicated.

EV differential concentrations for patient groups characterized by severe asthma (subdivided in just asthma or asthma + polyps) or non-severe asthma (all characterized also by polyps) are indicated in Figure 4, panels A-C. In general, we could observe that the lowest concentration of EV was found in the group of non-severe asthma + polyps, followed by severe asthma without polyps. Moreover, in the group of severe asthma, patients at the naïve status had the higher concentration of particles.

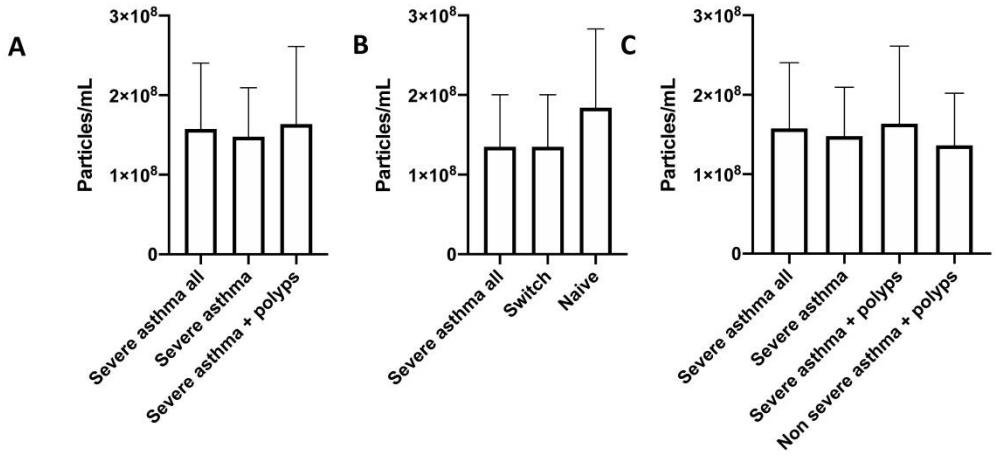

Figure 4. - EV concentration distribution obtained by NTA analysis: panel A represents severe asthma EV concentration, expressed by particles/mL, in the main group and in the 2 subgroups. Panel B illustrates EV concentration in patients with severe asthma subdivided according to the treatment status (naïve or switch). Panel C represents the main group of patients with severe or non-severe asthma + polyps.

The results of size distribution in EV extracted from patients' serum are illustrated in Figure 5. As a general observation, EVs from patients with polyps were in general larger, even if all in the range of exosomes (mean diameter <200 nm). We also analysed particles size by subdividing the patients according to their treatment status (at enrolment or the day of the change in the treatment-switch) and we observed an increased particle size in severe asthma patients undergoing a switch in the treatment in comparison with severe asthma naïve patients at enrolment in the project.

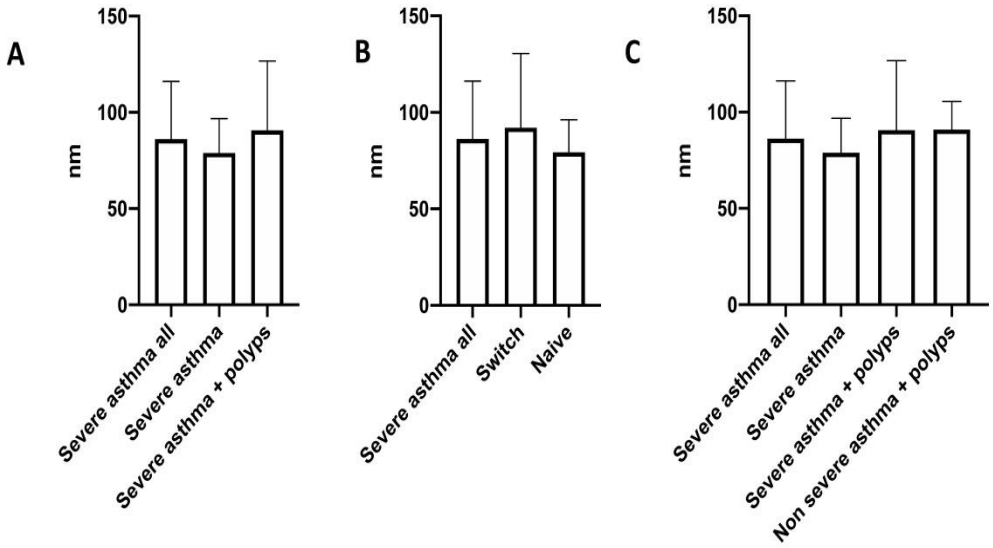

Figure 5 - EV size distribution obtained by NTA analysis: panel A represents severe asthma EV size, expressed by mean diameter of particles, in the main group and in the 2 subgroups. Panel B illustrates EV size in patients with severe asthma subdivided according to the status of naïve or switch. Panel C represents the main group of patients with severe or non-severe asthma + polyps.

Serum cytokine analysis

Several cytokines were measured in serum samples from the patients enrolled in this study: TGFβ1, IL-4, IL-5, IL-6, IL-10, IL-17A, IL-33, IL-13, eotaxin-3, and IL-25. Some were also measured in NLF samples, if available. In general, IL-17A, IL-4 and IL-33 were hardly detectable in all the samples investigated and, for most of the samples, NLF concentrations were lower than the corresponding values in serum sample. Moreover, for NLF, only IL-5, IL-6, eotaxin-3 and IL-25 were measurable. In the following graphs the mean values and standard deviation (SD) of the concentration measured are represented. The serum TGFβ1 trend in the different pathological groups investigated and in particular in patients with severe asthma, subdivided in the respective subgroups (naïve, switch, etc.) is illustrated in Figure 6.

