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Differences and similarities in inflammatory mechanisms between asthma and chronic obstructive pulmonary disease (COPD)

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ABSTRACT

Asthma and chronic obstructive pulmonary disease (COPD) are the two most common respiratory chronic diseases worldwide, which are increasing in prevalence and have an enormous impact on the lives of patients and their caregivers. Both diseases are characterized by chronic inflammation of the lung, but the nature of the inflammatory response differs between diseases and shows a high degree of heterogeneity within each disease, accounting for a large number of endotypes and phenotypes. During my PhD various lines of research have been explored either by the research group in Padova or in partnerships with both national and international colleagues. We performed two clinical "real life" studies on two large cohorts of adult asthmatics and patients with COPD followed at our Centre, observing the existence of multiple phenotypes and inflammatory endotypes of these two diseases. In adult asthmatics, we differentiated patients with early onset asthma (EOA) from late onset asthma (LOA). In EOA patients, we observed a crucial role of allergic rhinitis and eosinophilic inflammation on disease control and pulmonary function. Conversely, in patients with LOA, rhinitis was not linked to poor asthma control while obesity, associated with a neutrophilic profile, had a major impact. In the cohort of COPD patients we observed that blood lymphopenia was related to a more severe disease with a predominant emphysematous component, while high levels of neutrophils and monocytes were related to chronic bronchitis and bronchiectasis.

My main research field was the investigation of the immunopathology of asthma and its etiology at the beginning of the natural history of the disease. We performed a series of studies on a cohort of wheezing children who underwent bronchoscopy for clinical purposes in whom bronchial biopsies were available for research. We first observed that airway remodeling and inflammatory hallmarks of adult asthma are also present in preschool wheezing and that phenotypes and endotypes of asthma are already recognizable. In particular, we observed that basement membrane thickening, a putative sign of epithelial-mesenchymal activation, is a very early sign that may precede inflammation and predict asthma development. Furthermore, we highlighted the presence of the eosinophilic non-atopic (T2H-nA) endotype where type 2 inflammation is present in absence of atopy. In these patients we described the presence of innate lymphoid cells type 2 (ILC2) as major drivers of type 2 inflammation. Of note, ILC2 infiltration was associated with persistence of asthma in a longitudinal follow-up, while we observed a shift toward the development of atopy with growth. In subsequent analyses we investigated the effects of air pollution and viral infections on the pathological hallmarks of asthma. We observed a direct relationship linking type 2 inflammation to both outdoor air pollution exposure and antiviral innate immune response impairment hypothesizing a possible existence in asthma of a pathogenetic intersection between atopy, air pollution and viral infections. Finally, we also described in a cohort of COPD patients that underwent lung surgery a deranged interferon-response mediated by NK, whose impairment was related to increased disease severity. These observations complement those of a different study, in which we studied the involvement of innate lymphoid cells type 1 (ILC1). Finally, addressing the interaction between COPD and viral infections we investigated the risk factors for the development and severity of COVID-19 in patients with COPD. We reported that cardio-metabolic conditions were the main risk factors for development of COVID-19 in COPD patients. Emphysema and low DLCO, were related to a more severe disease and need for intensive care, remarking a possible relationship between COPD severity and impaired antiviral responses.

ABSTRACT (ITA)

L'asma e la broncopneumopatia cronica ostruttiva (BPCO) sono le due malattie respiratorie croniche più frequenti, la cui prevalenza è in aumento e che hanno un enorme impatto sulla vita dei pazienti e dei caregiver. Entrambe le malattie sono caratterizzate da un'infiammazione cronica del polmone, ma la natura della risposta infiammatoria varia da una malattia all'altra e presenta un elevato grado di eterogeneità all'interno della stessa malattia per la presenza di diversi endotipi e fenotipi. Durante il mio dottorato di ricerca ho portato avanti diverse linee di ricerca sia con il gruppo di ricerca di Padova sia con altri gruppi di ricerca internazionali e nazionali. Abbiamo condotto due studi clinici "real life" su due ampie coorti di asmatici adulti e pazienti con BPCO seguiti presso il nostro Centro. In questi studi abbiamo osservato l'esistenza di molteplici fenotipi ed endotipi infiammatori di queste due malattie. Negli asmatici adulti abbiamo differenziato i pazienti con asma a esordio precoce (EOA) da quelli con asma a esordio tardivo (LOA). Nei pazienti con EOA, abbiamo osservato un ruolo cruciale della rinite allergica e dell'infiammazione eosinofila sul controllo della malattia e sulla funzione respiratoria. Al contrario, nei pazienti con LOA, la rinite non era associata ad un peggior controllo dell'asma ma era l'obesità, spesso associata a un profilo infiammatorio neutrofililico, ad avere un impatto maggiore. Nella coorte con BPCO abbiamo identificato la linfopenia periferica come fattore che correlava con la gravità della malattia nei pazienti con predominante componente enfisematosa, mentre valori elevati di neutrofili e monociti erano associati a bronchite cronica e bronchiectasie.

Il mio principale campo di ricerca è stato lo studio dell'immunopatologia dell'asma e dei fattori eziologici all'inizio della storia naturale della malattia. Abbiamo condotto quindi una serie di studi su una coorte di bambini con wheezing sottoposti a broncoscopia per scopi clinici nei quali sono state analizzate delle biopsie bronchiali a scopo di ricerca. Abbiamo innanzitutto osservato che rimodellamento ed infiammazione delle vie aeree tipici dell'asma nell'adulto sono presenti anche nel wheezing prescolare dove sono già riconoscibili differenti fenotipi ed endotipi di malattia. In particolare, abbiamo osservato che l'ispessimento della membrana basale, riconducibile all'attivazione dell'unità epitelio-mesenchimale, è un segnale molto precoce che può precedere l'infiammazione stessa e che può predire lo sviluppo dell'asma durante lo sviluppo. Inoltre, abbiamo evidenziato la presenza dell'endotipo eosinofilico non atopico (T2H-nA), in cui l'infiammazione di tipo 2 è presente in assenza di atopia. In questi pazienti abbiamo rilevato l'aumento selettivo delle cellule linfoidi innate di tipo 2 (ILC2), che rappresentano le principali cellule all'origine dell'infiammazione di tipo 2. Abbiamo poi associato l'infiltrazione di ILC2s alla persistenza dell'asma in un follow-up longitudinale, con l'osservazione molto interessante che c'era un'evoluzione di questi soggetti verso lo sviluppo di atopia nel tempo. Negli studi successivi abbiamo analizzato gli effetti di altri fattori predisponenti all'asma, come l'inquinamento atmosferico e le infezioni virali, sulle caratteristiche patologiche di questa coorte. Abbiamo osservato una relazione diretta tra infiammazione di tipo 2, esposizione all'inquinamento atmosferico e la compromissione della risposta immunitaria innata antivirale, ipotizzando la possibile esistenza nell'asma di un'intersezione patogenetica tra atopia, esposizione ad inquinanti atmosferici ed infezioni virali.

Inoltre, in uno studio condotto su pazienti affetti da BPCO sottoposti a chirurgia polmonare, abbiamo descritto un'alterata risposta antivirale con alterazione del meccanismo degli interferoni, la cui compromissione è stata correlata a una maggiore gravità della malattia ed è mediata dalle cellule NK; queste osservazioni sono complementari ad un altro studio in cui abbiamo studiato il coinvolgimento delle cellule linfoidi innate di tipo 1 (ILC 1) nella BPCO.

Infine, affrontando l'interazione tra BPCO e infezioni virali, abbiamo studiato i fattori di rischio per lo sviluppo e la gravità della COVID-19 nei pazienti con BPCO. Abbiamo rilevato che la presenza di concomitanti condizioni cardio-metaboliche era il principale fattore di rischio per lo sviluppo di COVID-19 nei pazienti con BPCO. L'enfisema e la bassa DLCO sono risultati correlati a una malattia più grave e alla necessità di cure intensive, evidenziando una possibile relazione tra la gravità della BPCO e l'alterazione della risposta antivirale.

INTRODUCTION

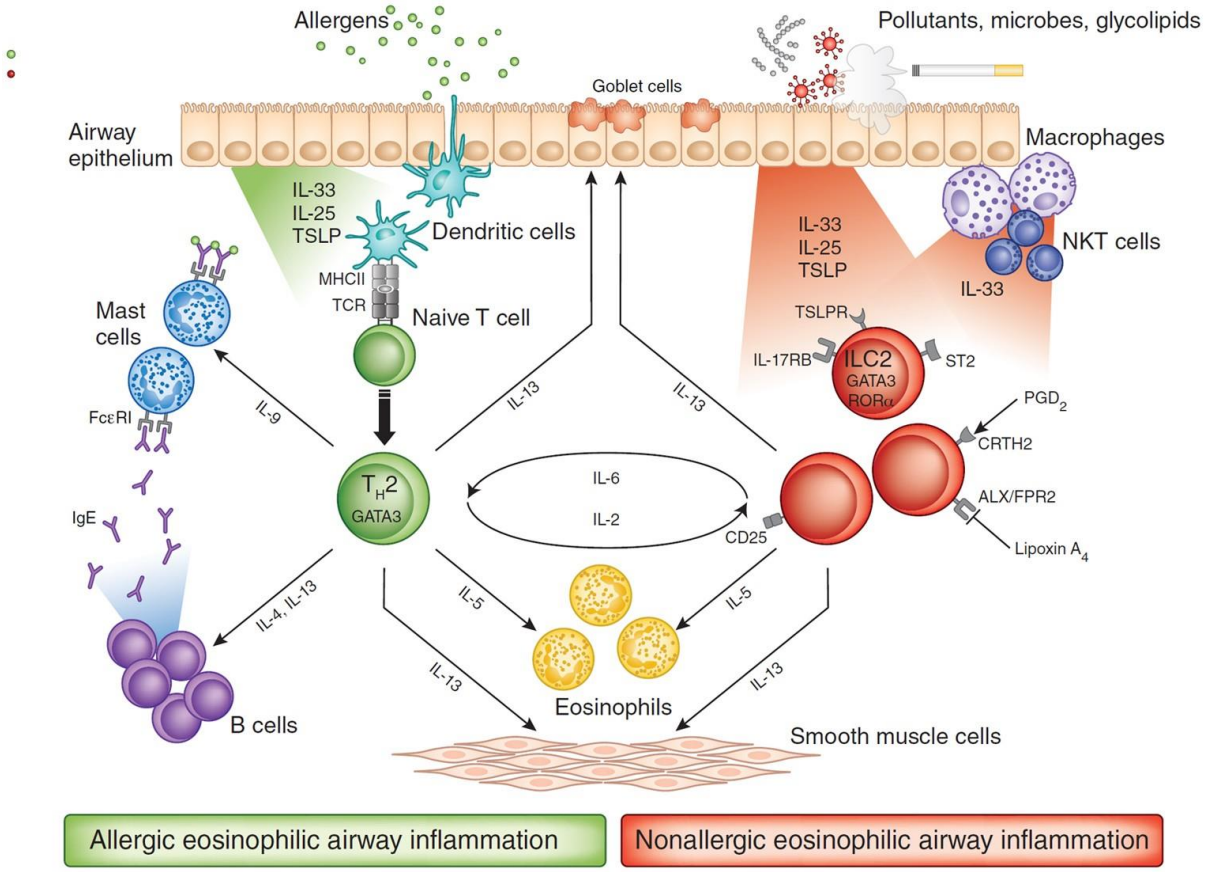
Asthma and chronic obstructive pulmonary disease (COPD) are the two most common respiratory chronic diseases worldwide, both are increasing and have an enormous impact on the lives of patients and their caregivers. They both cause airflow obstruction, which is variable in asthma and fixed in COPD and they have similar symptoms that include shortness of breath, cough, and sputum, and these similarities can often cause difficulties in making the correct diagnosis especially in older age patients (1). Both diseases are characterized by chronic inflammation of the lung, but the nature of the inflammatory response differs between diseases and shows a high degree of heterogeneity within each disease, accounting for a large number of endotypes (2).
It is important to distinguish between asthma and COPD to secure appropriate treatment: indeed asthma inflammation is usually responsive to inhaled corticosteroids (ICS); conversely, most cases of COPD are corticosteroid-resistant, necessitating a search for alternative anti-inflammatory therapies.

Asthma

Asthma is an heterogeneous disease usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheezing, shortness of breath, chest tightness and cough that vary over time, together with expiratory airflow limitation. Airflow limitation, typically reversible in asthma, may become persistent in a subset of patients (3).

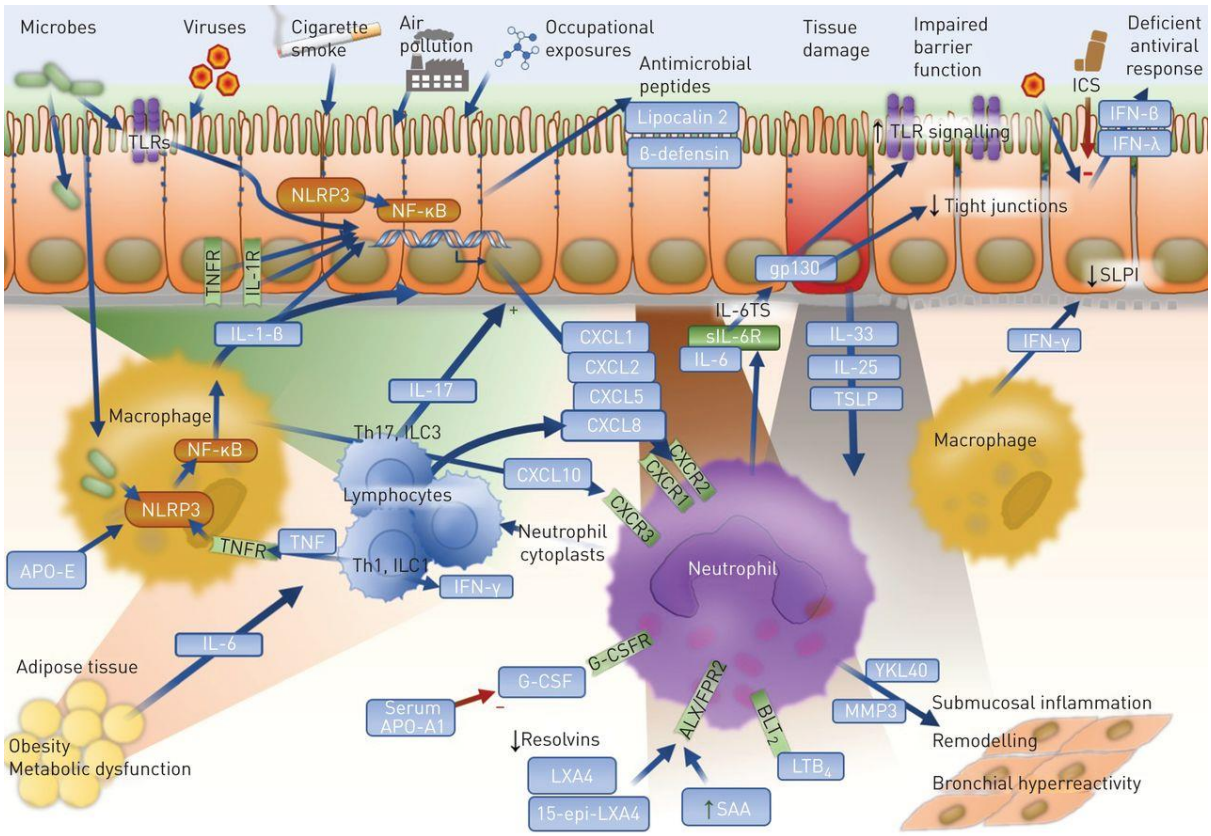
Asthma has risen to become one the most prevalent chronic diseases in developed countries, affecting over 10% of adults. The majority of asthma patients are atopic and have an allergic pattern of inflammation in their airways, which extends from the trachea down to peripheral airways (4). However, asthma can occur also in non-atopic patients highlighting the heterogeneity of this disease. Indeed several phenotypes of asthma have been recognized, characterized by different severity grades, degrees of airway obstruction, extent of response to treatment and involvement of comorbidities. The molecular mechanisms underlying phenotypes (endotypes) are also different: we commonly discriminate asthma endotypes between T2-High and T2-Low on the basis of the presence (or absence) of an active type 2 inflammatory response, whose effector cell is the eosinophil (5). T2-High asthma then subdivides into atopic and non-atopic, depending on the activation of adaptive immunity represented by T-helper2 lymphocytes with a mechanism of allergic hypersensitivity (Figure 1). The absence of type-2 inflammatory traits defines T2-Low asthma which is, therefore, a diagnosis of exclusion and which may include several endotypes like neutrophilic (Figure 2) and pauci-granulocitic asthma with involvement of activation of T-helper 1 and T-helper 17 mediated inflammatory pathways (6).

Figure 1. Molecular pathways in T2 High asthma



Two different pathways lead to eosinophilic airway inflammation in asthma. In allergic asthma, dendritic cells present allergens to CD4+ T cells, inducing TH2 cells, which produce IL-4, IL-5 and IL-13, and leading to IgE switching in B cells, airway eosinophilia and mucous hypersecretion. In non-allergic eosinophilic asthma, air pollutants, microbes and glycolipids induce the release of epithelium-derived cytokines, including IL-33, IL-25 and TSLP, which activate ILC2s in an antigen independent manner via their respective receptors (IL-17RB, ST2 and TSLPR). Activated ILC2s produce high amounts of IL-5 and IL-13, leading to eosinophilia, mucous hypersecretion and airway hyperactivity. 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; PGD2, prostaglandin D2; RORα, retinoic acid receptor–related orphan receptor α. From ref 5.