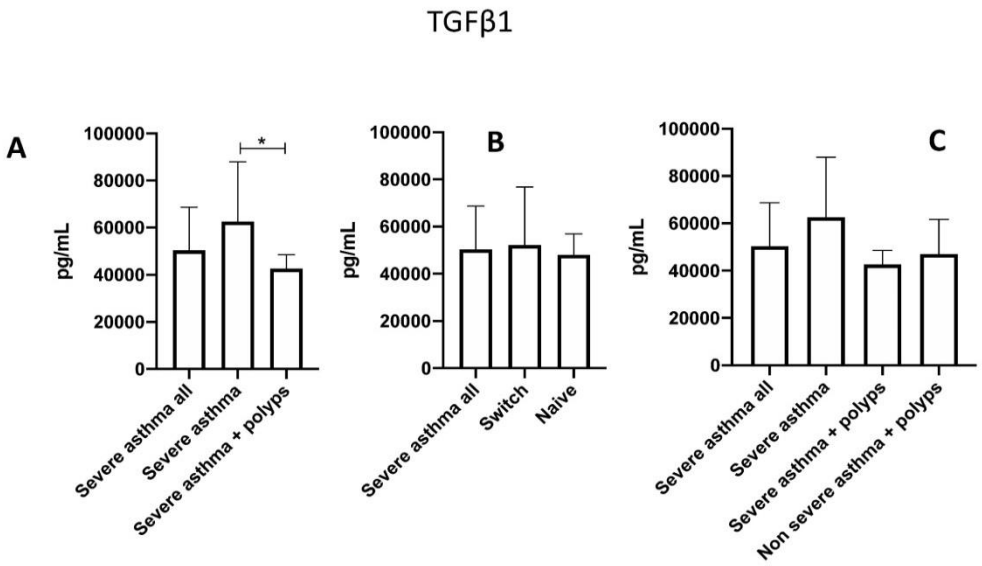

Figure 6 - TGFβ1 trend in serum samples of patients:

A) mean values of TGFβ1 in severe asthma;

B) patients with severe asthma subdivided into naïve and switch;

C) patients with severe asthma and non-severe asthma + polyps.

TGFβ1 concentrations were higher in severe asthma patients, and the difference between severe asthma and severe asthma + polyps was statistically significant.

In Figure 7, we show IL-5 concentrations in the different groups. The group of patients with severe asthma + polyps presented the highest concentration values. Moreover, patients undergoing a switch in therapy also had higher values than naïve patients. Differences were statistically significant only for samples illustrated in panel A but not in the other situations due to the high SD between samples.

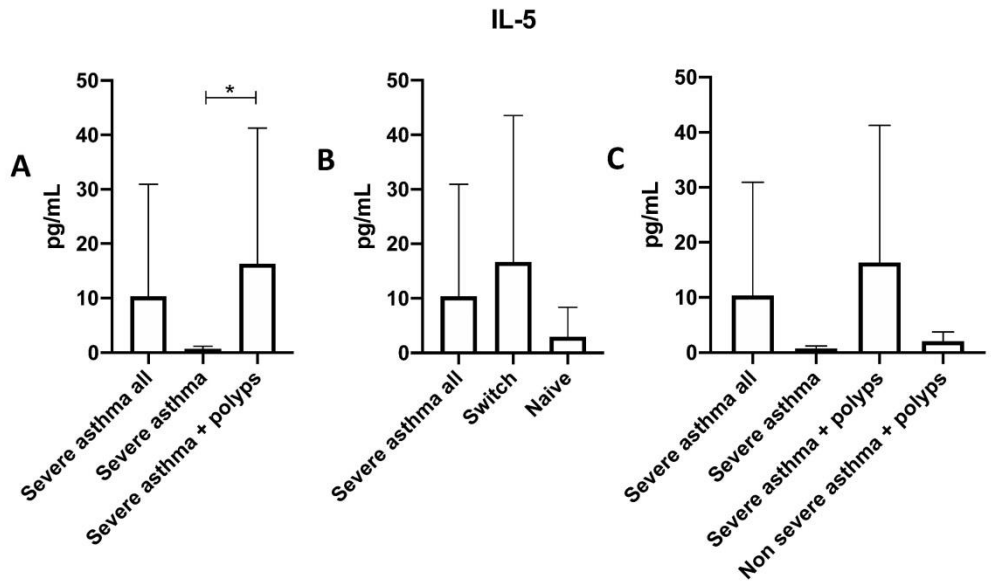

Figure 7 - IL-5 behaviour in serum samples of patients:

A) mean values of IL-5 in the three experimental groups investigated;

B) patients with severe asthma subdivided into naïve and switch;

C) patients with severe asthma and non-severe asthma + polyps.

The IL-6 trend in serum samples from patients is illustrated in Figure 8. Differences were not always statistically significant due to the high SD between samples, but the group characterized by severe asthma + polyps presented the lowest values, with $p<0.05$. Patients at the naïve status had values higher than subjects switching to another treatment.

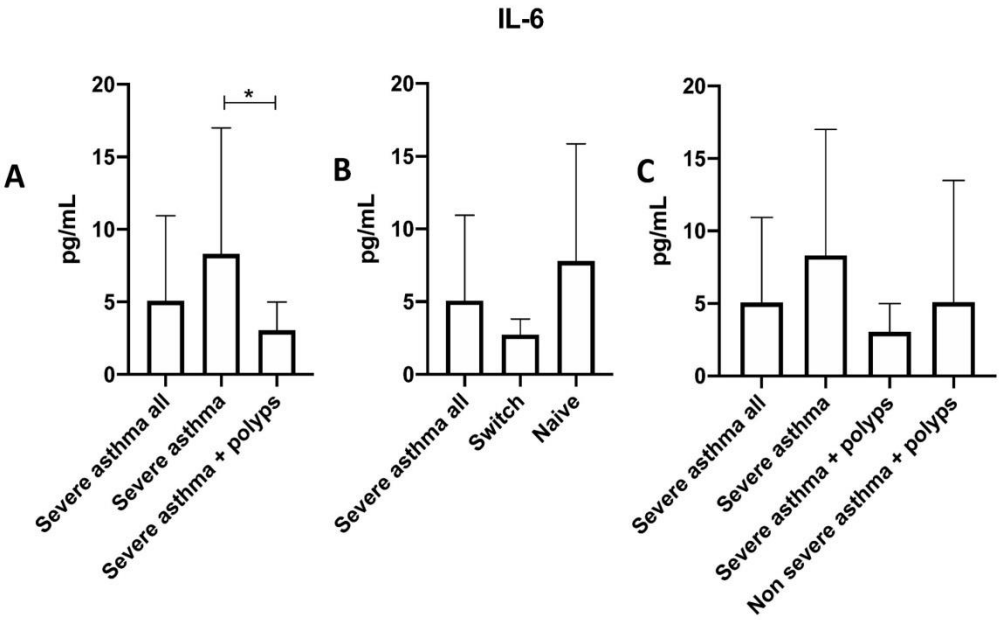

Figure 8 - IL-6 trend in serum samples of patients:

A) mean values of IL-6 in the three groups of severe asthma;

B) patients with severe asthma subdivided into naïve and switch;

C) patients with severe asthma and non-severe asthma + polyps.

IL-10 was also analysed and the results are shown in Figure 9. The only significant difference was found in patients with severe asthma between naïve and switch status. Patients switching to another treatment presented lower concentrations of IL-10.

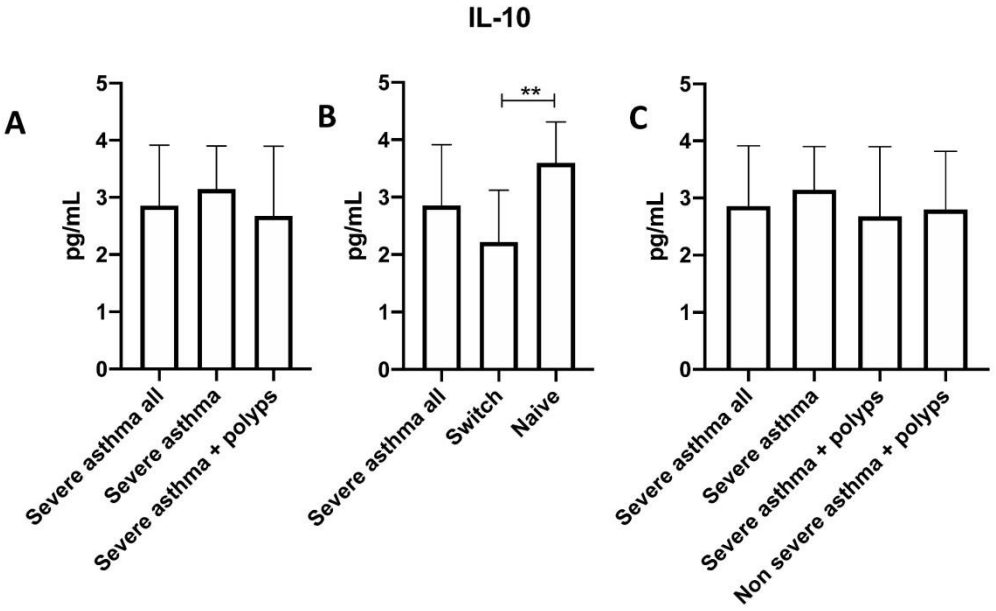

Figure 9 - IL-10 trend in serum samples of patients:

A) mean values of IL-10 in the three groups of severe asthma;

B) patients with severe asthma subdivided into naïve and switch;

C) patients with severe asthma and non-severe asthma + polyps.

IL-13 showed a specific trend, as illustrated in Figure 10. Patients affected by non-severe asthma + polyps showed the highest concentration while patients affected by severe asthma without polyps showed the lowest, even if the differences were not statistically significant.

Eotaxin-3 and IL-25 were analysed in a smaller group of patients and analyses are still in progress. The results obtained so far are shown in Figures 11-12. For eotaxin 3 the highest values were measured in serum of patients with non-severe asthma + polyps. Moreover, significant differences were observed in the group of patients with severe asthma between patients with or without polyps as illustrated in Figure 11.

IL-25 showed no statistically significant differences among groups, but, as a general observation we measured lower values for patients with non-severe asthma + polyps.

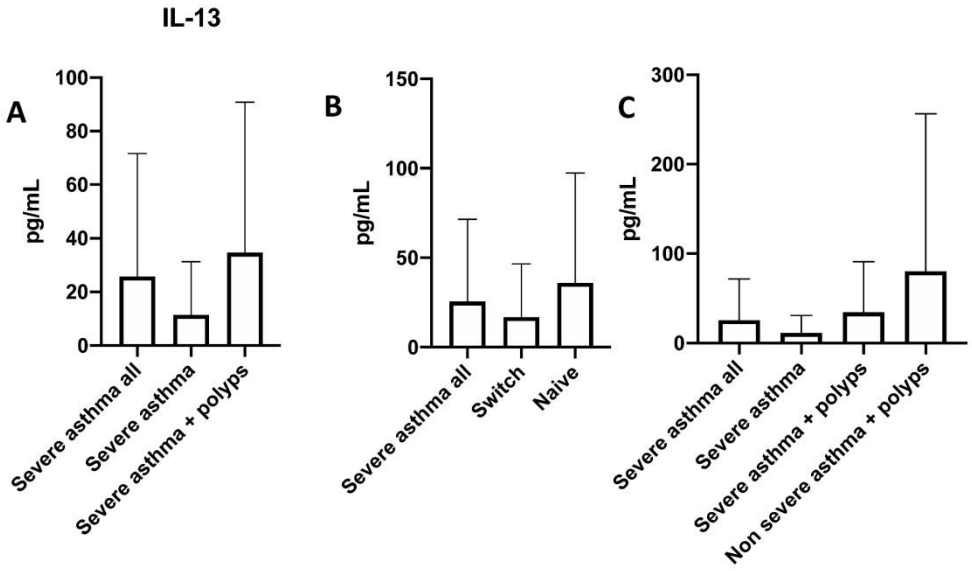

Figure 10. IL-13 trend in serum samples of patients:

A) mean values of IL-13 in the three groups of severe asthma;

B) patients with severe asthma subdivided into naïve and switch;

C) patients with severe asthma and non-severe asthma + polyps.

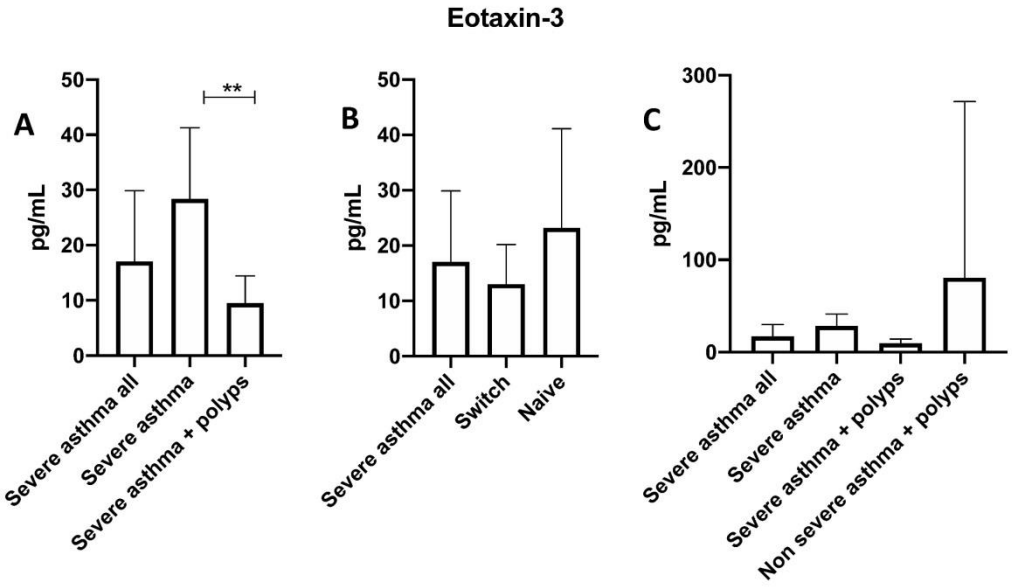

Figure 11. Eotaxin-3 trend in serum samples of patients:

A) mean values of eotaxin-3 in the three groups of severe asthma;

B) patients with severe asthma subdivided into naïve and switch;

C) patients with severe asthma and non-severe asthma + polyps.