Figure 2. Molecular pathways in T2 Low neutrophilic asthma

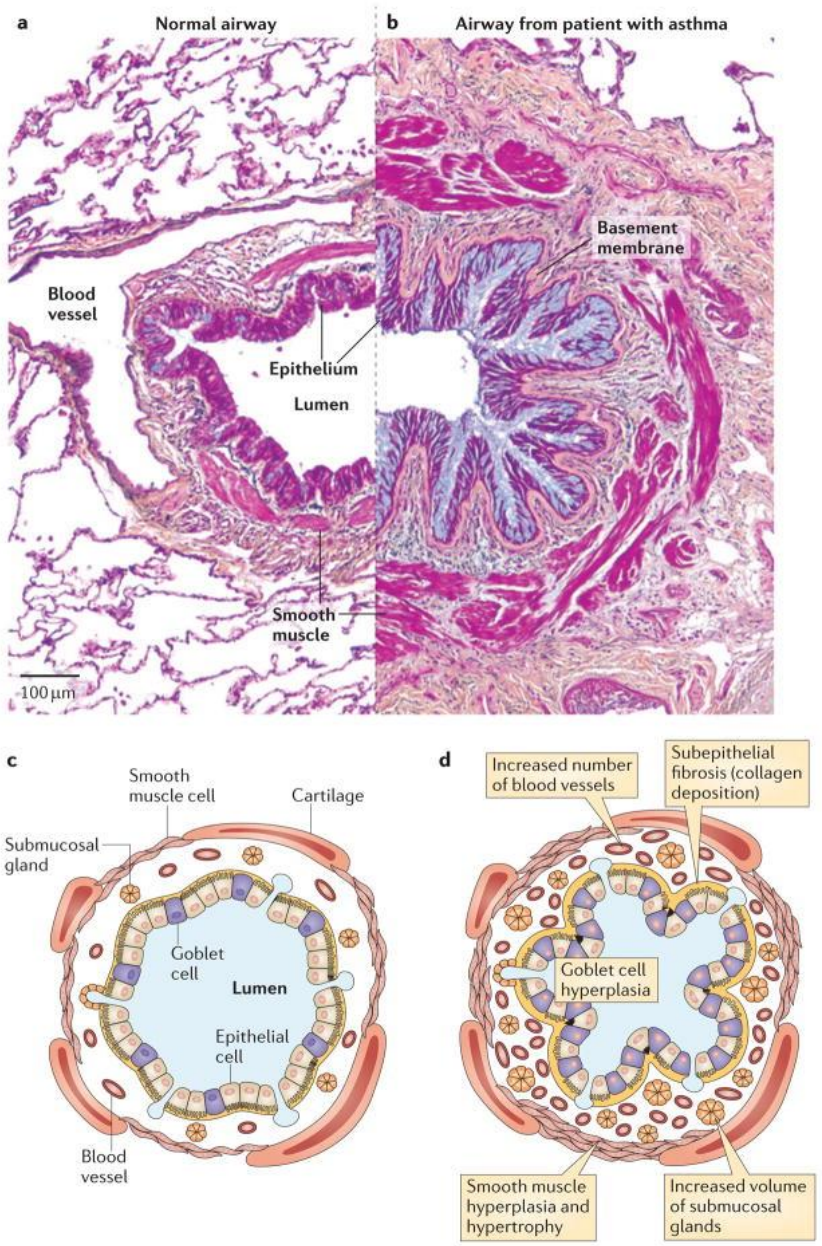


Key pathways mediating neutrophilic airway inflammation in type 2-low asthma. APO: apolipoprotein; FPR: N-formyl peptide receptor; G-CSF: granulocyte colony-stimulating factor; G-CSFR: granulocyte colony-stimulating factor receptor; gp130: glycoprotein 130; ICS: inhaled corticosteroid; IFN: interferon; IL: interleukin; ILC: innate lymphoid cell; IL-6TS: interleukin-6 trans-signalling; LXA: lipoxin; NLRP: nucleotide-binding oligomerisation domain, leucine-rich repeat, and pyrin domain containing; sIL-6R: soluble interleukin-6 receptor; SAA: serum amyloid A; SLPI: secretory leukocyte protease inhibitor; Th: T helper CD4+ lymphocyte; TNF: tumor necrosis factor; TSLP: thymic stromal lymphopoietin. From ref 6.

The main pathological feature during an asthma attack is airway narrowing, which is largely due to contraction of airway smooth muscle, but vascular congestion and airway edema from leaky bronchial vessels may also contribute. In addition, there are chronic structural changes to the airways even in stable conditions, also known as airway remodeling, such as basement membrane thickening, mucous cell metaplasia and increased airway smooth muscle bulk that may result in irreversible narrowing (Figure 3, ref 7). Mucus plugs, comprising mucus glycoproteins and plasma proteins may occlude the airways in fatal asthma (8). The inflammation in asthmatic airways also promotes airway hyper-responsiveness (AHR), one of the defining physiological abnormalities of asthma, which accounts for airway narrowing in response to various environmental triggers and produces the characteristic variable symptoms of asthma, including nocturnal worsening. The mechanisms of AHR are still not certain, but are likely to relate to increased release of mediators from inflammatory cells (particularly mast cells), increased contractility of airway

smooth muscle, increased sensitivity of airway sensory nerves and to existing airway narrowing for geometric reasons.

Figure 3. Airway remodeling in asthma



Airway structure in a healthy subject (part a; a schematic representation is depicted in part c) and in a patient with asthma (part b; a schematic representation is depicted in part d). The airways in asthma show considerable structural remodeling, including goblet cell hyperplasia/metaplasia, subepithelial fibrosis (basement membrane thickening), increase in smooth muscle volume and in the number of blood vessels (neoangiogenesis). From ref 9.

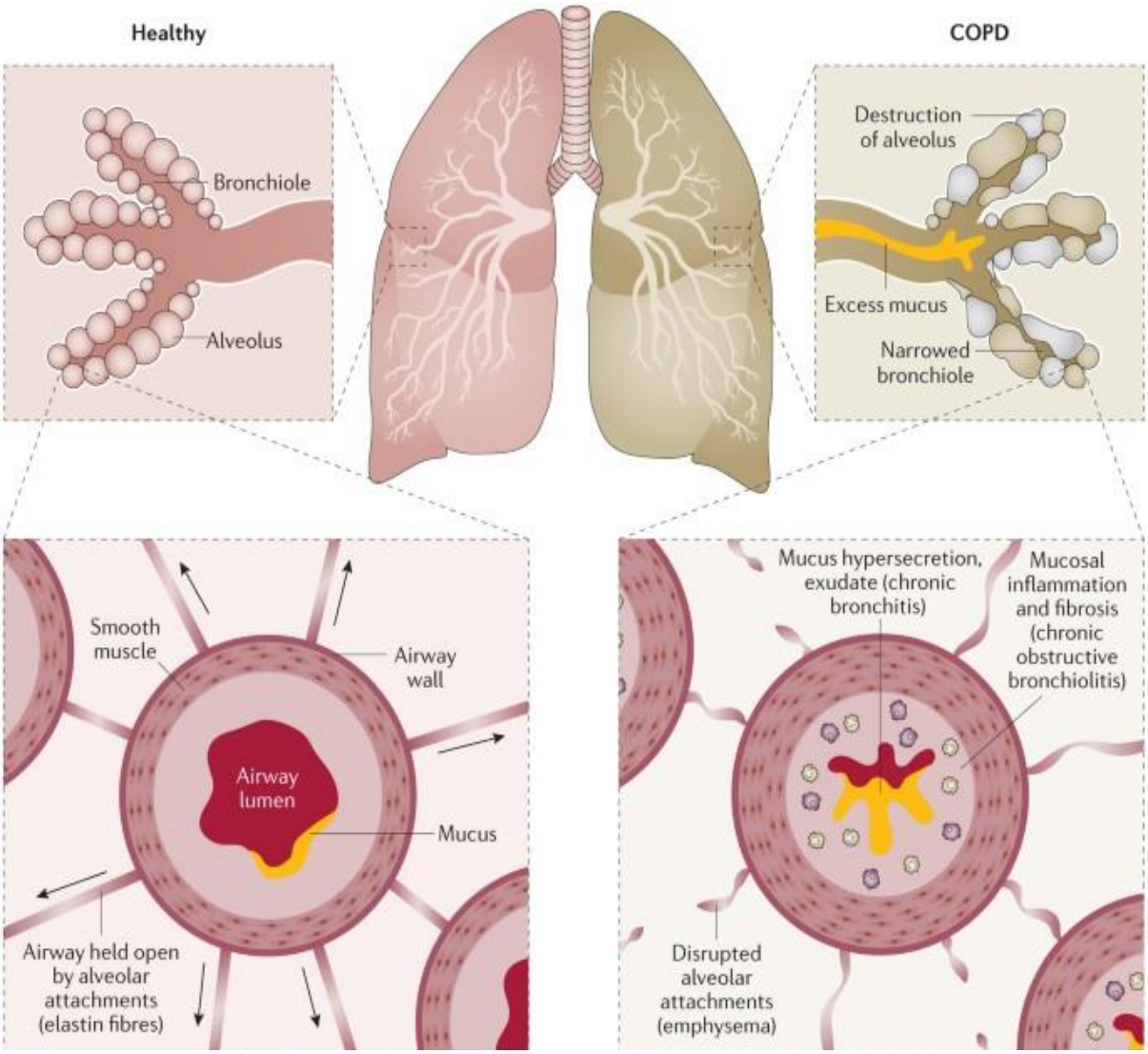
COPD

COPD is a common, preventable and treatable disease that is characterized by persistent respiratory symptom and airflow limitation, that is due to alveolar/airways abnormalities usually caused by significant exposure to noxious particles or gasses (10). It has been estimated that the global prevalence of COPD is around 11% among the population over the age of 40 years (11) and it is considered to be the third cause of death in the United States, after ischemic heart and stroke (12).

There are clearly different clinical phenotypes of COPD, with some patients having predominantly small airway disease, whereas others have mainly increased alveolar wall destruction (emphysema) and hyperinflation. Other differences include age of onset, rate of progression, frequency of exacerbations and the association with other diseases, such as chronic cardiovascular and metabolic diseases (comorbidities). Although attempts have been made to segregate COPD patients into different clusters based on clinical and radiological characteristics, it has been difficult to identify these phenotypes in different populations and there has been no definite link to underlying disease mechanisms (endotypes) (13,14).

Many biological processes characterize the development of COPD. Inhalation of noxious particles (i.e. cigarette smoke) causes airway and lung inflammation in all subjects; however in COPD patients the degree of inflammation is greatly amplified, it rapidly progresses with tissue structural changes and it is often accompanied by systemic inflammation. Indeed, only about 20% of heavy smokers develop COPD suggesting a probable role of genetic predisposing factors (15). Pathological features of COPD are represented in figure 4: chronic bronchitis, emphysema and small airways disease (15). These affect the both large and the small airways and lung parenchyma, and contribute to the development chronic airflow obstruction (as measured by a reduction in forced expiratory volume in one second (FEV1) or the ratio of FEV1 to forced vital capacity) through increases in the resistance of the conducting airways and lung compliance. Chronic bronchitis is due to the increase of bronchial secretions, caused by submucosal glandular hypertrophy and hyperplasia. Emphysema is the permanent destructive enlargement of distal airspaces, causing a loss of lung elastic recoil (16). Chronic inflammation and remodeling of the small airways and particularly of the terminal bronchioles (epithelial metaplasia, increased airway smooth muscle goblet cell hyperplasia, destruction of alveolar attachments) are features related to the severity of disease characteristic of COPD (17).

Figure 4. Small airway remodeling in COPD



In healthy lungs, the small airways (bronchioles) are held open by alveolar wall attachments that contain elastin fibers. In chronic obstructive pulmonary disease (COPD), the small airways are narrowed through thickening of the bronchiolar peripheral wall by inflammation and fixed narrowing (as a result of fibrosis), disruption of alveolar attachments (as a result of emphysema) and luminal occlusion by mucus and inflammatory exudate. From ref. 18.

INFLAMMATORY MECHANISMS OF ASTHMA

Since the 1980s, numerous papers have described the cell and inflammatory mediators implicated in the immunopathology of bronchial asthma. The mechanisms of interaction between different cells (endotypes) and how they will determine the clinical, functional and histological features typical of the disease (phenotype) is still the object of research. Indeed, over the years, by acknowledging the role and function of various cellular pathways, we gained more and more awareness of the heterogeneity of asthma in real life. Therefore, rather than describing the immunopathology of asthma as a single disease, the current approach will point out to different immunopathological mechanisms of disease in relation to the phenotype they determine.

Eosinophils

The presence of an eosinophilic inflammatory infiltrate characterizes the T2 High endotype. Eosinophils play a starring role in both the acute and chronic phases of inflammation and can be considered the "ultimate effectors" of the inflammatory response. Recruitment of eosinophils in the asthmatic airways is promoted by IL-5 (produced by both Type 2 CD4+ T lymphocytes and by innate lymphoid cells type 2-ILC2) and is mediated by eotaxins (CCL11) (19) Eosinophils secrete a number of injurious and oxidant substances as preformed mediators (e.g. major basic protein-MBP, eosinophil cationic protein-ECP, eosinophil peroxidase-EPO, eosinophil-derived neurotoxin-EDN), but they also produce a wide range of regulatory cytokines and growth factors. The release of cationic protein, major basic protein and free radicals oxygen species may have an hypersensitization action on the nerve endings, contributing to the development of bronchial hyperresponsiveness. The eosinophils also produce and release leukotrienes, responsible for bronchoconstriction, and other lipid mediators implicated in vasodilation and in mucus hypersecretion (20). According to some studies, eosinophils, would also be able to promote airway remodeling through the production of several growth factors growth factors involved in fibrogenesis and angiogenesis (TGF-beta, IL-11, IL-17, IL25, TIMP-1, MMP-9) (21).

Neutrophils

A neutrophilic infiltrate is frequently observed in T2 low asthma, particularly in patients with late onset disease and in association with obesity (22). Neutrophils are recruited and activated by Th1 and Th17 lymphocytes through cytokines such as IL-8, IL-17, IL-27, IFN-γ and TNF-α (23). The activated neutrophil releases mediators such as: oxygen free radicals, nitric oxide, elastase, MMP-9, LTB4 and amplifies the release of IL-8 in order to induce an autocrine loop (24).

Mast cells

Mast cells definitely play a crucial role in the acute response to the triggers. Not only allergens but also chemical and physical stimuli can activate mast cells (acting

directly on chemoreceptors or indirectly through the release of substance P). Once activated, mast cells release preformed mediators (such as histamine) and lipid mediators (such as leukotrienes), which trigger acute inflammation and induce bronchoconstriction (25). Mast cells also release inflammatory mediators, such as cytokines, chemokines, growth factors and neurotrophins. Accordingly to some studies, eosinophils, would also be able to promote the airway remodeling through the production of several growth factors growth factors involved in fibrogenesis and angiogenesis (TGF-beta, IL-11, IL-17, IL25, TIMP-1, MMP-9) (26).

Macrophages

The role of macrophages in asthma is currently debated as they may have either proinflammatory and anti-inflammatory effects. Tissue macrophages can be polarized toward an M1 type with pro-inflammatory activity (with secretion of cytokines such as IL-1β and TNF-α) or an M2 type, with tissue-repair activity (and release of cytokines such as IL-10 or growth factors such as TGFβ). In vitro studies observed that type 2 cytokines, particularly IL4 and IL-13 are able to stimulate macrophage differentiation toward the M2 type thus hypothesizing a role in airway remodeling (27). Other studies (28) demonstrated that macrophages may be activated by allergens via low-affinity IgE receptors (FcεRII).

T helper lymphocytes

CD4+ T lymphocytes traditionally represented the key cell in the immunopathogenesis of asthma: they orchestrate the sensitization to aeroallergens, the acute inflammatory response to trigger factors as well as chronic inflammation. Th2 lymphocytes have a crucial role in T2-High allergic asthma producing a pattern of cytokines that orchestrate the characteristics of the inflammatory response. They secrete IL-5 and GM-CSF which act on eosinophils by inducing their production from the bone marrow, activation and prolonged survival. In addition, they produce IL-4 and IL-13 which are active on B lymphocytes by inducing isotype switch toward IgE production and activation, and also participate in airway remodeling by acting on fibroblasts, epithelial and bronchial smooth muscle cells (29).

In T2-low asthmatics, a predominance of Th1 lymphocytes have been observed (30). The Th1 lymphocyte differentiates under the influence of IL-12 and IFN-γ. Th1 lymphocytes may secrete IL-8, INF-γ, IL-27 and TNF-α which have important pro-inflammatory effects, particularly in the recruitment and activation of neutrophils (31). Th17 is another subpopulation that has raised recent interest in asthma. This lymphocyte subtype produces mainly IL-17 (32). The role of this cell and its cytokine product is still controversial; it has been shown in humans and animals that Th17 is a pro-inflammatory cell that promotes neutrophilic inflammation acting synergistically with Th1 (33).

Innate lymphoid cells (type2)

This subpopulation of innate lymphoid cells has been recently discovered in bronchial wall-associated lymphatic tissue (BALT) of asthmatic patients. Although

scarcely represented they may have an important role in asthma pathogenesis given the strength of their cytokine production. They are activated by IL-25, IL-33 and TSLP, cytokines that are produced by the bronchial epithelium in response to chemical and physical damage but also to infection (32). They produce the same cytokine spectrum as the Th2 lymphocyte, especially IL-5 and IL-13, under the transcriptional control of GATA3 (33). The role of these cells is still controversial: they are thought to have either an amplifying effect on the Th2 response or an alternative function based on their ability to stimulate eosinophils regardless of Th-2 lymphocytes, with a probable role in T2-High non-allergic asthma endotypes (34).

Asthma endotypes

T2-High Asthma

T2-High asthma, according to the most recent estimates, comprises 60-70% of all forms of adult asthma and over 80% of pediatrics (33,35,36). It includes both allergic asthma (atopic T2-high) and several non-allergic phenotypes, all of which are accumulated by the presence of eosinophilic inflammation enhanced by type 2 cytokines. As clearly illustrated in Figure 1, all asthma phenotypes that fall into the T2-high family have a common downstream pathway in the inflammatory cascade: the release of IL-5 and IL-13 which recruit and feed eosinophilic inflammation and stimulate bronchial muscle thickening and remodeling (36).