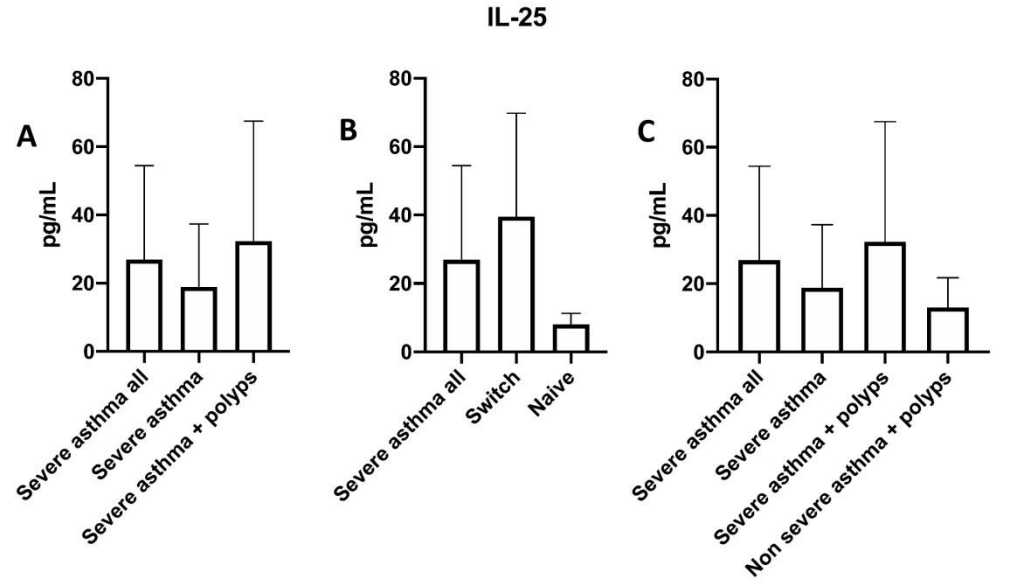

Figure 12. IL-25 trend in serum samples of patients:

A) mean values of IL-25 in the three groups of severe asthma;

B) patients with severe asthma subdivided into naïve and switch;

C) patients with severe asthma and non-severe asthma + polyps.

Moreover, for some of the same patients, we also measured some cytokines in NLF samples. We tested IL-5, IL-6, IL-10, eotaxin-3 and IL-25 and the results are illustrated in Figure 13. Generally, NLF concentrations were lower than the corresponding serum levels but, in some cases, NLF concentration of some cytokines were higher than serum. Despite observing great individual variability of these measurements, some comparisons reached statistical significance (IL-6, Eotaxin-3, IL-25).

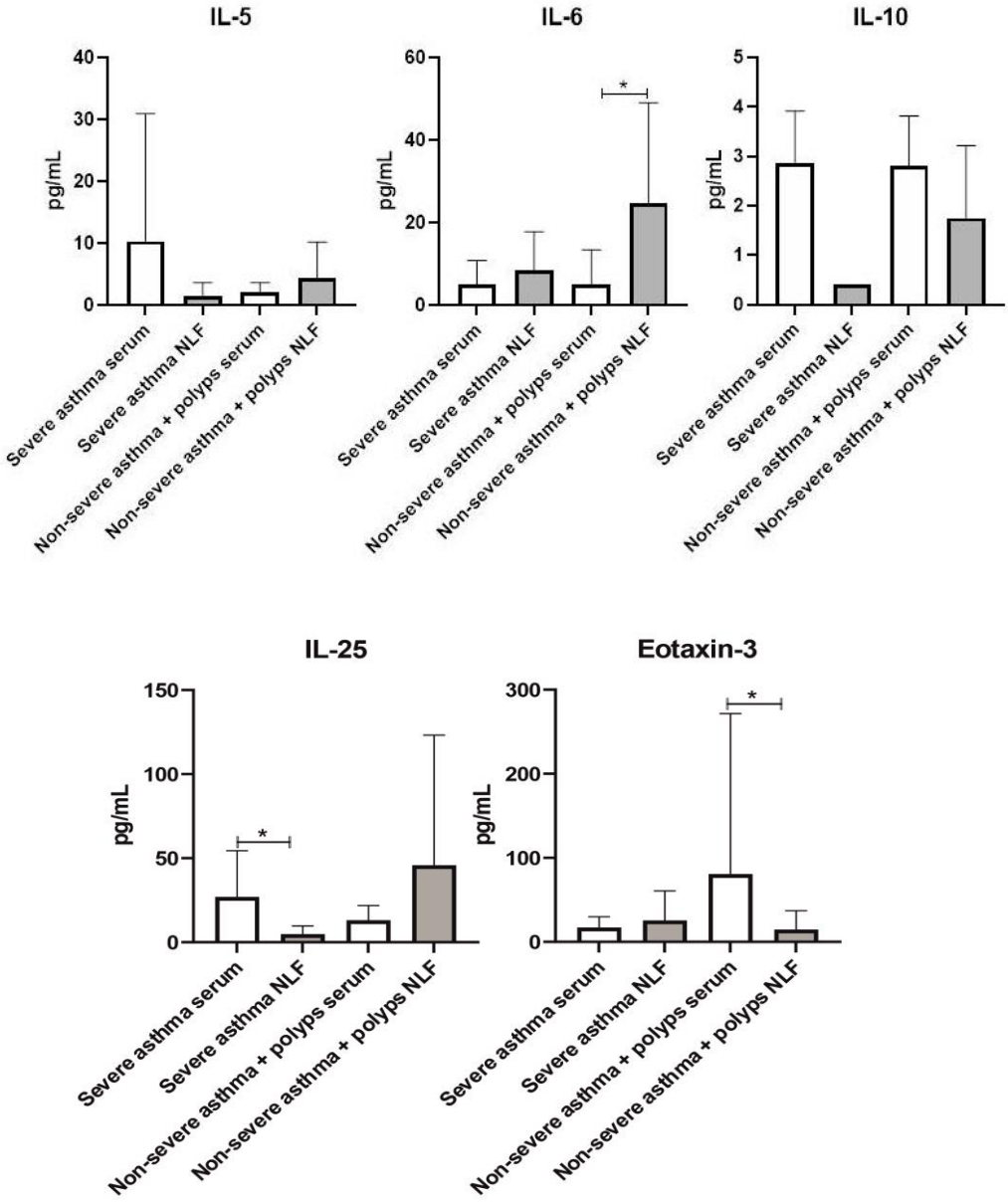

Figure 13. IL-5, IL-6, IL-10, IL-25 and Eotaxin-3 concentrations in serum and NLF samples from patients with severe asthma and with non-severe asthma + polyps.