Allergic asthma represents the prototypical type 1 hypersensitivity reaction. As summarized in Figure 1, the central cell in the immunologic cascade is represented by the type 2 T helper lymphocyte. After the dendritic cells provide allergen presentation to lymphocytes, lymphocytes become activated and shift toward a Th2 immunological profile, producing the cytokines of the type 2 response. IL-4 and IL-13 activate B lymphocytes and promote isotype switching toward IgE production. Moreover, IgE produced by the B lymphocyte and IL-9 produced by the Th2 lymphocyte are responsible for activation of tmast cells (33,37). In recent years, a possible role of the bronchial epithelium as an active part of this process has been highlighted after it has been shown to be capable of producing alarmins such as IL-33 and IL-25 and thymic stromal lymphopoietin (TSLP), which can effectively attract and stimulate lymphocytes (38)

The absence of atopy in many patients with T2-High asthma suggests that the immunopathogenic mechanism differs from that of allergic asthma. Numerous studies have shown that type 2 cytokines are increased also in non allergy-mediated immunopathological processes such as cancer, ulcerative rectocolitis, and Crohn's disease (37), demonstrating that type 2 inflammation and eosinophilia can also be present in the absence of allergy. In recent years, it has been demonstrated that eosinophilic response may be supported by innate type 2 lymphoid cells (ILC2) (33-38). Indeed, it has been reported in mouse models lacking lymphocytes, that ILC2

are nonetheless capable of inducing eosinophilia and bronchial hyperresponsiveness (33,39). Their role as an "alternative promoter" of eosinophilia has been demonstrated in chronic rhinosinusitis (40) and is a matter of key interest in bronchial asthma. As illustrated in Figure 1, alarmins produced by the bronchial epithelium such as IL-33, IL-25, and TSLP(25), may stimulate ILC2 to produce Th2-associated cytokines such as IL-13 and IL-5 that act, similarly to allergic asthma, by recalling and activating eosinophils and promoting remodeling (41,42). A relatively recent study has shown that TSLP and ILC2 levels are directly related to the steroid resistance in severe asthma (38).

T2-Low Asthma

In 20-30% of asthmatic subjects, eosinophilic (Th2) airway inflammation is not detectable. These patients are usually adults with mild-moderate asthma with adult onset and they often show steroid resistance. Features of neutrophilic, mixed (eosinophilic-neutrophilic) as well as absence of inflammation (pauci-granulocytic asthma) are often found. Indeed, T2- low asthma represents a collective of several endotypes characterized by non-type2 immunopathological pathways: without (or with poor) production of IL-5, IL-13, IL-4 and eosinophilic inflammation. The identification of T2-low asthmatic subjects is therefore an exclusion process given the absence of immunopathological features of type 2 inflammation. Because of this extreme heterogeneity, T2-low asthma currently represents one of the major challenges in asthma research, for the definition of the various endotypes and phenotypes as well as for the development of "precision" drugs for severe T2-low asthma (43).

Figure 2 illustrates the hypothetical immunopathogenic mechanism underlying neutrophilic asthma. Neutrophils and macrophages are recruited and activated by IL-8. Some studies reported a possible role of both Th1 and Th17 lymphocytes with production of IFN-γ and IL-17, respectively. Moreover, it has also been shown that eosinophilia and neutrophilia (mixed inflammatory endotype) may coexist in some subjects (43).

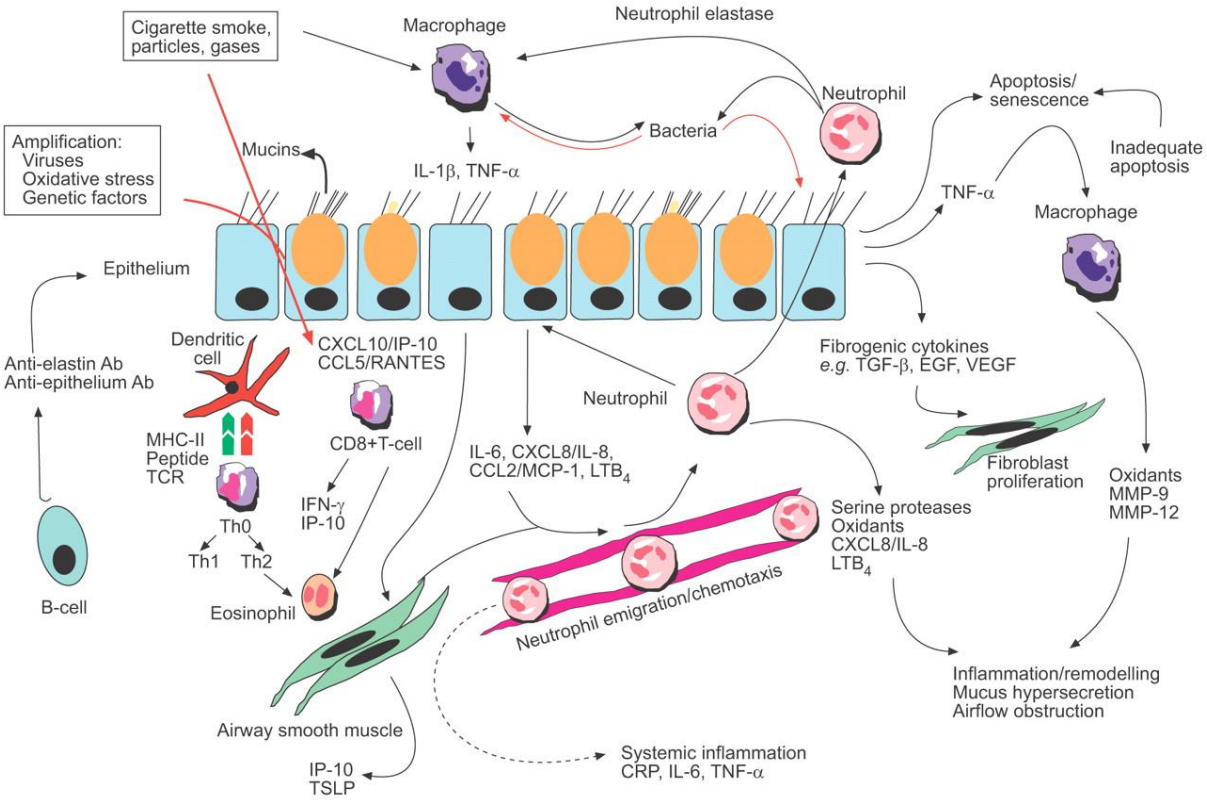
Another putative endotype is the biological mechanism underlying asthma in obese subjects: it is now recognized that in subjects with BMI > 30, there is systemic proinflammatory state that stimulates the production of certain distinctive mediators such as leptin, TNF-α, and IL-6 (43). In mouse models, obesity results in increased bronchial hyperreactivity and bronchial neutrophilic inflammation mediated by elevated levels of IL-17 (44). However, it is still unclear what the primary source of interleukin 17 is in obese subjects. It is speculated that it may be driven by Th17 lymphocytes but type 3 innate lymphoid cells may also play a role. In a recent study on obese asthmatic subjects, it was observed that weight loss is associated with an improvement in clinical control of symptoms together with a "restoration" of signs of type 2 inflammation (45).

Some asthmatics show neither signs of T2-high nor neutrophilic inflammation. These patients, who by definition have eosinophil levels < 2% and neutrophil levels <61% in induced sputum (140) are called pauci-granulocitic. In these patients, airway remodeling and bronchial hyperresponsiveness are possibly due to the activation of neural pathways and other non-inflammatory mediators that would be able to stimulate growth factors such as TGF-beta by fibroblasts (46). The biological plausibility of this "inflammation-free" endotype has also been confirmed in the animal model. It is speculated that this form of asthma may be the one that could receive the most benefit from bronchial thermoplasty, which would have a neurolytic effect on the plexuses intercalated with the bronchial wall responsible for the production of the mediators at the origin of this form of asthma (47).

INFLAMMATORY MECHANISMS OF COPD

Chronic inflammation of COPD is characterized by an accumulation of macrophages, neutrophils and T-lymphocytes (mainly CD8+), lymphocytes are also present as organized lymphoid aggregates with B-cells. Unlike asthma in COPD inflammation is predominantly located in the peripheral airways and lung parenchyma (48) and the degree of inflammation increases with the severity of disease (49).

Figure 5. Summary of inflammatory and structural cell interactions in COPD



Inflammatory and cellular pathways linking chronic cigarette exposure to the chronic inflammation of chronic obstructive pulmonary disease (COPD). Activation of neutrophils, macrophages, epithelial cells, dendritic cells, T-cells, B-cells, fibroblasts and airway smooth muscle cells leads to release of cytokines, chemokines and proteases. Amplification signals are important in augmenting the inflammatory responses that underpin COPD. Ab: antibody; Th: T-helper cell; MHC: major histocompatibility complex; TCR: T-cell receptor; CXCL: CXC chemokine ligand; IP: interferon (IFN)-γ-inducible protein; CCL: CC chemokine ligand; RANTES: regulated on activation, normal T-cell expressed and secreted; TSLP: thymic stromal lymphopoietin; IL: interleukin; TNF: tumor necrosis factor; MCP: monocyte chemotactic protein; LT: leukotriene; CRP: C-reactive protein; TGF: transforming growth factor; EGF: epidermal growth factor; VEGF: vascular endothelial growth factor; MMP: matrix metalloproteinase. Adapted from ref. 50.

Neutrophils

Neutrophils are considered one of the most important inflammatory cell subtypes in COPD. Neutrophils are localized particularly to the bronchial epithelium, bronchial glands (51) and in bronchial smooth muscle (52). Sputum neutrophilia is associated with the presence of greater airflow obstruction and with an accelerated decline in

lung function (53). Neutrophil recruitment in the lung is the consequence of the production of chemoattractants. Two neutrophil chemoattractants are particularly important in this respect, C5a generated by cleavage of complement C5 in interstitial fluid, and interleukin IL-8 synthesized by cells in the lung, e.g. macrophages, epithelial cells, endothelial cells, smooth muscle cells and neutrophils themselves (54). As a neutrophils migrate, serine proteinases, including neutrophil elastase (NE), cathepsin G and proteinase 3 (PR3), are released from azurophil granules into the extracellular space, and some active enzyme is retained on the plasma membrane (55). Substrates for these proteinases include elastin, collagen and fibronectin, which are major components of the extracellular matrix, and their degradation is linked to all clinical facets of COPD (56). Cigarette smoke extract impairs the phagocytic ability of neutrophils, through suppression of caspase-3-like activity in the neutrophils, an impairment that does not lead to a suppression of spontaneous apoptosis (57). These abnormalities may underlie the increased risk of respiratory infections in smokers and COPD patients.

T-cells

CD8+ T-cell numbers are increased throughout the airways, the lung parenchyma and pulmonary vasculature (58) and have also been localized in airway smooth muscle (52). Despite their prominence in COPD, their role still remains elusive. Lung parenchymal cells may be damaged by the release of lytic substances, such as perforin and granzyme, from CD8+ T-cells, and studies of COPD CD8+ T-cells reveal increased cytotoxic activity of these cells with higher concentrations of perforin present in sputum (59). There is an association between the numbers of structural cells undergoing apoptosis and CD8+ T-cell numbers in alveolar walls (60), indicating a possible induction of apoptosis of epithelial and endothelial cells by CD8+ T-cells.

Activated CD4+ T-cell numbers are also increased in the small airways of smokers with COPD (61), and they appear to have a Th1 profile, with expression of the chemokine CXCL10, which may control the release of elastolytic matrix metalloproteinases (MMPs) (62). In murine models, emphysema has been shown to develop in the presence of adaptively transferred pathogenic CD4+ T-cells. In severe emphysema, T-cells isolated from lung tissues showed oligoclonal expansion to conventional antigenic stimuli (63). Anti-elastin antibody, an autoantigen that may underlie an autoimmune response, has been reported in COPD patients (64). In addition, significantly fewer regulatory T-cells were found in the lungs of patients with COPD, with reduced gene expression of forkhead box protein (FOX) P3, a transcription factor crucial for the development of regulatory T-cells, and less IL-10 secretion, which could permit clonal expansion of elastin-specific Th1 cells (64). Finally, enhanced cytotoxicity activity of NK has been demonstrated in COPD from lung DC–mediated priming via IL-15 trans-presentation on IL-15Rα (65).

B-cells

An increased number of B-cells have been observed in advanced COPD (49).

Moreover bronchus-associated lymphoid tissue has been described in COPD (66). This evidence suggests an involvement of the adaptive immune response in the pathogenesis of the disease. Lymphoid follicles were demonstrated in both the parenchyma and bronchial walls of patients with emphysema (67) and an oligoclonal antigen-specific reaction of the B-cells has been described. Plasma cells, which are derived from maturation of B-cells, were found in subepithelial and submucosal glands in COPD compared with asymptomatic smokers (68).

The idea of autoimmunity as a mechanism for the chronic inflammatory and emphysematous damage is also supported by the detection of circulating autoantibodies, such as anti-elastin antibodies and anti-epithelial antibodies, in smoking patients with COPD (64,69). Further support for the autoimmune pathogenesis comes from the development of an emphysema model in mice immunized with anti-epithelial antibodies.

Dendritic cells

The accumulation of dendritic cells in small airways of patients with severe COPD supports the involvement of adaptive immunity (70). This increase correlates with sputum levels of CC chemokine ligand (CCL), macrophage inflammatory protein (MIP)-3α, a chemoattractant for DCs. Studies in mice report that chronic cigarette exposure led to an increase in DCs, associated with increased levels of CCL-2/monocyte chemotactic protein (MCP)-1 and CCL20/MIP-3α (71). Moreover, dendritic cells sustain CD8+ recruitment and retention in lung tissue (72).

Eosinophils

The role of eosinophils in the pathogenesis of COPD is unclear but they may differentiate an enodotype of COPD. Increased eosinophil numbers have been reported in sputum, BAL and the airway wall, with increased levels of eosinophil cationic protein in BAL and sputum in some studies (73,74). Expression of IL-4 and -5, typical T-helper cell (Th)-2 cytokines associated with eosinophilia in asthma, has also been reported (68). Moreover, IL-8, in addition to its neutrophil chemotactic effects, also has eosinophil chemotactic properties (75). Increased numbers of eosinophils in sputum and BAL fluid in COPD have been related to a good clinical response to corticosteroid treatment (74). Of importance, during acute exacerbations a marked increase in submucosal eosinophil numbers has been observed, particularly in exacerbations associated with a viral infection. (76). Moreover, a recent study reported that a preferential distribution of eosinophils towards the airway lumen characterized patients with high symptomatic COPD (77).

Monocytes/macrophages

Macrophages are derived from blood monocytes which traffic to the lungs and differentiate locally. Clusters of macrophages have been found around small airways associated with the peribronchiolar fibrosis in smokers and ex-smokers (78). There is heterogeneity of macrophages, with some macrophages having a proinflammatory profile (type M1), whereas others are anti-inflammatory (type M2), promote tissue

repair and are more phagocytic (79). In general, it is likely that “M1-like” proinflammatory macrophages predominate in COPD but further phenotyping is needed in the future (80). M2 macrophage markers, such as CD163 are increased in COPD lungs and a subset of M2 type macrophages may contribute to defective remodeling in COPD (81). Monocytes/macrophages are important effector cells in COPD through their release of ROS, extracellular matrix proteins and lipid mediators, such as leukotrienes, prostaglandins, cytokines, chemokines and MMPs (82). CD68+ macrophages are prominent and increase in number in the airways of COPD patients (83), and their numbers increase with increasing severity (49). Macrophages from COPD patients release more inflammatory proteins than macrophages from normal smokers and non-smokers, indicating increased activation (84). On the other side, alveolar macrophages from COPD patients also show reduced phagocytic uptake of bacteria and this may predispose to chronic colonization of the lower airways by bacteria such as *Haemophilus influenzae* or *Streptococcus pneumoniae* (85).

Epithelium

Bronchial epithelial cells have a central role in the pathogenesis of the disease in light of the fact that it is capable of producing mediators that can fan inflammation and promote remodeling. The epithelial response to cigarette smoke represents attempts by the airway epithelium to protect itself and repair the injury caused by cigarette smoke (86). Chronic injury leads to the development of squamous metaplasia, which is the reversible replacement of the columnar epithelium by squamous epithelium, and it has been correlated with airflow obstruction severity (87). Cigarette smoke induces the release by epithelial cells of IL-1β and -8 and granulocyte colony-stimulating factor (G-CSF) (88), causing neutrophil and monocyte chemotactic recruitment and activation (89). Moreover, it has been demonstrated that epithelial cells from COPD patients release profibrotic factors such as TGF-β, EGF, VEGF, and FGF-1 and -2 (90,91). Epidermal growth factor receptor (EGFR) cascade is also overexpressed in bronchial epithelial cells from COPD (92).

Airway smooth muscle

Airway smooth muscle cells are also capable of releasing cytokines, chemokines, growth factors and proteases and can participate in the inflammatory and remodeling process (93). Cytokines and chemokines, which may be released from airway smooth muscle, include IL-6 and IL-8, MCP-1, -2 and -3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (50). IL-1β, TNF-α and bradykinin induce the release of IL-8 (94), a potent neutrophil chemoattractant and activator, whereas IFN-γ and TNF-α induce the release of IP-10 (95). a potent chemoattractant for human monocytes, neutrophils, natural killer cells and T-cells, preferentially Th1 cells. Finally, also thymic stromal lymphopoietin is expressed by smooth muscle of patients with COPD (96) and may trigger DC-mediated Th2 inflammatory responses. Finally, airway smooth muscle cells are an important source of connective tissue growth factor (CTGF) and TGF-β (97), promoting remodeling.

AIM OF THE THESIS

The aim of my PhD project was to investigate inflammatory mechanisms in pathogenesis of asthma and COPD, with the purpose to assess the role of the immune system in the etiology, pathogenesis, clinical presentation and outcomes in these two conditions, with a particular focus to the distinct disease phenotypes and endotypes.

Early-onset and late onset asthma represent phenotypic variants of the disease; the former is generally thought to be associated with male gender and allergic T2-high inflammation, the latter with a female predominance and more diverse mechanisms (98). Starting from this assumption, we first studied the clinical characteristics and comorbidities of a "real life" cohort of 175 asthmatic adult patients followed in our outpatient clinic stratified by the age of disease onset (STUDY I). The primary outcome of the study was to evaluate how different comorbidities would affect symptom control in the two distinct groups of patients with Early Onset and Late Onset asthma. Moreover, to understand the mechanisms driving phenotypic differences, we explored whether reported comorbidities were associated with lung function and inflammatory parameters.

My main line of research was then focused on exploring the first steps in pathogenesis of early onset asthma, at the beginning of its natural history, to unravel the significance of early airway alterations and the link with predisposing factors such as airway infections and air pollution. We took advantage of a cohort of 120 children that underwent bronchoscopy for clinical reasons and performed bronchial biopsies for research purposes. We could thus assess the airway pathology in children with wheeze, a common condition during the first years of life that could be defined as a "pre-asthma" state since these children are at high risk (50% will develop asthma). Structural traits (basement membrane thickness and destroyed epithelium) and inflammatory infiltrate (eosinophils, lymphocytes, macrophage and neutrophils) typical of adult asthma were quantified in bronchial biopsies from these children. We then performed a 5 year longitudinal follow-up with the purpose to identify which pathological traits were associated with asthma development implicating a pivotal role in the pathogenesis of the disease (STUDY II). Given the widely recognized importance of eosinophils and type 2 inflammation in the pathogenesis of early onset asthma (99), we investigated the presence of blood biomarkers that could detect airway eosinophilia (STUDY III).

Next, because we observed that about 50% of children with wheezing and airway eosinophilia lacked allergic traits, we aimed to assess whether innate lymphoid cells type 2 can be promoters of type 2 inflammation in wheezing children as in adults. In collaboration with the Laboratory for Translational Research of Obstructive Pulmonary Diseases, Ghent University (BE), we developed an immunohistochemical staining method for staining ILC2s in biopsies (Figure 6). We first applied this

method in a small group of patients with asthma from TDI (a model of non-allergic eosinophilic asthma, STUDY IV) and then to our own cohort of children with wheeze (STUDY V).

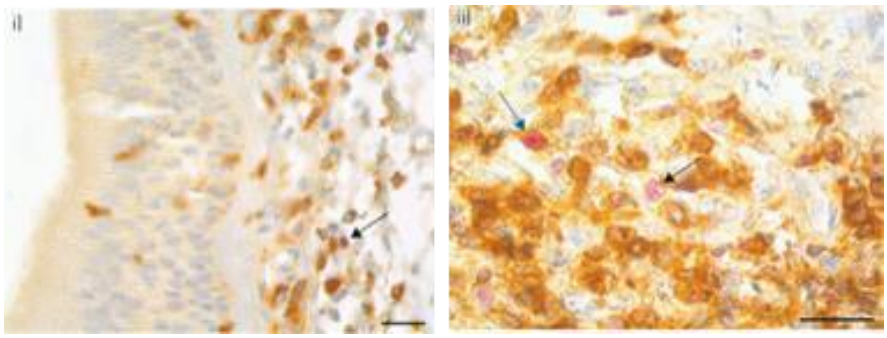


Figure 6. Micro-photography x60 of double staining immunohistochemistry (GATA3 and CD3) bronchial biopsies of children from study V. GATA3 marker is colored in red while CD3 is colored in brown. T lymphocytes are CD3+ and GATA3+/- cells (left picture, arrow). ILC2 are CD3- and GATA3+ cells (right picture, arrows).

Since epidemiological data suggest that wheezing episodes associated with viral infections early in life are a major risk factor for the development of asthma (100), we then investigated, in a subset of our wheezing cohort, whether the impairment of the antiviral innate response at baseline could lead to asthma development at follow-up (STUDY VI). Finally, since a causal link between long-term exposure to outdoor air pollution and asthma onset has been recently confirmed (101) we sought to assess the impact of long and short term exposure to outdoor air pollutants (PM and NO2) to airway pathology (STUDY VII) and to antiviral innate response (STUDY VIII) in wheezing children.

On the line of research in COPD, in collaboration with Respiratory Disease Clinic of Treviso City Hospital, we are performing a pilot study on a large “real life” cohort of 400 patients with COPD followed in our outpatient clinic, investigating the relation between blood cell counts including eosinophils, as systemic biomarkers, and clinical outcomes, particularly exacerbations (STUDY IX). On the same line, since respiratory viral infections are frequent causes of exacerbations of COPD (102), we investigated the activation of antiviral innate responses in lung samples from patients with COPD (and appropriate smoking and nonsmoking controls) and correlate it with the most important outcomes of the disease (STUDY X). Given the putative role of ILCs in innate response, in particular of ILC1, we collaborated with the group at Ghent University to an analysis of the different ILCs subsets in bronchial biopsies from patients with COPD(STUDY XI).

Finally, due the intercurrent outbreak of the Sars-Cov-2 pandemic, we wanted to assess the impact of COVID-19 in COPD, examining the prevalence and the risk factors for development/clinical progression of COVID-19 in COPD patients followed at our outpatient clinics (STUDY XII).

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RESULTS ACROSS THE STUDIES

Studies on asthma

STUDY I

Differences Between Early- and Late-Onset Asthma: Role of Comorbidities in Symptom Control.

Turrin M, Rizzo M, Bonato M, Bazzan E, Cosio MG, Semenzato U, Saetta M, Baraldo S. J *Allergy Clin Immunol Pract. 2022 Dec;10(12):3196-3203.*

BACKGROUND

Heterogeneity is a central feature of asthma (1,3,103). Indeed, several phenotypes have been proposed according to the inflammatory profile or clinical and functional characteristics (1,3,103). A key point in defining phenotypes is the influence of age at disease onset. In many cases, asthma symptoms begin in childhood (98). Patients with early-onset asthma (EOA) are mainly males with atopy and a history of sensitization to inhalant allergens (98,104,105). Although in some cases symptoms may resolve in adolescence, no improvement of the disease with growth occurs in many patients, so that early-onset persistent asthma occurs in a considerable proportion of patients routinely seen in asthma clinics (104). Asthma can initiate in adulthood as well, particularly in women after the menopausal period. Patients with late-onset asthma (LOA) are reported to have more severe disease, with persistent airflow obstruction and rapid lung function decline (105). In patients who develop asthma at an older age, the pathogenetic mechanisms seem to differ from those with early-onset asthma (EOA) (106). Delineation of comorbidities is an important share in the disease burden, because comorbidities can contribute to poor control of asthma and may affect the natural history of the disease and the therapeutic approach. Comorbidities with a major impact are (A) rhinitis (particularly in its allergic form), which causes inflammation of the upper airways and can contribute to exacerbate asthma (107) ; (B) gastroesophageal reflux (GERD), which may aggravate airway inflammation (108) ; (C) obesity, which not only affects respiratory mechanics but induces a systemic proinflammatory status (109); and (D) bronchiectasis, which exposes the patient to an increased risk for exacerbations owing to pulmonary infections (110).

The treatable traits approach has caused a recent reappraisal of the focus on comorbidities, especially the need to consider and treat comorbidities carefully as part of a multidimensional therapeutic approach tailored to the patient's phenotype. However, the interaction between comorbidities and other characteristic traits of asthma in defining the clinical and functional outcomes of the disease is still underappreciated. In particular, both age at onset and inflammatory patterns are key underlying factors in asthma with well-recognized involvement in the evolution of the disease.

AIM OF THE STUDY

The aim of our study was to understand disease characteristics, focusing on the burden of comorbidities, in adult asthmatic patients stratified by age at disease onset. The primary outcome of the study was to evaluate how different comorbidities would affect symptom control in two distinct groups of patients with EOA and LOA. The secondary aim was to explore whether reported comorbidities were associated with lung function and inflammatory parameters, to understand the mechanisms driving phenotypic differences.

METHODS AND MATERIALS

Study population

We conducted a cross-sectional real-life study recruiting a convenience sample of adult asthmatic patients attending the outpatient asthma clinic of our institution (Respiratory Disease Clinic, Padova University Hospital) from January 2018 to January 2020. Minimal inclusion and exclusion criteria were defined per protocol to avoid selection bias. The study included all patients aged at least 18 years with a diagnosis of asthma made according to Global Initiative for Asthma Guidelines (GINA 2022) (more than 1 year before study entry). All patients attended our clinic for routine health care assessment and were not specifically selected for this study. Patients were categorized into two groups according to the age at onset of the disease: EOA, when symptoms onset occurred before age 12 years of age (111); and LOA, when symptom onset occurred after age 40 years (112) Exclusion criteria were limited to a lack of informed consent and the presence of other major pulmonary conditions apart from asthma, which may affect symptom control. Patients with intermediate onset (aged 12-40 years) were also excluded from this analysis.

Patient evaluation and outcomes measurement

At the visit, we recorded the clinical history, results of the physical examination, pulmonary function tests, and Asthma Control Test, weight and height, and treatment during the previous 12 months. Blood cell counts and serum IgE levels (total and specific) of the most recently available tests were also collected. Outcomes considered were (A) clinical (i.e., symptom control: patients were defined as controlled when they had no more than one episode of bronchospasm per month and no nocturnal respiratory symptoms); (B) pulmonary function tests: FEV 1 , FEV 1 /FVC, FVC and FEF25-75 (C) blood biomarkers: total serum IgE (kU/L), eosinophils, neutrophils, and lymphocytes (cells × 10^9/L).

Statistical analysis

All analyses were performed using SPSS software (version 25.0.0.1 for Windows, IBM, Armonk NY, USA). Patients' characteristics are expressed as means ± SDs or median (range) for continuous variables and counts and percentages for categorical variables. For continuous variables, normal distributions were tested using the

Shapiro-Wilk test. Comparisons among groups were evaluated with either Student t test or Mann-Whitney U test, as appropriate. Distributions of categorical variables were compared with Chi-square test or Fisher exact test when the sample size was small (n<5). Correlation coefficients were calculated using nonparametric Spearman's rank method.

RESULTS

The study cohort consisted of 175 adult asthmatic patients, mean age 57.5 ± 17.1 years, with a female predominance (59%). Most patients had never smoked (54.8%), whereas 39.5% were active smokers and 5.7% were ex-smokers (Table 1). When we stratified by age at onset, 77 of 175 patients (44%) had EOA (onset less than 12 years), whereas 98 patients (56%) had LOA (onset greater than 40 years). Early-onset asthma persisting into adulthood presented with low values of both FEV 1 and FVC that were significantly decreased compared with LOA (even if within the normal range), suggesting submaximal development of lung volumes in these patients. The FEV1/FVC ratio was normal and similar in the two groups. EOA patients showed higher levels of serum IgE (p=0.001) and blood eosinophils (p=0.007) while LOA showed higher levels of blood neutrophils (p=0.018).

In EOA patients, there was a significant correlation between serum IgE and blood eosinophils levels (r=0.42; p<.001). In addition, the FEV1/FVC ratio in EOA was negatively correlated to both IgE (r = –0.35; p=.002) and eosinophils (r=–0.40; p<.001). None of these correlations were observed in patients with LOA.

With regard to comorbidities 62.8% of patients in the whole cohort experienced allergic rhinitis, 60.5% GERD. In addition, 21.7% were obese, 6.2% had a history of bronchiectasis. We then analyzed and compared the impact of the individual comorbidities on symptom control, lung function, and inflammatory markers in the EOA and LOA groups.

Rhinitis

Rhinitis was highly prevalent in the whole cohort, early-onset asthma had a much higher prevalence of rhinitis compared with LOA (75.3% vs 53%; p=0.002) (Table 1). In EOA the prevalence of concomitant rhinitis was much higher in patients with uncontrolled asthma (39 of 41; 95%) than in those with controlled disease (17 of 36; 47%) (Figure 7A). Moreover, early-onset asthma patients with rhinitis had higher total serum IgE levels, higher blood eosinophils counts, lower FEV1 and FEV1/FVC (Table 2).

Table1. Clinical and functional characteristics, comorbidities, and blood parameters in the two age-related groups.

	Whole cohort	EOA (onset <12 y)	LOA (onset >40 y)	*P-value*
Subjects, n	175	77 (44%)	98 (56%)	
Sex, male (%)	72 (41%)	34 (44%)	32 (32.6%)	n.s.
Age, y	57.5 ± 17.1	45.7 ± 18.1	63.9 ± 11.9	.06
Smoking status, n (%): - active - former - never	 69 (39.5%) 10 (5.7%) 96 (54.8%)	 27 (35%) 3 (3.8%) 47 (61.2%)	 42 (42.8%) 7 (7.2%) 49 (50%)	n.s.
FEV_1 /FVC (%)	77.6 ± 10.6	77.0 ± 10.5	78.5 ± 10.5	n.s.
FEV_1 (% predicted)	94.37 ± 21.5	93.2 ± 21.5	102.7 ± 21.5	.004
FVC (% predicted)	102 ±17	100 ± 20.8	109.6 ± 22.4	.007
Rhinitis, n (%)	110 (62.8%)	58 (75.3%)	52 (53%)	.002
Gastroesophageal reflux, n (%)	106 (60.5%)	41 (53.2%)	65 (66.3%)	n.s.
Obesity, n (%)	38 (21.7%)	17 (22.3%)	21 (21.4%)	n.s.
Bronchiectasis, n (%)	11 (6.2%)	2 (2.6%)	9 (9.1%)	n.s.
Eosinophils (cells × 10^9 /L)	0.26 ± 0.23	0.31 ± 0.27	0.17 ± 0.14	.007
Lymphocytes (cells × 10^9 /L)	2.13 ± 0.8	2.23 ± 0.59	2.00 ± 0.68	n.s.
Neutrophils (cells × 10^9 /L)	3.83 ± 1.8	3.43 ± 1.4	4.15 ± 1.58	.018
Serum IgE (kU/L)	207 ± 155	321 ± 549	91 ± 114	.001

Results are expressed as means ± SDs.

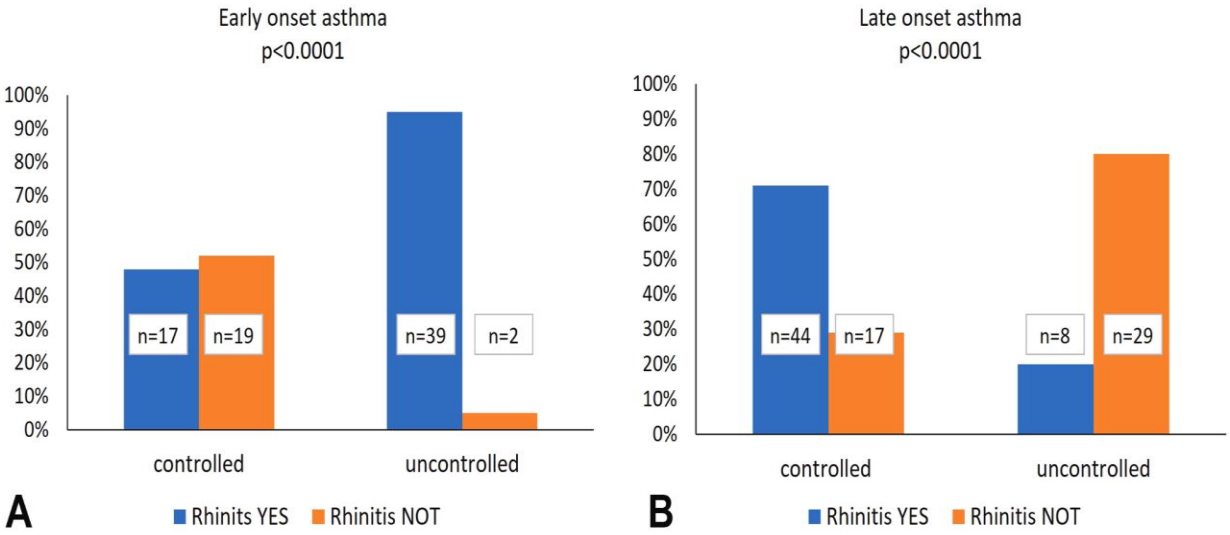

Figure 7. Association between presence of rhinitis and asthma control in (A) early-onset and (B) late-onset asthma. y axis indicates proportion of patients. Blue columns indicate patients with rhinitis, and orange columns, those without rhinitis. P values represent comparison with Chi-square test.

Conversely, in LOA patients, an opposite association was observed between rhinitis and clinical-functional severity of the disease. Indeed, late-onset asthma patients with concomitant rhinitis had significantly better respiratory function than did LOA patients without rhinitis (Table 2). Furthermore, in LOA, most patients with uncontrolled disease had no rhinitis (78% vs 28% in controlled asthma; Figure 7B). In this age group, rhinitis was not associated with increased IgE levels and eosinophil count.

Table 2. Impact of rhinitis on respiratory function and blood tests in the two age-related groups.

	EOA (onset <12 y)			LOA (onset >40 y)		
	Rhinitis (n = 58)	**No rhinitis (n = 19)**	***P-value***	**Rhinitis (n = 52)**	**No rhinitis (n = 46)**	***P-value***
FEV1 /FVC (%)	74.9 ± 10.8	83.5 ± 6.86	.01	82.57 ± 9.68	74.09 ± 9.67	<.0001
FEV1 (% predicted)	86.8 ± 22.1	96.9 ± 13	.04	106.6 ± 21.5	95.3 ± 23.3	.03
FVC (% predicted)	99.8 ± 18.2	100.6 ± 13.5	n.s.	105.25 ± 23.23	106.6 ± 23.25	n.s.
FEF25-75 (%predicted)	60.2 ± 28.9	78.9 ± 25.4	.007	82.9 ± 34	63 ± 31.4	.002
Total serum IgE (kU/L)	441 ± 635	71 ± 77	<.0001	106 ± 135	68 ± 65	n.s.
Eosinophils (cells × 10^9 /L)	0.36 ± 0.29	0.14 ± 0.1	.003	0.18 ± 0.11	0.17 ± 0.17	n.s.

Results are expressed as means ± SDs.

GERD

The prevalence of GERD was not significantly different in patients with EOA (53.2%) and LOA (66.3%). In the current cohort, we observed no relation between the presence of GERD and the severity of disease, respiratory function, or atopic markers (IgE and eosinophil count). Most patients were receiving therapy for reflux, which may explain these results.

Obesity

Obesity was an important comorbidity in this cohort (21.7% in the whole population). Although the prevalence of obesity was similar in patients with EOA (22.3%) and LOA (21.4%) (Table 1), the impact of obesity on asthma control was different in the two age-related groups. Whereas obesity was not related to disease control in patients with EOA (with the same prevalence in controlled and uncontrolled patients), it was significantly associated with disease control in patients with LOA. Concomitant obesity was present in 35% of patients with uncontrolled disease versus 12% in patients with controlled disease (Figure 8).