EV surface antigen analysis

Profiling of EV surface antigens was performed by flow cytometry after ultracentrifugation isolation from serum, according to manufacturer's instructions (MACS PLEX kit). EVs were analysed for the expression of 37 specific surface antigens. Expression levels of each EV surface antigen were normalized by mean fluorescence intensity (MFI) of CD9-CD63-CD81 measured in the respective sample. Each experimental group showed expression of surface antigen on EVs. In Figures 14-15, the results measured for EV extracted from patients subdivided according to severe or non-severe asthma and also status of treatment (naïve or switch) are illustrated.

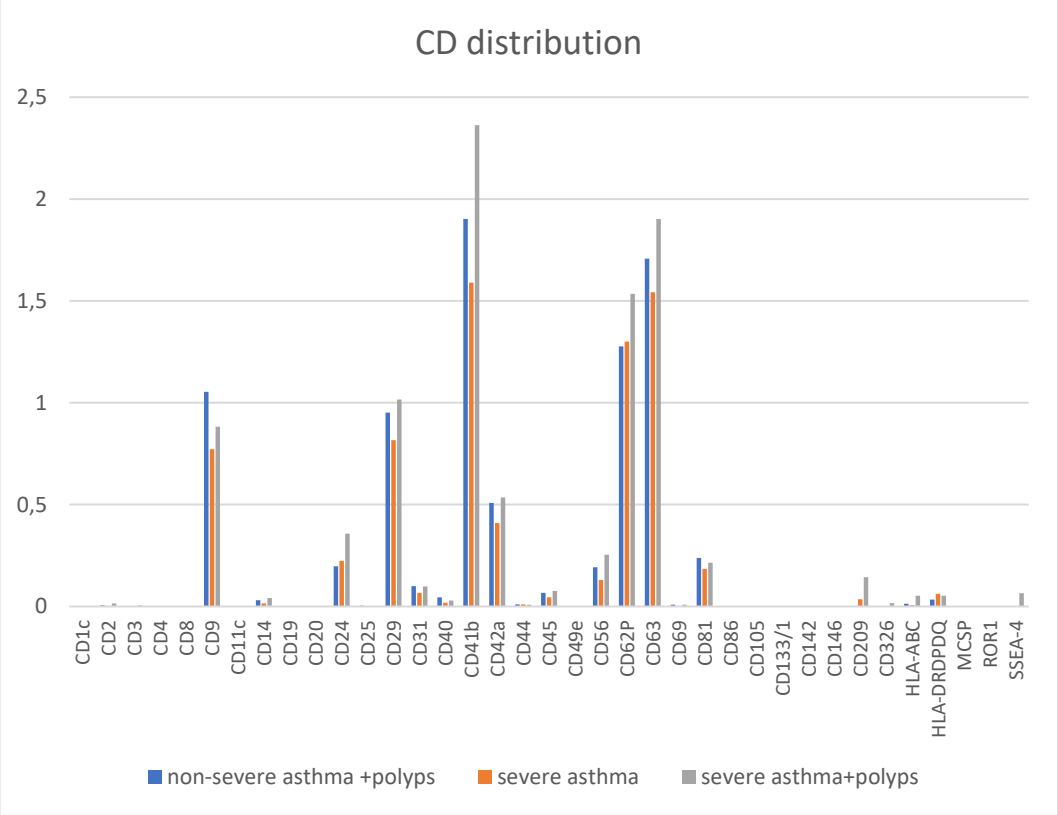

Figure 14 - Surface antigen distribution in patients affected by only severe asthma, severe asthma + polyps, non-severe asthma + polyps.

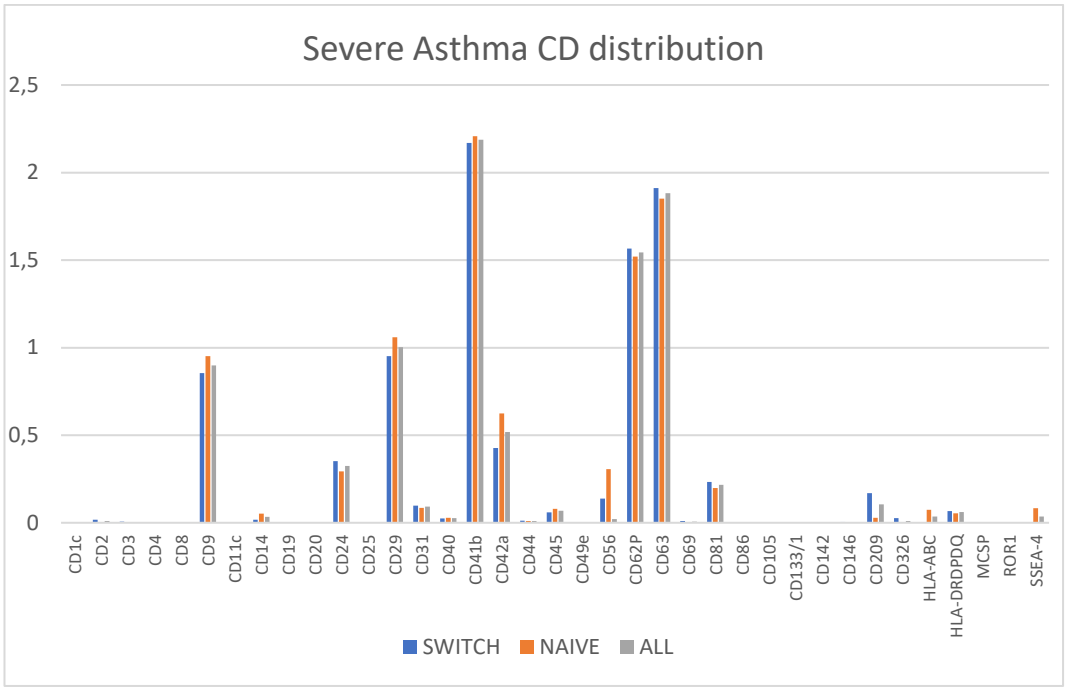

Figure 15 - Surface antigen distribution in patients affected by severe asthma, subdivided into switch and naive.