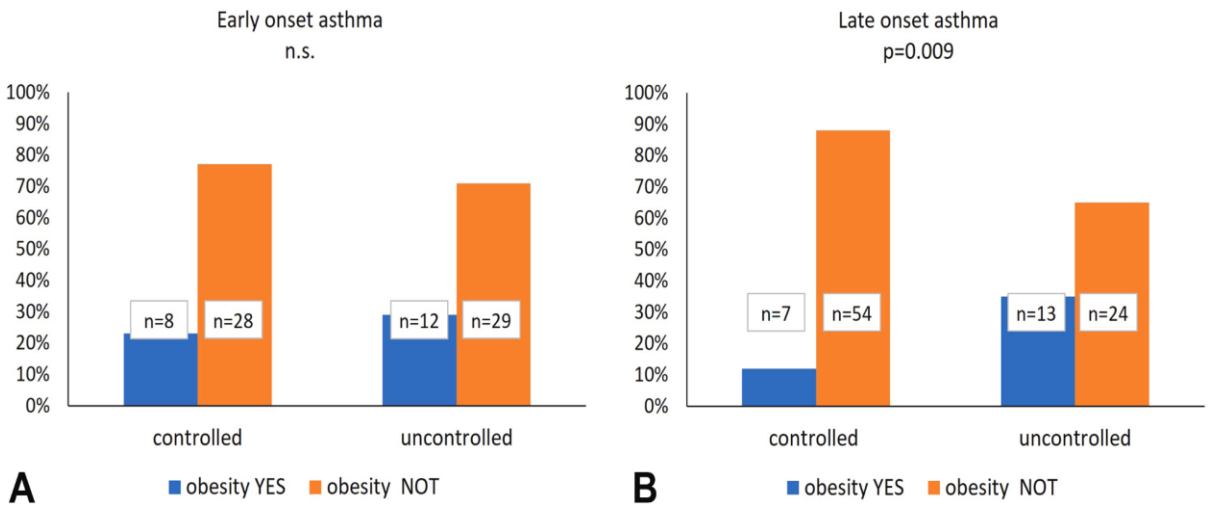

Figure 8. Association between presence of obesity and asthma control in (A) early-onset and (B) late-onset asthma. y axis indicates proportion of patients. Blue columns indicate patients with obesity, and orange columns, those without. P values represent comparison with Chi-square test.

Furthermore, EOA patients with concomitant obesity had impaired respiratory function compared with EOA patients with normal weight, mainly characterized by reduced FEV1 and FVC, with a normal FEV1 /FVC ratio (Table 3).

Table 3. Impact of obesity on respiratory function and blood tests in the two age-related groups.

	EOA (onset <12 y)			LOA (onset >40 y)		
	Obesity (n = 17)	**No obesity (n = 60)**	***P-value***	**Obesity (n = 21)**	**No obesity (n = 57)**	***P-value***
FEV1 /FVC (%)	75.62 ± 11	77.4 ± 10.4	.269	73.2 ± 9.5	80 ± 10.3	.009
FEV1 (% predicted)	78.5 ± 20.1	92.5 ± 19.7	< .0001	92.1 ± 25	103.8 ± 22.1	.003
FVC (% predicted)	86.5 ± 16.7	103.9 ± 15.2	< .0001	99.2 ± 21.2	109.9 ± 22.3	.087
FEF25-75 (%predicted)	58.3 ± 27	66.7 ± 18.2	.098	60.6 ± 24.7	75.4 ± 31.9	.02
Neutrophils (cells × 10^9 /L)	3.32 ± 1.15	3.87 ± 1.70	.178	4.20 ± 1.10	3.70 ± 1.6	.03

Results are expressed as means ± SDs.

Conversely, in LOA patients, obesity was related to an obstructive pattern (with reductions in FEV1 , FEV1/FVC, and FEF 25-75), with no difference in FVC. Finally, obese patients with LOA had a neutrophilic pattern of inflammation that was not observed in obese EOA patients (Table 3).

STUDY II

Clinical and Pathologic Factors Predicting Future Asthma in Wheezing Children. A Longitudinal Study.

Bonato M, Bazzan E, Snijders D, Tinè M, Biondini D, Turato G, Balestro E, Papi A, Cosio MG, Barbato A, Baraldo S, Saetta M. Am J Respir Cell Mol Biol. 2018 Oct;59(4):458-466.

BACKGROUND

Asthma is a disease with a highly variable clinical spectrum (1,3,103). Early-onset asthma represents a phenotypic variant generally determined by allergic T2-high endotype (113). Although it is known that asthma is influenced by age, sex, genetic background, and environmental exposure, the natural history of the disease is still poorly understood (114). Preschool wheezing is a common event in infants that may lead to asthma development during adolescence in among one third of children (115,116). The current knowledge about the evolution from wheezing to asthma originates mainly from epidemiological studies. Few is known about pathophysiological mechanisms underlying this transition.

AIM OF THE STUDY

Our aim in this study was to investigate the clinical characteristics and airways pathological features in preschool wheezing children which could herald the development of asthma later on in life. The results could lead to improved knowledge of the pathogenesis of early-onset asthma.

METHODS AND MATERIALS

Study population

Eighty preschool children (age<6 years) were recruited at the Department of Women's and Children's Health, University of Padova, Italy, from 2002 to 2014. All of the children underwent bronchoscopy for the appropriate clinical indications according to European Respiratory Society guidelines (117). Fiberoptic bronchoscopy was well tolerated by all of the children. Written consent was obtained from the children's parents. The study was performed according to the Declaration of Helsinki and was approved by the Ethics Committee of the Padova City Hospital.

Clinical-functional evaluation of patients at baseline

Wheezing condition was diagnosed at baseline by a respiratory pediatrician. He/she collected a detailed clinical history, visited the child, and administered parental interviews focused on the presence of respiratory symptoms, the treatment during the previous 12 months, and the presence of allergic manifestations.

The severity of wheezing was graded on a scale from 0 to 3 (0: no symptoms; 1: mild; 2: moderate; 3: severe), and the frequency of wheezing was graded on a scale from 0 to 6 (0: no episodes; 1: less than one episode/month; 2: one episode/month;

3: two to three episodes/month; 4: one episode/week; 5: more than one episode/week; 6: daily episodes).

At baseline, all children underwent routine blood tests, including complete blood cell counts and total and specific IgE levels The presence of atopy was defined by an increase in the total (higher than the age-related normal levels) and specific IgE (>0.35 kU/L; ImmunoCap, Phadia). In particular, specific IgEs for the following aeroallergens were investigated in all children: house dust mite (Dermatophagoides pteronyssinus and D. farinae), molds (Alternaria alternate), and cat dander and grass pollens (Lolium perenne, Poa pratensis, Phleum pratense, Dactylis glomerata, and Cynodon dactylon). Spirometry was performed only in children who were able to cooperate with the test.

Clinical-functional evaluation of patients at follow-up

At the follow-up visit, a respiratory pediatrician interviewed the children's parents or the study subjects and conducted a detailed clinical investigation to confirm or exclude asthma diagnosis. The asthma diagnosis was obtained by the respiratory pediatrician who regularly followed the children during the follow-up and was made according to clinical and lung-function criteria as recommended by current guidelines (3, 118) in children with a history of repeated episodes of wheezing, breathlessness, or cough—particularly at night or in the early morning—that were present even apart from colds, and were responsive to prescribed bronchodilators. At the follow-up visit, the typical symptoms (wheezing, shortness of breath, and cough) were to be associated with at least one of the following conditions: 1) treatment with regular or as-needed asthma medications, and 2) the presence of airflow obstruction that was reversible with bronchodilators. At the follow-up visit, pulmonary function tests (Superspiro; Micro Medical) and fractional exhaled nitric oxide (FeNO) measurements (NIOX VERO; Aerocrine) were performed.

Bronchoscopy and bronchial biopsies processing

Bronchoscopy with endobronchial biopsy and bronchoalveolar lavage were conducted at baseline in accordance with criteria laid down in the guidelines for fiberoptic bronchoscopy in children (117). Bronchoscopy with endobronchial biopsy and BAL was performed by using a flexible bronchoscope with an external diameter of 4.9 mm. Bronchial biopsy specimens were taken by using Olympus FB 19 C-1 bronchial forceps, which were inserted through the service channel of the bronchoscope (2-mm diameter). Biopsy specimens were gently extracted, fixed in 4% formaldehyde, and dehydrated through alcohol series. They were embedded in paraffin wax and processed for histochemical and immunohistochemical analysis. Bronchial biopsies were considered suitable for examination when there was at least 1.0 mm of basement membrane length and 0.1 mm^2 of subepithelial area. In particular, analysis of epithelial loss and reticular basement membrane thickness was performed on sections stained with hematoxylin–eosin (14, 15). The number of inflammatory cells (eosinophils, neutrophils, mast cells, macrophages, and CD4+ T

lymphocytes) were quantified in the subepithelium by immunohistochemistry. Mouse monoclonal antibodies were used for identification of eosinophils (anti-eosinophil cationic protein, Diagnostic Developments), neutrophils (ant-ielastase M752), mast cells (anti-tryptase M7052), macrophages (anti-CD68 M814) and CD4+ cells (anti-CD4 M834, all from Dako Ltd.). BAL fluid cell counts (total cell counts, lymphocytes, neutrophils, macrophages, and eosinophils) were performed by the Padova City Hospital Central Laboratory.

Statistical analysis

All analyses were performed using SPSS (v26, IBM Armonk, NY, USA) (level of significance $p < 0.05$). For continuous variables, normal distributions were tested using the Shapiro-Wilk test. Comparisons among groups were evaluated with either Student's t test or the Mann-Whitney U test as appropriate. Distributions of categorical variables were compared using the $\chi 2$ test or Fisher's exact test when the sample size was small ($n < 5$). Correlation coefficients were calculated using the nonparametric Spearman's rank method. Univariate logistic analyses, followed by a multivariate logistic regression, were performed to detect the strongest predictors of asthma at follow-up. The covariates included in the final models were those that were significantly different between children with and without asthma at follow-up in univariate analyses.

RESULTS

Our cohort included 80 children, all < 6 years of age (mean 3.8 ± 1 yr). The median follow-up duration was 5 years (range 1–13 yr). Table 4 reported a comparison of pathological characteristics in bronchial biopsy and BAL of children at baseline according to the presence of wheezing, as previously published by our group (119), wheezing children show airway pathology comparable to atopic asthma. Indeed, in comparison to non wheezing children, wheezing children had a significant basement membrane thickener, more destroyed epithelium, more eosinophils and mast cells.

Table 4. Comparison of Pathological Characteristics between wheezing and no wheezing children at baseline

	Wheezing at baseline	No Wheezing at baseline	*P* Value
Subjects, *n* (%)	41 (51)	39 (49)	
Epithelial loss, % (*n* = 80/80)	65 (12–100)	43 (0–100)	0.013
BM thickness, μm (*n* = 80/80)	4.7 (3.2–8.0)	3.7 (2.1–4.9)	<.001
Eosinophils, cells/mm^2 (*n* = 73/80)	55 (0–455)	12 (0–304)	.006
Neutrophils, cells/mm^2 (*n* = 71/80)	232 (0–1023)	139 (0–925)	n.s.
Mast cells, cells/mm^2 (*n* = 74/80)	324 (28–950)	104 (0–800)	.001
Macrophages, cells/mm^2 (*n* = 68/80)	113 (0–597)	94 (0–467)	n.s.
CD4+ lymphocytes, cells/mm^2 (*n* = 67/80)	239 (0–1014)	293 (0–1,108)	n.s.
BAL eosinophils, % (*n* = 74/80)	0 (0–10)	0 (0–9)	n.s.
BAL neutrophils, % (*n* = 74/80)	17 (0–88)	16 (0–91)	n.s.
BAL lymphocytes, % (*n* = 74/80)	7 (1–35)	4.5 (0–13)	n.s.
BAL macrophages, % (*n* = 74/80)	74 (6–95)	79 (4–100)	n.s.
ECP, μg/l (*n* = 77/80)	14 (2–200)	9 (2–200)	n.s.
IL-8, pg/ml (*n* = 55/80)	236 (2–4372)	351 (4.7–5728)	n.s.

Data are expressed as median (min-max). P values refer to the comparison between children with asthma and those with no asthma at follow-up.

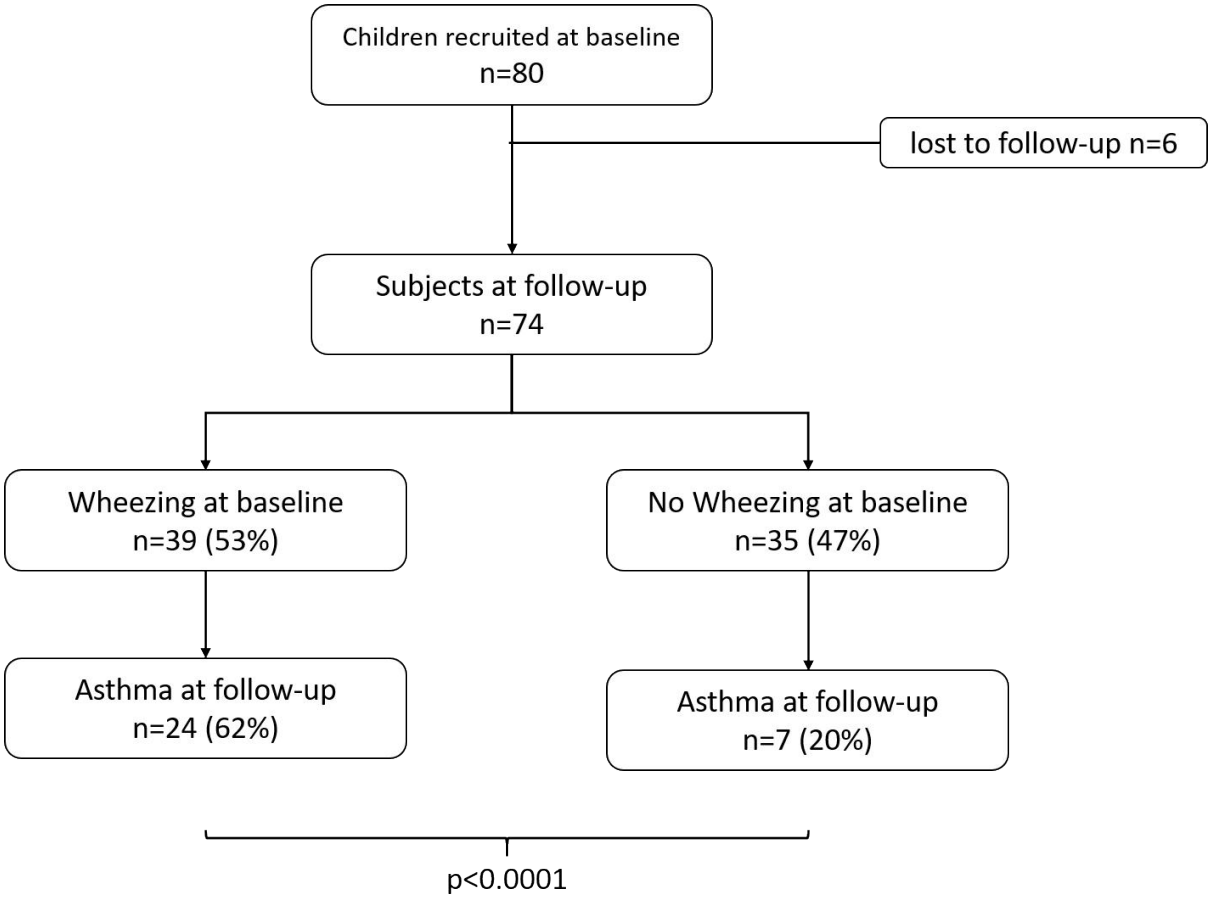

Figure 9. Flow diagram of the children included in our study stratified according to the presence of wheezing at baseline. The overall attendance rate was 92.5%.

Figure 9 summarizes the outcomes of the study. Follow-up data were available for 74 children: 54 out of 80 attended a follow-up visit, and 20 children who lived far away from our hospital completed a questionnaire by telephone and provided available clinical records. The clinical characteristics at baseline of the six children lost at follow-up were not different from those of the remaining seventy-four.

Table 5 illustrates the clinical characteristics of all of the children at baseline according to their asthma status at follow-up. At follow-up, 31 children in our cohort developed asthma (42%), and 43 did not (58%). Children with asthma at follow-up did not differ from those without asthma in sex distribution, age at baseline, age at symptom onset, and follow-up duration. Subjects who had asthma at follow-up were more likely to have wheezing at baseline (p=0.0002). Both the frequency and severity of wheezing at baseline were higher in children who developed asthma at follow-up (p=0.001, p=0.0008). There was a trend for children who developed asthma at follow-up to have increased IgE levels at baseline (p=0.07) compared with children who did not. Similarly, when we analyzed separately children with only one or two sensitizations (n = 15) and those with multiple sensitizations (n=20), we found that the percentage of children who developed asthma at follow-up was higher in multi-sensitized children (>2 allergens; 53%) than in children with 2 or less

sensitizations (35%) but the difference was not significant. Of interest, children with asthma at follow-up had lower birth weight and less breastfeeding than those without asthma (both p = 0.02). The two groups did not differ with regard to pulmonary function parameters, blood eosinophils, history of previous bronchiolitis, or parental smoking exposure.

Table 5. Clinical Characteristics at Baseline in Relation to Asthma at Follow-up

	Asthma at Follow-up	No Asthma at Follow-up	*P* Value
Subjects, *n* (%)	31 (42)	43 (58)	
Sex, male (%)	16 (51)	22 (51)	n.s.
Age, yr	3.7 ± 1	3.8 ± 0.9	n.s.
Age symptom onset, yr	1.5 ± 1.1	1.9 ± 1.1	n.s.
Follow-up duration, yr	5.2 ± 2.5	5.7 ± 2.8	n.s.
Wheezing at baseline, *n* (%)	24 (77)	15 (34)	.0002
Wheezing severity (0–3)	1 (0–3)	0 (0–3)	.0008
Wheezing frequency (0–6)	3 (0–6)	0 (0–5)	.001
FEV_1, % predicted*	103 ± 11	103 ± 13	n.s.
FEV_1/FVC, %*	93 ± 6.5	93 ± 8	n.s.
Allergic dermatitis, n (%) **	4 (13)	3 (7)	n.s.
Atopic rhinitis, n (%) **	5 (16)	7 (16)	n.s.
Blood IgE, kU/L	69 (0–3,647)	38 (0–2,188)	.07
Blood eosinophils, cells/µl	310 (0–1,760)	210 (0–990)	n.s.
Birth weight, g†	3,092 ± 631	3,542 ± 632	.02
Bronchiolitis, *n* (%)‡	7 (32)	12 (32)	n.s.
Breastfeeding > 3 mo, *n* (%)§	7 (39)	25 (71)	.02
Parental smoking, *n* (%)‖	7 (47)	11 (4)	n.s

*Definition of abbreviations: FEV1 = forced expiratory volume in 1 second; FVC = forced vital capacity; n.s. = not significant. Data are expressed as counts (percentages); mean ± SD or median (min-max). P values refer to the comparison between children with asthma and those with no asthma at follow-up. *Data at baseline were available for a subset of children (17/74), ** Data at baseline were*

available for a subset of children (54/74). †Data at baseline were available for a subset of children (46/74), ‡Data at baseline were available for a subset of children (59/74), §Data at baseline were available for a subset of children (53/74), ||Data at baseline were available for a subset of children (40/74).

Seventeen out of 74 children (23%) were treated with inhaled corticosteroids at baseline, with a higher proportion among children who developed asthma at follow-up (42%) than in those who did not (9%, p<0.0001). No difference was observed between the two groups in the proportion of children treated with oral corticosteroids (6% vs. 5%).

The results of the quantitative pathology in bronchial biopsies and BAL analysis performed at baseline according to the asthma status at follow-up are shown in Table 6. Of all parameters evaluated, only BM thickening (p=0.001) and eosinophils in bronchial tissue (p=0.026) were higher at baseline in children who developed asthma at follow-up compared with those who did not. The percentage of damaged epithelium and the number of neutrophils, macrophages, mast cells, and CD4+ lymphocytes in bronchial biopsies did not differ between the two groups of children, and neither did inflammatory cells and mediators in BAL.

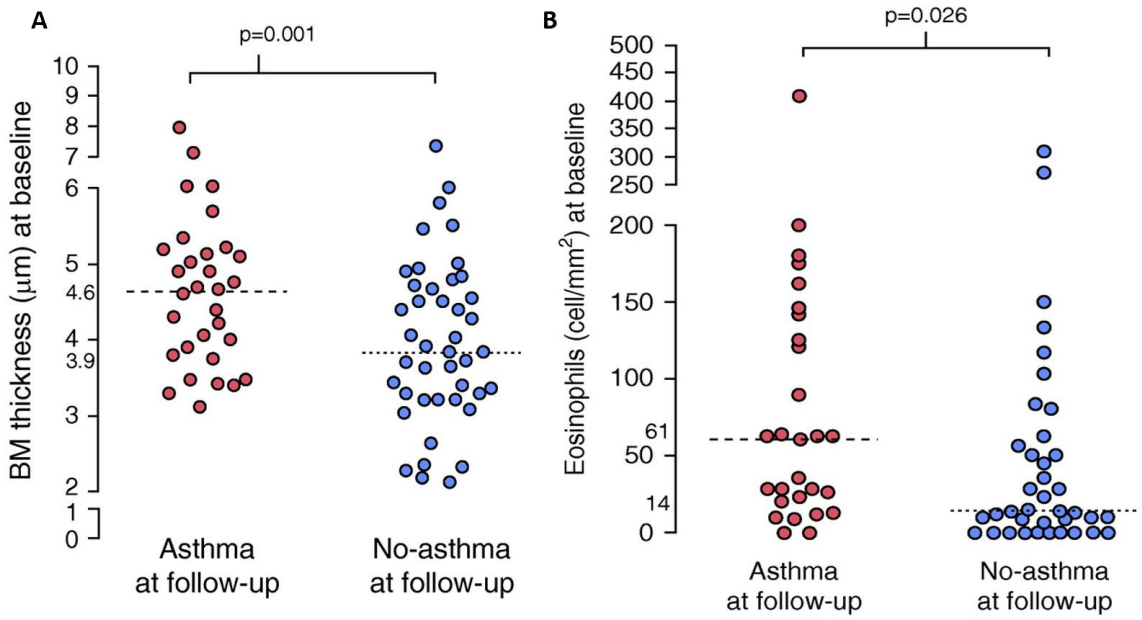

Figure 10. (A) Scatterplot reporting BM thickness in children with asthma (orange) and without asthma (blue) at follow-up. (B) Scatterplot reporting eosinophils in bronchial biopsies from children with asthma (orange) or without asthma (blue) at follow-up.

Table 6. Pathological Characteristics at Baseline in Relation to Asthma at Follow-up

	Asthma at Follow-up	No Asthma at Follow-up	*P* Value
Subjects, *n* (%)	33 (58)	31 (42)	
Epithelial loss, % (*n* = 74/74)	65 (12–100)	45 (0–100)	n.s.
BM thickness, μm (*n* = 74/74)	4.6 (3.12–8.08)	3.9 (2.1–7.4)	.001
Eosinophils, cells/mm^2 (*n* = 68/74)	61 (0–455)	14 (0–304)	.026
Neutrophils, cells/mm^2 (*n* = 70/74)	120 (0–1,023)	197 (0–925)	n.s.
Mast cells, cells/mm^2 (*n* = 63/74)	271 (28–950)	144 (0–800)	n.s.
Macrophages, cells/mm^2 (*n* = 67/74)	92 (0–597)	118 (0–467)	n.s.
CD4+ lymphocytes, cells/mm^2 (*n* = 67/74)	312 (0–1,014)	200 (0–1,108)	n.s.
BAL eosinophils, % (*n* = 69/74)	0 (0–10)	0 (0–9)	n.s.
BAL neutrophils, % (*n* = 69/74)	17 (0–68)	14 (0–91)	n.s.
BAL lymphocytes, % (*n* = 68/74)	5 (1–35)	6 (0–26)	n.s.
BAL macrophages, % (*n* = 68/74)	75 (22–97)	74 (4–100)	n.s.
ECP, μg/l (*n* = 71/74)	14 (2–200)	8 (2–200)	n.s.
IL-8, pg/ml (*n* = 51/74)	262 (2–3,000)	306 (36–5,728)	n.s.

Data are expressed as median (min-max). P values refer to the comparison between children with asthma and those with no asthma at follow-up.

Figure 10A illustrates the distribution of BM thicknesses at baseline, showing that children who developed asthma at follow-up had thicker BMs than those who did not. This finding was confirmed even when only children who were ≤3 years of age were considered (p=24; p=0.03). BM thickness was positively correlated with the frequency (r=0.49; p<0.0001) and severity of wheezing (r=0.4; p=0.0005) at baseline, and with the frequency of wheezing (r=0.41; p=0.0005) and use of bronchodilators (r=0.26; p=0.03) at follow-up. Because well-preserved airway smooth muscle was present in a minority of subjects in our study (n=10), we could not perform a complete analysis for this parameter. Figure 10B shows the distribution of tissue eosinophils in bronchial biopsies at baseline, indicating that children who developed asthma at follow-up had higher eosinophil numbers than children with no asthma at follow-up. No correlations were observed between levels of eosinophils in bronchial biopsies and other clinical characteristics at baseline or at follow-up. All these results have been confirmed independently from inhaled corticosteroid treatment at the moment of bronchoscopy.

STUDY III

Blood eosinophils relate to atopy and not to tissue eosinophils in wheezing children.

Bonato M, Bazzan E, Snijders D, Turato G, Biondini D, Tinè M, Cosio MG, Barbato A, Saetta M, Baraldo S. Allergy. 2020 Jun;75(6):1497-1501. doi: 10.1111/all.14170. Epub 2020 Feb 5. PMID: 31895479.

BACKGROUND

Asthma is a heterogeneous disease, with diverse underlying pathophysiology, usually characterized by an inflammatory response of the airways, often eosinophilic, which may differ among patients (99,120). There is a current need for accessible indicators predicting eosinophilic airway inflammation for an accurate asthma management and monitoring response to biologic therapies. Several indicators of airway eosinophilia have been proposed including BAL, sputum and blood eosinophils, BAL ECP, serum IgE and FeNO (121-123). Blood eosinophils are currently used as a proxy for airway eosinophils to differentiate eosinophilic and non-eosinophilic phenotypes even if their accuracy is at best modest. Several studies, demonstrating discordance between airway and systemic eosinophilia, have questioned the clinical utility of these biomarkers in predicting asthma outcomes (121-125). ICS therapy and atopy could be further confounding factors. The relationship between blood and tissue eosinophils has been scarcely investigated in children (126), in particular with regard to its prognostic value.

AIM OF THE STUDY

We sought to clarify the accuracy of blood eosinophils and other T2 markers (BAL-eosinophils, BAL-ECP, serum IgE) as indicators of airway eosinophilia.

METHODS AND MATERIALS

Study population

We investigated a cohort of 103 children undergoing bronchoscopy with bronchial biopsies for appropriate clinical indications.

Clinical-functional evaluation of patients at baseline

Clinical-functional evaluation protocol at baseline, including: questionnaire for symptoms and disease assessment, clinical records, spirometry and blood examination was the same in Study II.

Bronchoscopy and bronchial biopsies processing

Full details on the bronchoscopy, bronchial biopsy, and BAL procedures have been previously described in Study II. Inflammatory cells (eosinophils, neutrophils, mast cells, macrophages and CD4+ T lymphocytes) were quantified in the area extending 50 μm beneath the BM and were expressed as the number of positive cells per square millimeter of subepithelium. Basement membrane thickening and percentage of destroyed epithelium has been measured as in Study II. The presence of free

eosinophil granules was assessed using a semiquantitative score [0: no granules/scarce– 1 low/moderate density – 2 high density] and expressed as a percentage of the highest score.

Definitions

Definitions of wheezing and atopy were the same applied in Study II.

Based on published data a threshold of 23 eosinophils/mm^2 was used to define tissue eosinophilia in bronchial biopsies (127,128). Since there is no agreement on thresholds for blood eosinophilia in asthma, we considered a cut-off corresponding to the 75° percentile of our cohort (367 eosinophils/mcl).

Statistical analysis

All analyses were performed using SPSS (v26, IBM Armonk, NY, USA) (level of significance $p<0.05$). For continuous variables, normal distributions were tested using the Shapiro-Wilk test. Comparisons among groups were evaluated with either Student's t test or the Mann-Whitney-U test as appropriate. Distributions of categorical variables were compared using the Chi-square test or Fisher's exact test when the sample size was small ($n < 5$). Correlation coefficients were calculated using the nonparametric Spearman's rank method. ROC analysis with Younden correction was performed for assessing diagnostic accuracy of biomarkers considered.

RESULTS

One hundred and three children were recruited which characteristics are resumed in Table 7. According to definitions and cut-off for tissue and blood eosinophilia: 58 children had wheezing (56%), of them 42 (72%) had tissue eosinophilia (W-TisEos+) and there were no differences (Table 7, panel A) in demographic and clinical characteristics between W-TisEos+ and those with low tissue eosinophils (W-TisEos-, n=16).

Table 7. Parallel comparison of clinical-demographical and pathological characteristics of wheezing children. Panel A shows the comparison of children with and without tissue eosinophilia (W-TisEos+ vs W-TisEos-). Panel B shows the comparison of children with and without blood eosinophilia (W-BloodEos+ vs W-BloodEos-). Panel C reports data of non-wheezing children (NW).

	Panel A: TISSUE ANALYSIS			PANEL B: BLOOD ANALYSIS			PANEL C
	W-TisEos+ >23 cell/mm2	W-TisEos- <23 cell/mm2	p	W-TisBloodEos+ >367 cell/mcl	W-TisBloodEos- <367 cell/mcl	p	NW
Subjects, n (%)	42 (72)	16 (28)	n.s.	17 (29)	41 (71)	n.s.	45
Age (yrs)	5.9±3.0	4.3±2.1	n.s.	6.2±3.5	5.1±2.5	n.s.	4.9±2.6
Gender male, n (%)	17 (40)	7 (44)	n.s.	9 (52)	20 (49)	n.s.	25 (55)
Symptoms pattern (multitrigger/episodic)	33/9	11/5	n.s.	13/4	31/10	n.s.	n.a.
Symptom onset (erly/late)	29/13	13/3	n.s.	13/4	29/12	n.s.	n.a.
ICS treated, n (%) **Daily fluticasone (mcg)**	14 (33) 250 [200-400]	4 (25) 275 [100-1000]	n.s.	6 (35) 250 [200-600]	12 (29) 275 [100-1000]	n.s. n.s.	7 (15) 100 [50-1200]
OCS treated, n (%) **Daily bethametasone (mcg)**	1 (2) 1.5	0 (0) -	n.s.	0 (0) -	1 (2) 1.5	n.s.	4 (9) 1[0.5-1.5]
Atopy, n (%) **	13 (30)	6 (37)	n.s.	9 (52)	10 (24)	0.03	13 (28)
Serum IgE (kU/L)	411±862	393±841	n.s.	741±1264†	267±565	0.05	141±352
Rihinits, Y/N (%) n=71/103	7/22 (24)	4/9 (30)	n.s.	6/5 (54)£	5/26 (16)	0.01	5/24 (17)
Dermatitis, Y/N (%) n=96/103	7/31 (18)	2/14 (12)	n.s.	4/10 (28)	5/35 (12)	n.s.	12/30 (28)
Allergen sensitization, n	1 [0-3]	0 [0-3]	n.s.	1 [0-3]	0 [0-3]	0.08	0 [0-3]
Blood eosinophils cell/mcl	371±369	215±101	n.s.	617±422¥	185±87	<.0001	258±208
BAL							
Total cells, n*10^3 cells n=92/103	262 [2-1800]	184 [37-970]	n.s.	243 [12-1470]	153 [37-1800]	n.s.	200 [1-1680]
Eosinophils, % n=95/103	0 [0-10]	0 [0-4]	n.s.	0 [0-8]	0 [0-10]	n.s.	0 [0-9]
ECP, µg/L n=96/103	57±72	15±14	n.s.	69±77	36±57	n.s.	26±44
Neutrophils, % n=95/103	16 [0-88]	4 [1-44]	n.s.	16 [1-24]	11 [0-88]	n.s.	14 [0-91]
Lymphocytes, % n=94/103	6 [1-34]	5 [1-26]	n.s.	5 [1-28]	6 [1-34]	n.s.	4 [0-16]
Macrophages, % n=94/103	74 [6-95]	78 [49-95]	n.s.	74 [25-94]	76 [6-95]	n.s.	79 [4-100]
BIOPSY							
BM thickness, µm n=103/103	5.3±1.7*	4.8±1.3*	**n.s.**	5.2±0.9ψ	5.1±1.8ψ	n.s.	3.7±0.97
Destroyed epithelium, % n=97/103	66±26#	64±29#	**n.s.**	69±26$	67±27$	n.s.	44±32
Eosinophils, cell/mm^2 n=103/103	109 [25-620]§	7 [0-23]	**<.0001**	134 [0-448]¶	38 [0-620]¶	n.s.	12 [0-304]
Eos granules score, % n=103/103	50 [0-100]‡	0 [0-25]	**.0009**	50 [0-100]∀	25 [0-100]∀	n.s.	0 [0-75]
Mast cells, cell/mm^2 n=96/103	239 [0-800]	139 [12-650]	**n.s.**	279 [0-755]	117 [0-800]	n.s.	100 [0-650]
Lymphocytes, cell/mm^2 n=80/103	321 [0-1014]	124 [0-739]	**n.s.**	115 [0-1014]	216 [0-981]	n.s.	245 [0-1108]
Neutrophils, cell/mm^2 n=99/103	209 [0-1023]	141 [0-611]	**n.s.**	201 [0-1023]	114 [0-611]	n.s.	134 [0-925]
Macrophages, cell/mm^2 n=19/103	117 [0-597]	84 [0-253]	**n.s.**	126 [0-597]	100 [0-430]	n.s.	88 [0-467]

*Data are expressed as mean ± standard deviation, mean [range] or count (percentage). The p-values in table are referred to the Mann-Whitney-U or Chi-square/Fisher exact test parallel comparison between W-TisEos+ vs W-TisEos- and W-BloodEos+ and WBloodEos-. ** The presence of atopy was defined by an increase of total (higher than the age-related normal levels) and specific IgE. Comparison with the control group (NW): † p=0.005; £ p=0.02; ¥ p<0.0001; * p<0.0001 for W-TisEos+ and p=0.01 for W-TisEos-; ψ p<0.0001 for both W-BloodEos+ and W-BloodEos-; # p=0.001 for W-TisEos+ and p=0.027 for W-TisEos-; $ p=0.008 for WBloodEos+ and p=0.005 for W-BloodEos-; § p=0.0004; ¶ p<0.0001 for W-BloodEos+ and p=0.01 for W-BloodEos-; ‡ p=0.0003; ∀ p=0.03 for W-BloodEos+ and p=0.05 for W-BloodEos;*

Importantly both WTisEos+ and W-TisEos- showed airway remodeling features of asthma (basement membrane thickening and bronchial epithelial loss) when compared to children without wheezing (Figure 11A,B). Not surprisingly eosinophil degranulation in the airways was higher in W-TisEos+ than in W-TisEos- (50 [0-100] vs 0 [0-25]; p<0.0009). The accuracy of blood eosinophils, serum IgE, BAL eosinophils and ECP in predicting tissue eosinophilia was examined by ROC analyses and Spearman rank's correlation coefficient. All four indicators had similar predictive accuracies, which were overall modest (AUC range 0.56-0.64, Figure 11C, Table 8, Table 9) and were weakly correlated to tissue eosinophils (r values ranging from 0.22 to 0.34, Table 9). Furthermore, when we limited ROC analyses to wheezing children only none of the four potential indicators examined could predict tissue eosinophilia. As seen in Figure 11D only if blood eosinophils were above 367 cell/l was it highly probable that tissue eosinophilia would also be present. Conversely, lower levels of blood eosinophils did not predict low tissue eosinophils: children with blood eosinophils below 100 cell/l did have significant airway eosinophilic inflammation.

Table 8. ROC analysis

Indicators	Best cut-off	NPV	PPV	AUC	p
WHOLE COHORT					
Blood eosinophils, cell/mcl	220	0.45-0.60	0.56-0.75	0.64	0.009
Serum IgE, kU/L	19.4	0.36-0.51	0.62-0.81	0.63	0.02
BAL eosinophils, %	2	0.30-0.50	0.56-0.80	0.56	n.s.
BAL ECP, mcg/L	30.9	0.37-0.50	0.60-0.80	0.62	0.03
WHEEZERS					
Blood eosinophils, cell/mcl	450	0.21-0.34	0.77-1.00	0.63	0.07
Serum IgE, kU/L	19.4	0.29-0.50	0.50-0.71	0.57	n.s.
BAL eosinophils, %	2	0.14-0.31	0.51-0.87	0.46	n.s.
BAL ECP, mcg/L	63.5	0.21-0.36	0.75-1.00	0.62	n.s.

Table 9. Univariate and multivariate logistic regression analysis of the four indicators in predicting the tissue eosinophilia.