Some surface antigen markers were found to be regulated between the three experimental groups investigated. In particular, the following molecules were found to be regulated: CD29, CD41b, CD42a, CD62P, CD209.

We also traced the origin of circulating EVs by grouping surface antigens according to their cellular source. Markers considered for each cell populations are reported in the table below (Table 3).

T cells	CD2, CD3, CD4, CD8, CD25, CD40, CD45, CD86
B cells	CD11c, CD19, CD20, CD25, CD24, CD40, CD44, CD45
APC	CD1c, CD 11c, CD209, HLA-II
Macrophages	CD11c, CD209
Monocytes	CD11c, CD14, CD29, CD49e
NK cells	CD2, CD69
Progenitors/stem cells	CD105, CD133/1, SSEA-4
Endothelium	CD31, CD62P, CD105, CD146
Platelets	CD41b, CD42a, CD62P
Eosinophils	CD44, CD63, CD69, CD81, CD86
Other	CD326, ROR1, MCSP

Table 3. Markers considered for each cell population

Different cell populations were found to originate EVs for the different experimental groups investigated, as illustrated in Figures 16 and 17. As a general observation, our results show that platelets were the major contributors, followed by eosinophils, then endothelial cells.

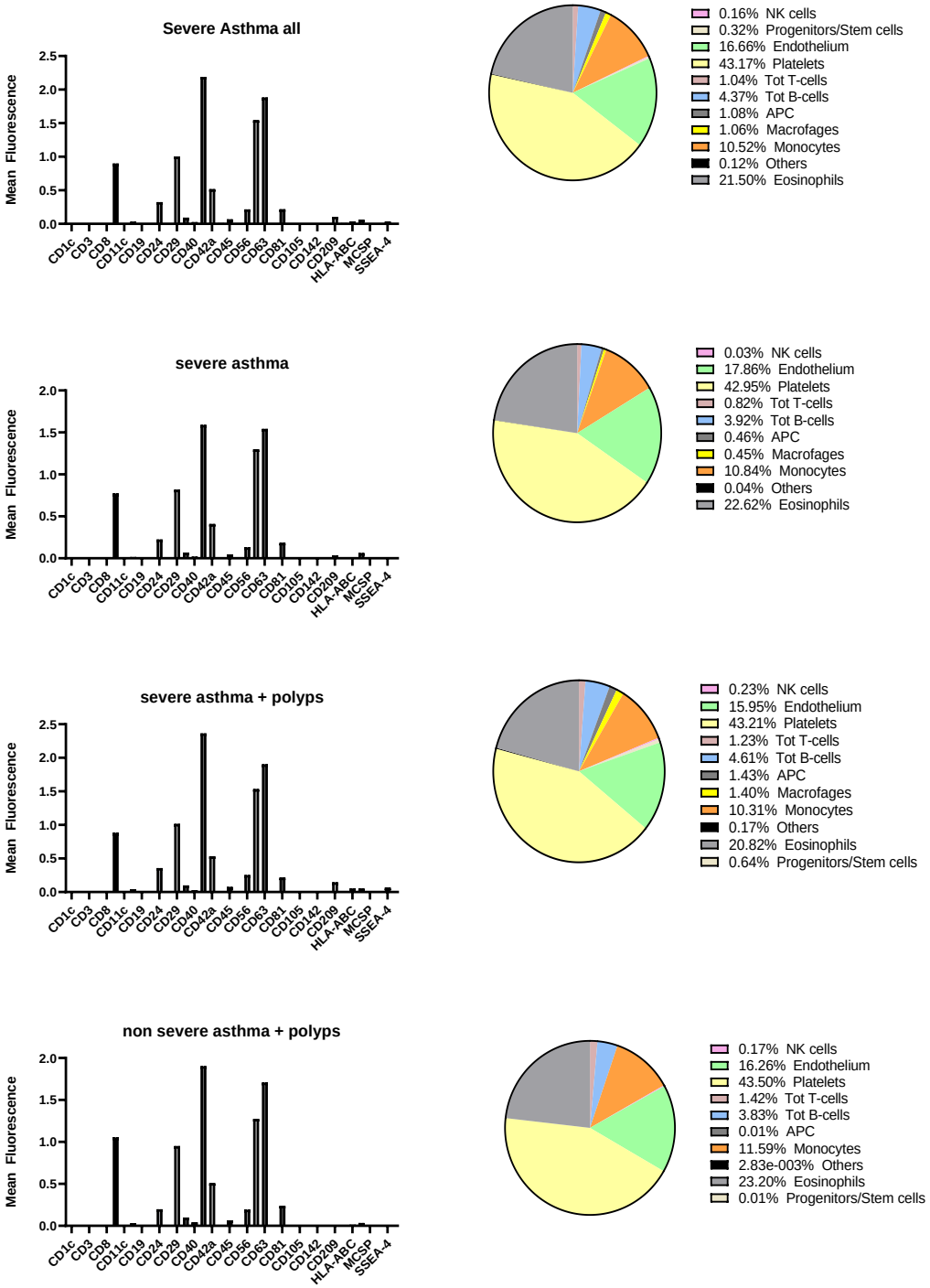

Figure 16 - Surface antigen distributions and cell origin in the group of asthma patients. The four main groups investigated are illustrated for comparison.

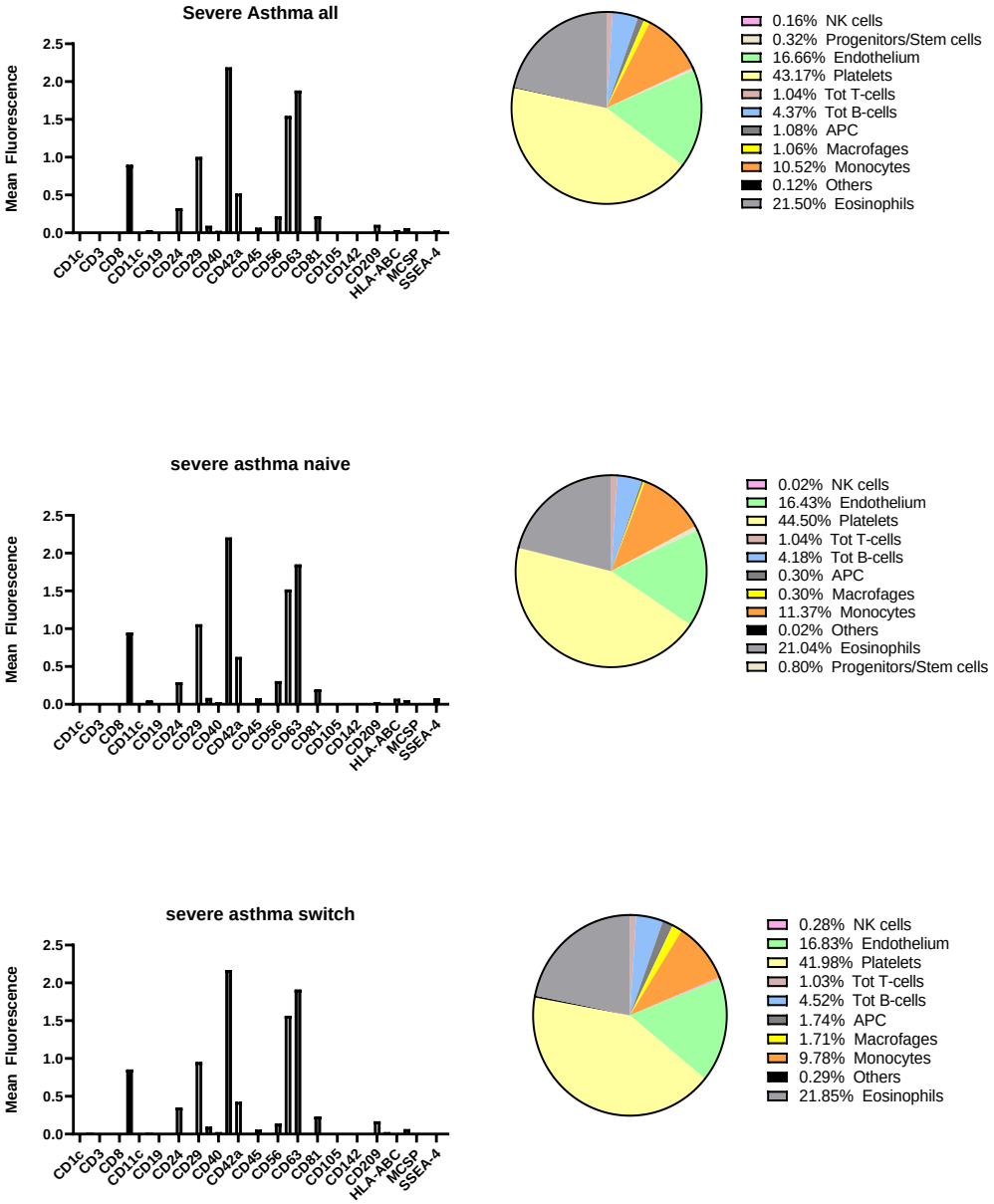

Figure 17 - Surface antigen distributions and cell origin in the group of severe asthma subdivided into switch and naive.

DISCUSSION

In this thesis project, we performed a molecular characterization of patients affected by asthma by analysing not only serum and NLF cytokines profiles but also by investigating some features of serum EVs.

The role of EVs and their cargo as functional biomarkers or as potential mediators in the development of severe asthma is a rapidly expanding area of research. Analysing the molecular and inflammatory profile of patients with severe asthma, we observed that 8 out of 13 patients were affected by CRSwNP, and thus we decided to investigate what happens from a molecular and inflammatory point of view in patients who present this phenotype in the absence of severe asthma. Indeed, as acknowledged in literature, CRSwNP has been reported as a frequent feature of patients with severe asthma: about 42% of severe asthmatic patients were affected also by CRSwNP, with poor asthma control, a high use of OCS and a defective QoL. As reported in the reslizumab trial by Castro et al., the presence of CRSwNP can be considered as a factor that influences the clinical outcomes of some biological therapies in patients affected by severe asthma [102]. Therefore, as reported above, in this project, clinical patterns of severe asthma were explored and further supported by the evaluation of CRSwNP, identifying different clinical patterns: the group of severe asthma, divided into two subgroups of patients affected only by severe asthma and patients affected by severe asthma + polyps.

Regarding analysis of the cytokine profile we performed in the enrolled patients, it is important to highlight that while T2 inflammation is related to the production of so called “T2 cytokines”, it should be noted that these cytokines are often an expression of different clinical manifestations, and therefore an individual, and not common, approach turns out to be fundamental for clinical pathological realities where T2 driven pathways are involved [18].

As is known, at the basis of asthma airway inflammation there is a network of mutually interacting cytokines and growth factors, secreted by inflammatory cells, structural tissue components, including epithelial cells, fibroblasts and smooth muscle cells. It is not simple to classify all the cytokines involved in asthma, both

due to their pleiotropic nature and to their overlapping properties. However, they can be subdivided into groups: lymphokines, such as IL-2, IL-3, IL-4, IL-5, IL-13, IL-15, IL-16, IL-17; pro-inflammatory cytokines, such as IL-1, TNF, IL-6, IL-11, GM-CSF, SCF; anti-inflammatory cytokines, such as IL-10, IL1ra, IFN-α; chemotactic cytokines, known as chemokines, as regulated upon activation, normal T cell expressed and secreted (RANTES), monocyte chemoattractant protein-1 (MCP-1), MCP-2, MCP-3,MCP-4, MCP-5, macrophage inflammatory protein-1 α (MIP-1α), eotaxin, IL-8; growth factors, such as platelet-derived growth factor (PDGF), TGF- α, fibroblast growth factor (FGF), and epidermal growth factor (EGF). [179,180].

Our data on cytokine analysis were very interesting and showed some agreement with literature.

Regarding TGFβ1, we observed concentration levels, albeit not significantly different among the analysed experimental groups, higher in the severe asthma group, compared to the other two groups, that is, severe asthma + polyps and non-severe asthma + polyps. Aubert J-D. et al. reported that the expression of TGFβ1 is similar in lungs from normal and asthmatic subjects [181]. Brown SD. et al. reported that TGFβ1 plays a role in asthma remodelling and its expression may be related to an excessive burden of ROS and oxidant stress. In particular, the authors reported that severe asthmatic children have higher total airway concentrations of TGFβ1 that are related to increased protein and mRNA expression of TGFβ1 in airway macrophages [182].

Therefore, our finding of higher concentrations of TGFβ1 in severe asthma correlates with what is reported in literature. We could thus hypothesize that the presence of nasal polyps does not affect the molecular profile in relation to concentration of TGFβ1 as they are all high across the groups.

Regarding IL-5, we observed higher concentrations in the severe asthma + polyps group than in the other two groups. As already known, the concept of the “united airways diseases” that highlights how, in the context of T2-high inflammation, eosinophils and IL-5 promote and sustain upper and lower airways diseases, allowed us to better understand our data regarding IL-5 [58].