UNIVARIATE			
Indicators	**Rho**		***p***
Blood eosinophils, cell/mcl	0.27		0.006
Serum IgE, kU/L	0.28		0.005
BAL eosinophils, %	0.34		0.009
BAL ECP, mcg/L	0.22		0.03
MULTIVARIATE			
Indicators	**OR**	**95%CI**	***p***
Blood eosinophils, cell/mcl	1.6	-	n.s.
Serum IgE, kU/L	0.06	-	n.s.
BAL eosinophils, %	1.0	-	n.s.
BAL ECP, mcg/L	2.6	0.001-0.004	0.009

We then compared (Table 7, panel B) wheezing children with high blood eosinophils (W-BloodEos+, n=17; 29%) to those with low blood eosinophils (W-BloodEos- , n=41; 71%): no differences in demographic characteristics between the two groups were found. A finding in our study that could explain in part the dissociation between blood and tissue eosinophils is that wheezing children with high blood eosinophils had higher prevalence of atopy (52 vs 24%; p=0.03), higher levels of total serum IgE (741±1264 vs 267±565; p=0.008), increased prevalence of rhinitis (54 vs 16%; p=0.01) and tended to have more sensitizations (1 [0-3] vs 0 [0-3]; p=0.08) in comparison to W-BloodEos-. Moreover, blood eosinophils were significantly correlated to serum IgE in the whole cohort (r=0.47; p<0.0001) independently to the presence of wheezing and tissue eosinophilia (Figure 11E). These findings suggest that blood eosinophils in children are largely a marker of atopy and not of tissue eosinophilia. Along these lines, stratification by higher levels of blood eosinophils, independently from tissue eosinophils, results in the definition of an atopic phenotype with higher IgE, more rhinitis and more sensitizations, which importantly was transversal to the whole cohort, being present both in wheezing and in non-wheezing children (data shown in original article). Finally, since wheezing is often transient in childhood, we looked at persistence of asthmatic symptoms after a five years follow-up. Among children who had wheezing at baseline, 59% still reported asthma symptoms at follow-up. Emphasizing the probable discrepant role of tissue and blood eosinophils was the finding that tissue, but not blood eosinophils, were associated with persistence of symptoms over time in our population (STUDY II). Furthermore,

tissue, but not blood eosinophils, correlated with FeNO levels at follow-up (Figure 12).

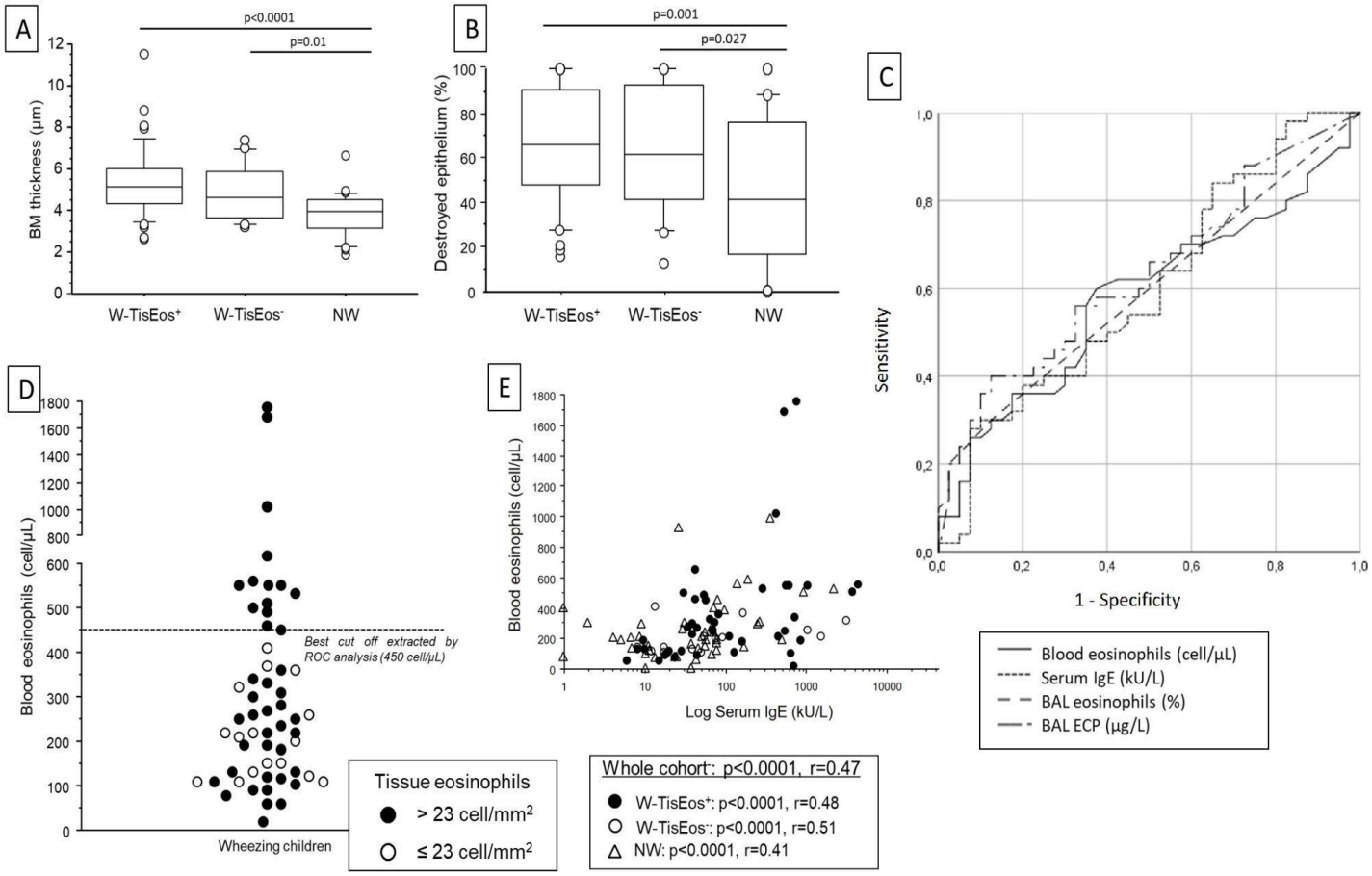

Figure 11. (A-B) Boxplots reporting values of BM thickness and destroyed epithelium in W-TisEos+, W-TisEos-. and non-wheezing children (Nw). Bottom/top of box-plot: 25th/75th percentile; solid line: median; brackets: 10th/90th percentile. (C) Receiver Operating Characteristic (ROC) curve of peripheral blood eosinophils, serum IgE , BAL eosinophils count and ECP levels in predicting bronchial biopsy eosinophilia (>23 cell/mm2). AUCs/best cut-offs in Table 1. (D) Scatterplots reporting values of blood eosinophils in wheezing children stratified according to presence of biopsy eosinophilia. Black dots represent W-TisEos+, white dots represent W-TisEos- . (E) Relationship between eosinophils in peripheral blood and serum IgE levels (expressed on logarithmic scale).

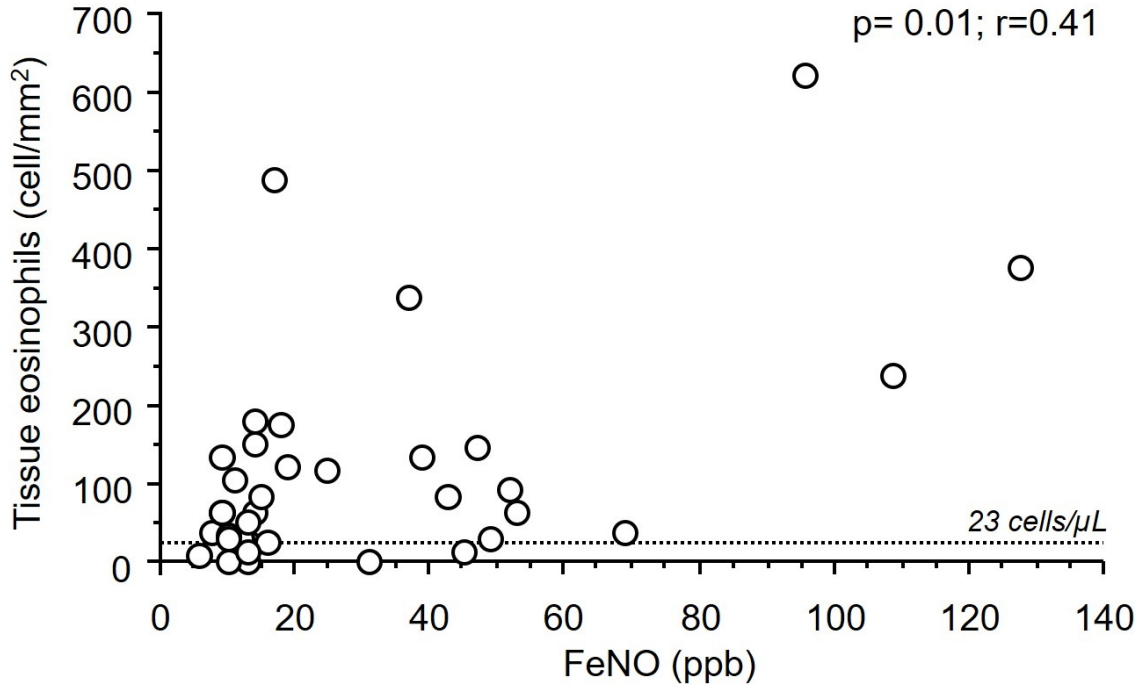

Figure 12. Relationship between eosinophils in airway submucosa at baseline and FeNO levels at follow-up in wheezing children. A significant, but modest, positive correlation emerged between baseline tissue eosinophils and FeNO levels at follow-up.

STUDY IV

Innate lymphoid cells in isocyanate-induced asthma: role of microRNA-155.

Blomme EE, Provoost S, Bazzan E, Van Eeckhoutte HP, Roffel MP, Pollaris L, Bontinck A, Bonato M, Vandenbroucke L, Verhamme F, Joos GF, Cosio MG, Vanoirbeek JAJ, Brusselle GG, Saetta M, Maes T. Eur Respir J. 2020 Sep 24;56(3):1901289.

Partnership: Laboratory for Translational Research of Obstructive Pulmonary Diseases, Ghent University (BE)

BACKGROUND

Asthma is a heterogeneous airway disease with many phenotypes. Whereas the mechanisms of early onset allergic asthma are well understood, the mechanisms leading to late onset asthma are less known. One important example of late onset asthma is occupational asthma caused by workplace exposures to respiratory sensitizers or inhaled irritants. Occupational asthma is a growing public health burden in industrialized countries. Respiratory sensitizers encompass high molecular weight (HMW) agents (e.g. wheat flour, natural rubber latex, animal proteins) and low molecular weight (LMW) agents (e.g. acrylates and diisocyanates). Toluene 2,4-diisocyanate (TDI) is an industrial intermediate which is processed into polyurethanes, used for the manufacture of foams, adhesives, paints and varnishes (129). Approximately 5-20% of workers long-term exposed to TDI develop asthma symptoms and have an accelerated decline in lung function (130). TDI-induced occupational asthma generally develops within 2-3 years since the first exposure and may be the outcome of both an inflammatory reaction and non-specific airway hyperresponsiveness (AHR) (131). Both type 1 and type 2 immune responses can be induced (131). The response to TDI exposure in sensitized asthmatics can be very severe and removal from the workplace is often the only effective approach to prevent asthma attacks (132). Elucidation of the pathogenesis of TDI-induced asthma is necessary for prevention and treatment. MicroRNAs (miRNAs) are short single stranded RNAs that negatively regulate gene expression by inhibiting mRNA translation or by degrading mRNA targets. Altered miRNA levels have a regulatory role in biological processes including stress response and inflammation. Several miRNAs have already been identified in the disease pathogenesis of asthma (133). One particular miRNA of interest is miR155, yet contradictory findings regarding its expression in airway samples and role in inflammatory response in mouse models (134) have been reported. Recently it was demonstrated that miR-155 is a critical regulator for IL-33 signaling (135) and affects innate lymphoid cell type 2 (ILC2) expression (135). ILC, the innate counterpart of T helper cells, are present in mucosal tissues and produce cytokines upon activation by alarmins (e.g. IL-33 and TSLP (thymic stromal lymphopoietin)) released by damaged epithelium. The importance of ILC2 has been investigated in several human studies and mouse models of atopic asthma (135), but little is known about this cell population in

isocyanate-induced asthma. However, whether miR-155 and ILCs contribute to airway responses towards isocyanates remains to be elucidated.

AIM OF THE STUDY

In this paper we used a mouse model of TDI-induced asthma to investigate changes in ILC and T cell subsets upon isocyanate exposure. The presence of ILC2 was examined in bronchial biopsies of TDI-induced asthmatic patients. In addition, we exposed miR-155 knockout (KO) and wild-type (WT) mice to TDI to investigate whether miR-155 contributes to isocyanate-induced airway inflammation and hyperresponsiveness.

METHODS AND MATERIALS

Murine experiments

Mice:

Male B6.Cg-Mir155tm1.1Rsky/J mice (n=6-10 per group, 5-8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred at Charles River (France). Male C57BL/6J wild-type mice were obtained from Charles River. The experimental protocol was approved by the Animal Ethical Committee of the Faculty of Medicine and Health Sciences (Ghent University (ECD 16/34)) and was carried out in accordance with institutional guidelines for animal care.

Exposure protocol:

On day 1 and 8, mice were dermally sensitized on the dorsum of both ears to 20 µl of 2% TDI (v/v) dissolved in a vehicle (a mixture of acetone and olive oil; AOO, ratio 2:3) or sole vehicle. On day 15, 22 and 29, isoflurane-anaesthetized mice were challenged (oropharyngeal administration) with 20 µl 0.01% TDI or vehicle (AOO ratio 1:4). On day 31, the animals were euthanized (Figure 14A). The AOO/AOO control group (also called vehicle group) was neither sensitized nor challenged with TDI, while the TDI/TDI group received TDI both dermally and oropharyngeally. Also an AOO/TDI group receiving no sensitization but only TDI oropharygeally was included in the experiments. Toluene 2,4-diisocyanate, acetone and olive oil were purchased from Sigma-Aldrich.

Bronchoalveolar lavage and tissue harvest:

Bronchoalveolar lavage was performed and cell counts were measured. Lungs and auricular lymph nodes were harvested and single cell suspensions were prepared (136).

Flow cytometry:

All staining procedures were performed in PBS without Ca2+ and Mg2+ containing 5 mM EDTA and 1% BSA. BAL cells were preincubated with FcR blocking antibody (anti-CD16/CD32, clone 2.4G2). Cells were stained with a combination of anti-mouse fluorochrome-conjugated monoclonal antibodies against: CD3 (145-2C11), CD5 (53-7.3), CD4 (GK1.5), CD45 (30-F11), CD11b (M1/70), CD11c (N418), CD45R (RA3-

6B2), CD90.2 (30-H12), Ly6G (1A8), Siglec-F (E50-2440), NK1.1(PK136), KLRG1(2F1/KLRG1), Ly-6G/Ly-6C(Gr-1)(RB6-8C5), FcεRIα(MAR-1), CD127(A7R34) and TCR-β (H57-597). For cytoplasmatic IFN-γ (XMG1.2), IL-13 (eBio13A), IL-17 (17B7) or matched isotype staining, cells were stimulated with ionomycin and phorbol 12-myristate 13-acetate, supplemented with brefeldin A and monensin at 37°C for 4 hours. Cell subsets were analyzed using an LSR Fortessa cytometer and FlowJo software. CD45+ and CD45- lung cells were sorted (>95% purity, data not shown) using an OctoMACS separator and CD45 microbeads (Miltenyi Biotec). To distinguish the T-helper cell subsets as well as the three ILC subsets (IFNγ-producing ILC1, IL-13-producing ILC2 and IL-17-producing ILC3), BAL cells were stimulated with PMA/ionomycin to measure intracellular cytokine production (Figure 13F).

Protein measurements:
Blood was collected from the retro-orbital plexus and centrifuged (10 min, 2500 rpm) for the isolation of serum. Total IgE measurement (BD OptEIA set mouse IgE) on serum was performed. IL-13, IL-33, IL-1 and CCL5 levels were determined by ELISA (R&D systems) in supernatants of LN cultures, in total lung homogenate and BAL supernatants respectively.

qRT-PCR:
RNA was extracted using the miRNeasy mini kit (Qiagen) and cDNA was prepared with the miScript II RT kit. miR-155 expression was determined with the miScript System (Qiagen) on a LightCycler 96 detection system (Roche) and normalized based on the expression of SNORD68 and SNORD95. The expression of mouse IL33 and HPRT was analysed with TaqMan Gene Expression assays.

Histology:
To quantify eosinophils and goblet cells, lung sections were stained with Congo Red or periodic acid-Schiff (PAS) staining respectively. Quantitative measurements were performed on an Axio Imager running AxioVision software. Hematoxylin/eosin staining was executed according to standard protocols (137).

Airway Hyperresponsiveness:
48 hours after the last instillation, airway hyperresponsiveness was measured (136). A “snapshot perturbation” maneuver was imposed to measure the resistance (R) of the whole respiratory system.

Analysis on human samples:
Study population:
Patient characteristics can be found in Table 10. Bronchial biopsies from 9 nonatopic subjects, non-smokers, with TDI-induced occupational asthma exposed to TDI at work were studied. Biopsies from 7 subjects were taken at time of diagnosis, 2-22 days after their last TDI exposure. Biopsies from 2 subjects were taken six months

after cessation of exposure to TDI. From 1 patient, biopsies were obtained both at time of diagnosis and at six months after the last TDI exposure. The study conformed to the declaration of Helsinki, and informed written consent was obtained from each subject.

Human sample processing and immunohistochemistry:
Biopsies were processed as previously described (138). ILC2 were detected with specific antibodies anti-CD3 and anti-GATA3. Sections were counterstained with hematoxylin. The number of positive cells was counted in the submucosal area up to 100 µm below the basement membrane. Positively stained cells were expressed as the number of cells per mm^2 of examined area.

In vitro human bronchial epithelial cell culture:
Primary normal human bronchial epithelial cells (HBEC) were purchased from Lonza and primary HBEC were in-house isolated (see Online repository material) from lung resection specimens from 3 different never-smoking donors obtained at the Department of Respiratory Medicine, Ghent University Hospital. Cells were grown in air-liquid interface (ALI) culture and on day 26 (donors) and 29 (Lonza), cells were exposed for 25 min to TDI (predissolved in ethylene glycol dimethyl ether (EGDME)) or to vehicle (1/50 EGDME in HBSS) on the apical side of the culture. Cells were harvested for RNA extraction and supernatant was collected 6h post exposure.