About IL-6, we observed higher serum concentrations in the severe asthma group, particularly in naive patients, compared to patients with severe asthma + polyps. On the other hand, in the NLF we saw a concentration of IL-6 higher than the serum concentrations always found in patients with non-severe asthma + polyps. Yang Y. et al. reported that IL-6 gene expression and serum IL-6 levels may be influenced by ethnic diversity, geographical differences and distinct dietary habits [183]. In agreement with these observations, in our results we could confirm a high variability between individuals, especially regarding the NLF/serum comparison. Moreover, it is important to highlight that literature reports an increased release of IL-6 from alveolar macrophages from asthmatic patients after allergen challenge and increased basal release compared with non-asthmatic subjects [184]. An increase of IL-6 levels was measured in nasal washings of children following a rhinovirus infection [185].

Regarding IL-10, our data showed higher serum levels of this cytokine in the severe asthma group than in the severe asthma + polyps group and also in the non-severe asthma + polyps group. Nevertheless, the most significant difference was found in severe asthma, with higher IL-10 values in the serum of naive patients than in switch patients. In accordance with this view, Robinson DS. et al. indicated increased numbers of macrophages and T cells expressing IL-10 mRNA in the BAL fluid of asthma patients [186].

Huang K. et al., exploring the cytokine patterns of patients with uncontrolled asthma with or without chronic rhinosinusitis, did not report significant differences in terms of IL-10 and IL-25 among three clusters: non-eosinophilic asthma without CRS (cluster 1), asthma with mild airflow limitation and chronic rhinosinusitis (cluster 2) and eosinophilic asthma with severe airflow limitation and chronic rhinosinusitis (cluster 3) [187]. Similarly, in our project, to date, we have not found any significant differences in the serum levels of IL-25 among the experimental groups investigated. However, in the severe asthma group, we found higher values in serum than in NLF.

On the other hand, with regard to IL-13, we did not observe significant differences between the various groups; we can just report a trend consistent with a higher serum level in non-severe asthma + polyps group compared to the severe

asthma group and the severe asthma + polyps group. Therefore, for IL-13 and severe asthma we can say that, currently, it may not represent a significant inflammatory marker. Also in the study conducted by Huang K. et al., the authors reported increased serum IL-13 values in cluster 3, patients with eosinophilic asthma with severe airflow limitation and chronic rhinosinusitis (46.5% with nasal polyps), compared to the other two clusters [187].

In relation to eotaxin-3, we found higher serum values in the severe asthma group than in the severe asthma + polyps group. By comparing the levels of eotaxin-3 in serum and in NLF we observed that in patients with non-severe asthma + polyps the highest values were found in serum compared to NLF. Coleman JM. reported that eotaxin-2 and eotaxin-3 are higher in patients with asthma and severe asthma, in association with poor asthma control and sputum eosinophilia [188].

Moreover, we performed a systematic characterization of circulating EVs in enrolled patients and tracked their cellular origin through a standardized flow cytometric assay, which allowed the simultaneous evaluation of 37 antigens expressed on EV surfaces. Through analysis of the various surface CDs presented by the exosomes obtained in our samples, we found that the main origin cells of exosomes in the groups enrolled, all characterized by high T2 inflammation, are represented by platelets, eosinophils and endothelial cells. In accordance with what is present in literature, exosomes have a heterogeneous molecular composition and their cargo composition is variable in relation to cellular origin and physiological state. In particular, some proteins are common to all exosome types such as CD63 and CD9, often used as markers of exosomes in general, defining also peculiar populations of vesicles [135]; other proteins are often used, as in our project, as exosome markers and to identify exosome cell origin. Eosinophils, one of the key players in asthmatic pathophysiology, elevated in the airways of asthma patients, are confirmed in our work as among the cells that most produce exosomes in patients suffering from asthma. Canas JA. et al. reported that eosinophil exosomes are autonomous molecules with the capacity to modulate and enhance the pathophysiology of asthma acting both on eosinophils and on structural lung cells [152]. In support of this view, Mazzeo C. et al. have clearly shown how eosinophils have the capacity to release

exosomes, also reporting that exosome secretion is higher in eosinophils from asthmatics [150]. In our project, however, we saw no difference in concentration of EVs between the different groups investigated. Akuthota P., using TEM, nanoscale flow cytometry and protein electrophoresis, also confirmed the presence of CD63 and CD9 in exosomes released by eosinophils [189]. Alhamwe et al. reported that platelets, which play a key role in the pathophysiology of asthma, can exert effects through EVs [190]. Few data are available in literature regarding exosomes of platelet origin in patients with asthma. To support this feedback, in literature, it has been reported that plasma EVs, many of them of platelet origin isolated from asthma patients, are able to induce a reduction of the endothelium-dependent relaxation in response to bradykinin and an increase in the acetylcholine-induced contraction of the trachea muscle, highlighting a potential role in airway smooth muscle dysfunction characteristic of asthma [191,192]. Moreover, Duarte D. et al. reported that levels of circulating platelet microparticles (PMPs) are increased in asthma patients [193]. Furthermore, the third cell type most frequently involved in the production of exosomes in our study were endothelial cells, which are included in the structural cells of the airways. In relation to type 2-high inflammation of the airways and in particular to asthma, the structural cells of the airways most commonly described in literature as producing exosomes are bronchial fibroblasts and lung epithelial cells [194].

Considering that clinically severe asthma is a highly heterogenous disorder with many differences in clinical features and symptoms, and factors triggering exacerbations, it can be understood how a single phenotype is associated with a variety of distinct molecular characteristics and pathomechanisms known as endotypes. Therefore, "individual variability" is the key to a clinical diagnostic approach aimed at precision medicine in chronic inflammatory diseases such as severe asthma.

However, these results will be expanded by increasing the sample size, and evaluated and compared with follow up data at 3, 6 and 12 months after the start of biological therapy, in order to be able to identify molecular and inflammatory differences in the various groups considered, getting more information and

insights on the role of exosomes in asthma disease. Moreover, analyses carried out to date during biological therapy in the enrolled patients, might allow us also to identify possible predictive biomarkers of response to biologic therapy.

PERSPECTIVES

Circulating EVs can be considered as active biovectors in patients affected by severe asthma, but the mechanisms by which they are involved in the development/pathogenesis of asthma inflammation are not completely understood. Further studies are necessary to investigate the direct involvement of the release of EVs by endothelium, inflammatory cells, and platelets, and the molecular mechanism which, in turn, may lead to high T2 inflammation, helping target treatment toward precision medicine. Indeed, the identification of molecular biomarkers of asthma through omics approaches may reveal the potential for multi-omics defined endotypes to decipher therapy approach and eligibility for targeted biological therapies.

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