Protein measurements and qRT-PCR:
Human IL-33 and TSLP mRNA levels and reference genes HPRT1 and RPL13A were measured in HBEC with Bio-Rad PrimePCR SYBR Green Assays. miR-155/RNU48 cDNA was obtained with the TaqMan MicroRNA Reverse Transcription Kit (Thermofisher Scientific). LC480 Probes Master (Roche) and Taqman microRNA assay primers were used for the PCR reactions.

Data analysis:
Statistical analysis was performed with SPSS, version 25.0. Non-parametric tests (Kruskal-Wallis and Mann-Whitney-U) were used to compare different groups, according to the standard statistical criteria. Values were reported as mean ± SD. P values < 0.05 (*) were considered as statistically significant.

RESULTS

Isocyanate exposure in mice induces a predominant type 2 airway inflammation
Mice exposed to TDI/TDI had higher numbers of total cells and eosinophils in BAL compared to mice exposed to vehicles (Figure 13B,C), which was also confirmed by flow cytometry. Mice exposed to TDI/TDI also displayed higher numbers of CD4+ T cells and ILCs (Figure 13D,E). All examined T cell and ILC subpopulations were elevated after TDI/TDI exposure, with a predominant increase in IL-13+ CD4+ Th2 cells and IL-13+ ILC2s (Figure 13G,H). Both %ILC2 as well as %Th2 cells were significantly positively correlated with the % eosinophils in BAL. In the AOO/TDI

group, most inflammatory markers tended to be intermediate between the fully challenged group (TDI/TDI) and the vehicle-exposed group. In lung tissue, TDI exposure increased the numbers of peribronchial eosinophils and airway goblet cells (Figure 13I-L).

GATA3+CD3- cells are present in bronchial biopsies of isocyanate-induced asthma patients

Since the presence of ILC2s had not yet been reported within the context of isocyanate-induced asthma, we wanted to evaluate whether ILC2 are also present in airway samples of TDI-exposed human subjects. Therefore we performed an immunohistochemical double staining for GATA3 (expressed both in ILC2 and Th2 cells) and CD3 (specific for T cells) to identify putative ILC2s in precious bronchial biopsies from patients diagnosed with isocyanate-induced asthma (n=9). Clinical characteristics are reported in table I. In biopsies taken at the time of diagnosis of TDI-induced asthma (within 2-22 days after the last TDI exposure), GATA3+CD3- cells -compatible with ILC2s- were detected in the airway submucosa in 5 out of 7 patients (Figure 14A,B). High numbers of CD3+ cells were present in biopsies (Figure 14C).

Remarkably, GATA3+CD3- cells could not be demonstrated in biopsies from patients (n=3) after cessation of TDI exposure for at least 6 months. In the patient from whom specimens were available at both time points, GATA3+ CD3- cells, presumably ILC2, were detectable at diagnosis, but disappeared after 6 months cessation of TDI exposure, while the number of eosinophils was reduced.

Table 10. Characteristics of study population

	Median [IQR]
Number	9
Gender ratio (male/female)	6/3
Age (years)	39 [26.5-47]
Atopy (yes/no)	0/9
Onset of asthma (years)	2 [0.5-4.5]
Exposure to TDI (years)	2.5 [2.25-25.75]
Interval between last exposure and biopsy (days)	21 [10.5-26]

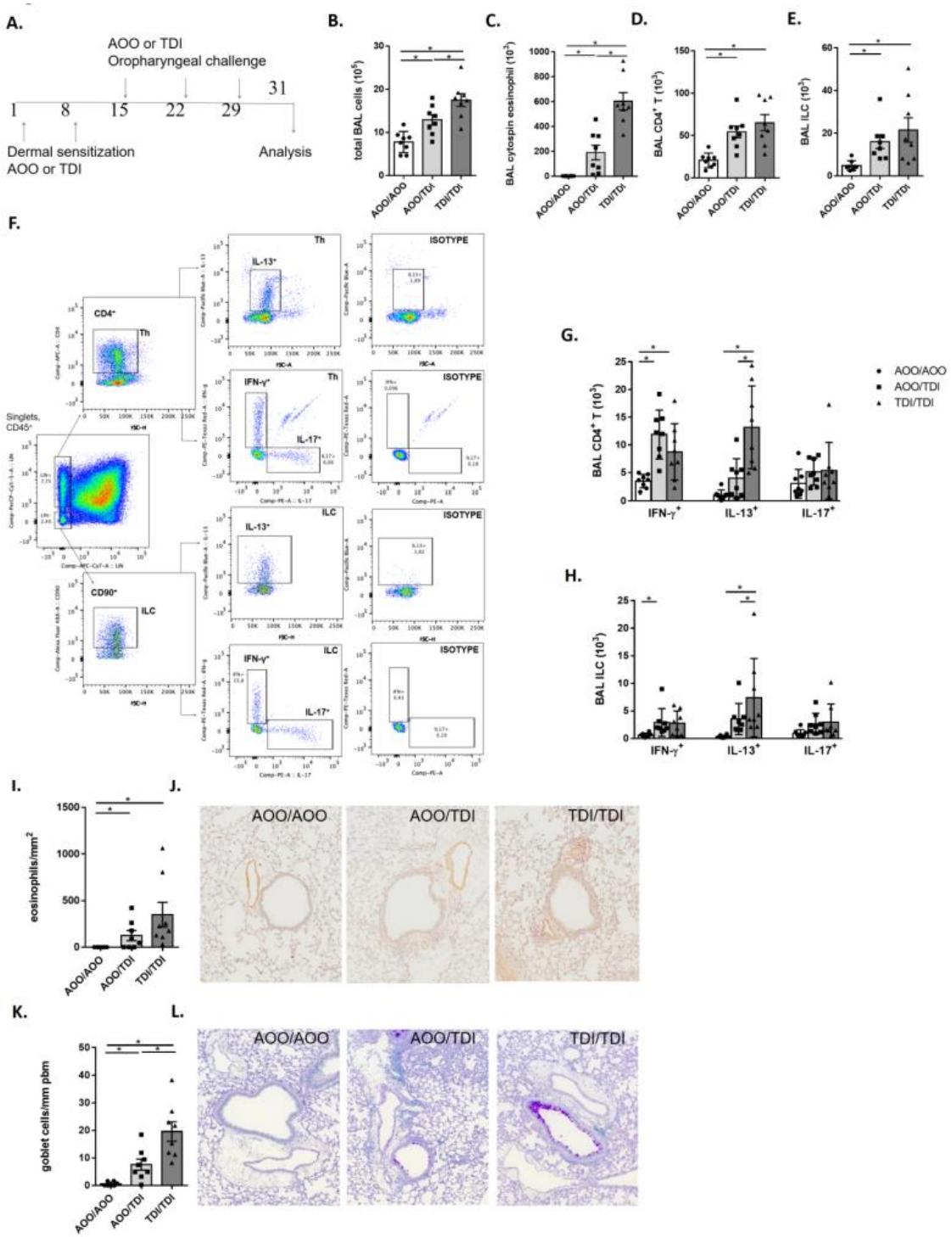

Figure 13: Exposure to TDI induces airway inflammation. WT mice were exposed to AOO/AOO (white bars), AOO/TDI (light gray bars), or TDI/TDI (dark gray bars). Exposure protocol in days (A), total BAL cells (B) and eosinophils (C), B and C were determined by cytospin. BAL cells were stimulated for 4 hours with phorbol 12-myristate 13-acetate/ionomycin + protein transport inhibitors, intracellular labeled and analyzed using flow cytometry (D-H) CD4+ T cells (CD45+ , CD11c-, CD11b-, CD45R-, CD5+, TCRβ+, CD4+) (D), ILCs (CD45+, Lin-(CD11c-, CD11b- , CD45R-, CD5-, TCRβ-), CD90+) (E), gating strategy (F), IFN-γ+(IL-17-) CD4+ Tcells, IL13+CD4+ T cells, IL-17+(IFN-γ-) CD4+ T cells (G), IFN-γ expressing ILC, IL-13+ILC andIL-17+ILC (H) are shown. Data are representative of three independent experiments. Representative photomicrographs and quantification of congo-red stained peribronchial eosinophils (I-J) and periodic acid–Schiff–stained mucus-producing goblet cells (K-L).

*Airways with a perimeter of the basement membrane larger than 800 µm and smaller than 2000 µm are included. Data are expressed as mean ± SD. N =8. * P <0.05.*

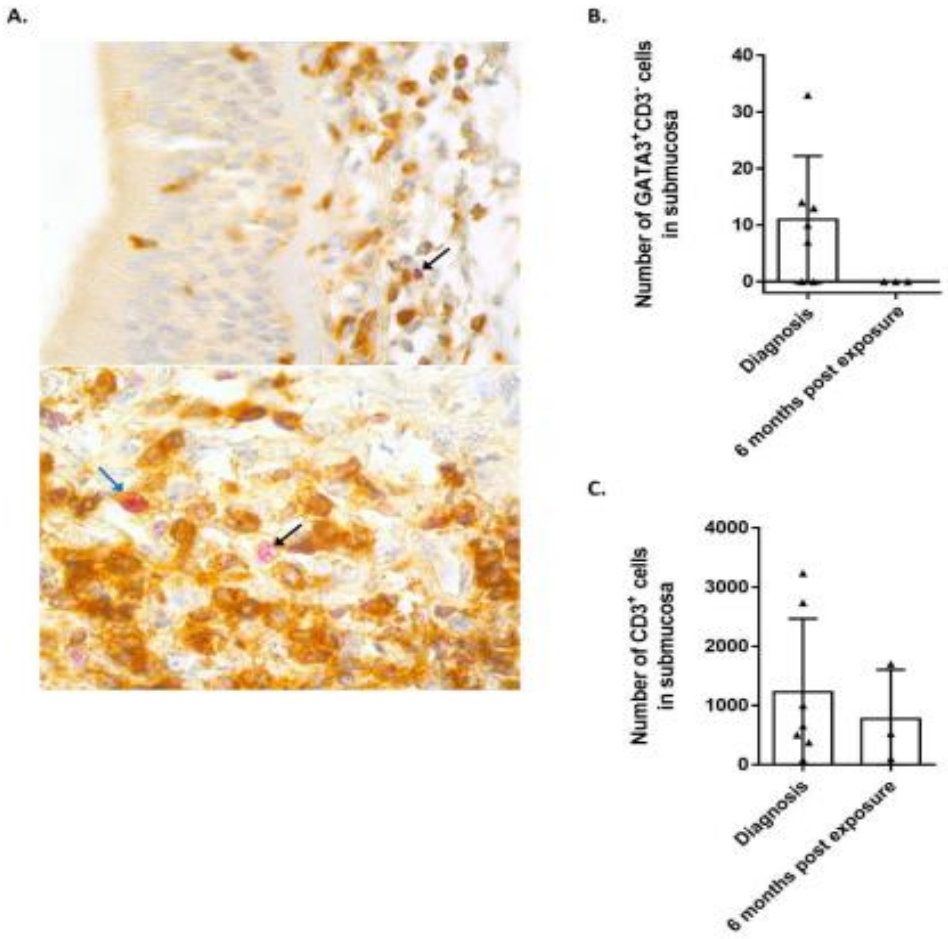

Figure 14: Immunohistochemical detection of GATA3+CD3-cells and T cells in human lung tissue. GATA3 in pink, CD3 in brown. Upper panel: representative examples of immunohistochemistry for GATA3+CD3-cells (in pink, black arrow) and CD3+cells (in brown) at time of diagnosis in the submucosa of an asthmatic subject who developed asthma symptoms after a period of exposure to TDI. Original magnification 63X. Lower panel: GATA3+CD3+cell (blue arrow) and GATA3+CD3- cell (black arrow), CD3+cells in brown. Original magnification 100X. (A). Quantification of GATA3+CD3-cells (B) and CD3+cells (C) at time of diagnosis and 6 months post exposure. Data are expressed as mean ± SD. N =3-6

<u>Isocyanate exposure can induce IL-33 expression in human bronchial epithelial cells and in the mouse model of TDI asthma</u>

Given that IL-33 and TSLP activate ILC2s and promote the induction of type 2 responses, we next assess whether TDI can activate the epithelial-driven IL-33 or TSLP pathway by exposing human bronchial epithelial cells (HBEC) to TDI or

vehicle. TDI exposure in commercially available HBEC (Lonza) led to an induction of IL-33 mRNA levels compared to vehicle control (Figure 15A). Of note, in in-house isolated primary HBEC, the impact of TDI on IL-33 expression varied between donors, as a similar trend was only observed for 1 out of 3 donors. TSLP mRNA expression either tended to increase or decrease upon TDI exposure, depending on the donor (data not shown). In murine lung homogenates, IL-33 protein levels were significantly increased in response to TDI (Figure 15B). Murine IL-33 mRNA was predominantly expressed in the non-hematopoietic (CD45-) compartment and was highest in the AOO/TDI group (Figure 15C).

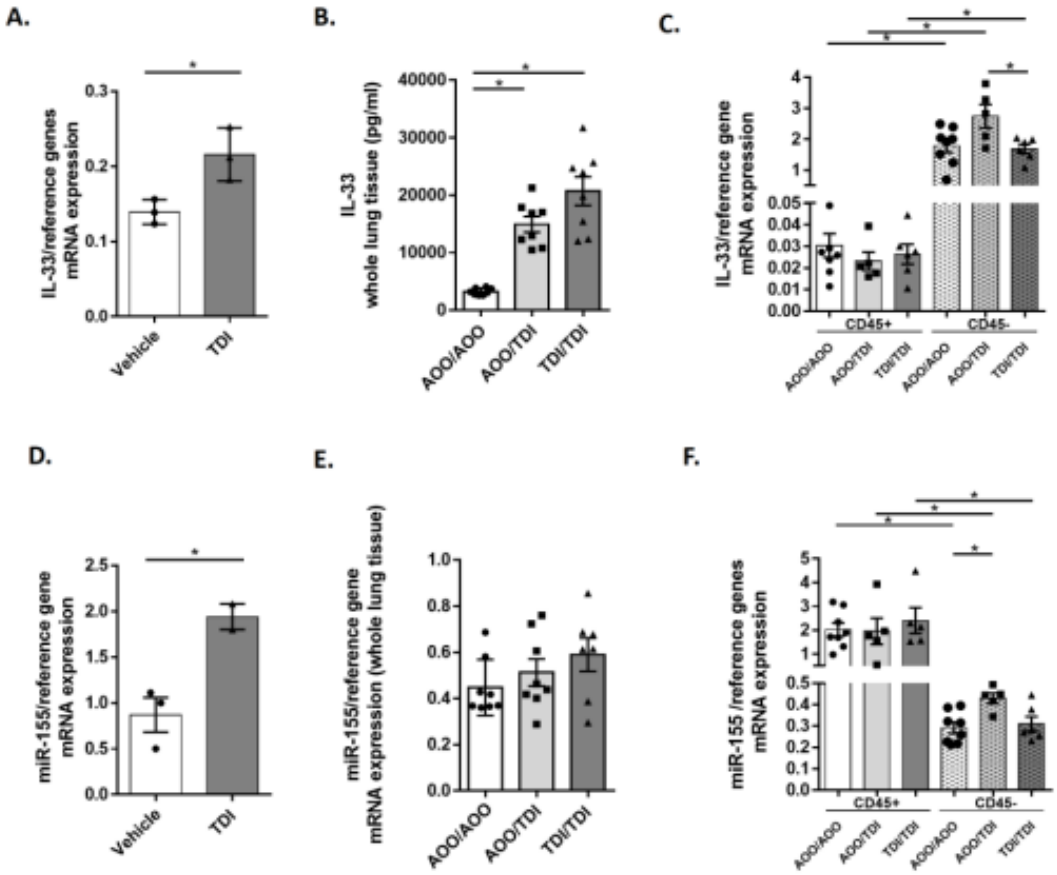

*Figure 15: IL-33 and miR-155 expression following TDI exposure. IL-33 mRNA levels were determined in vitro in HBEC (N=3 technical replicates) and normalized to HPRT and RPL13A. Vehicle=1/50 EGDME in HBSS (A) Murine IL-33 protein levels in lung homogenate (B) and IL-33 mRNA expression in murine sorted CD45+ and CD45- lung cells normalized to HPRT (C). miR-155/RNU48 mRNA expression in HBEC (D), miR-155/snorD95 mRNA expression in murine whole lung tissue (E) miR155 mRNA expression in murine sorted CD45+ and CD45- lung cells normalized to SNORD95 and SNORD68 (F). Murine data are expressed as mean ± SD. N =6-8. * P <0.05.*

<u>Isocyanate-induced airway inflammation is miR-155 dependent in mice</u>

Since proliferation of lymphoid cells –including ILC2s- is regulated by miR-155, a microRNA associated with allergic airway inflammation, we evaluated the expression of miR-155 in HBEC and in the mouse model of TDI asthma. TDI exposure in commercially available HBEC (Lonza) led to an induction of miR-155 expression

compared to vehicle control (Figure 15D), yet this was less apparent using the in-house generated HBEC. In mice, the expression of miR-155 was detectable in total lung tissue and significantly higher in the hematopoietic (CD45+) cell compartment compared to the non-hematopoietic (CD45-) cell compartment (Figure 15E,F). Interestingly, in the CD45- cell compartment miR-155 expression was upregulated in the AOO/TDI group. However, the miR-155 expression in total lung tissue or LN was not significantly modulated by TDI exposure (Figure 15E). Considering the controversial role of miR-155 in asthma, we subsequently evaluated the effect of miR-155 deficiency on isocyanateinduced type 2 airway inflammation by exposing miR-155 KO and WT mice to vehicle or TDI. The number of TDI-induced eosinophils was strongly reduced in miR155 KO mice (Figure 16A). Moreover, the TDI-induced increase in total and IL-13 producing CD4+ T cells and ILCs was significantly attenuated in TDI-exposed miR155 KO mice compared to WT mice (Figure 16B-E). An attenuated increase in the number of IFN-γ or IL-17 producing T-cells and IL-17 producing ILCs was also observed. In lung tissue, peribronchovascular eosinophilic inflammation and airway goblet cell metaplasia upon TDI exposure were significantly reduced in miR-155 KO mice compared to the corresponding WT mice (Figure 16F,G). The TDI-induced inflammation in lung tissue in WT mice, visualized by Haematoxylin & Eosin-staining, was absent in miR-155 KO mice (Figure 16H).

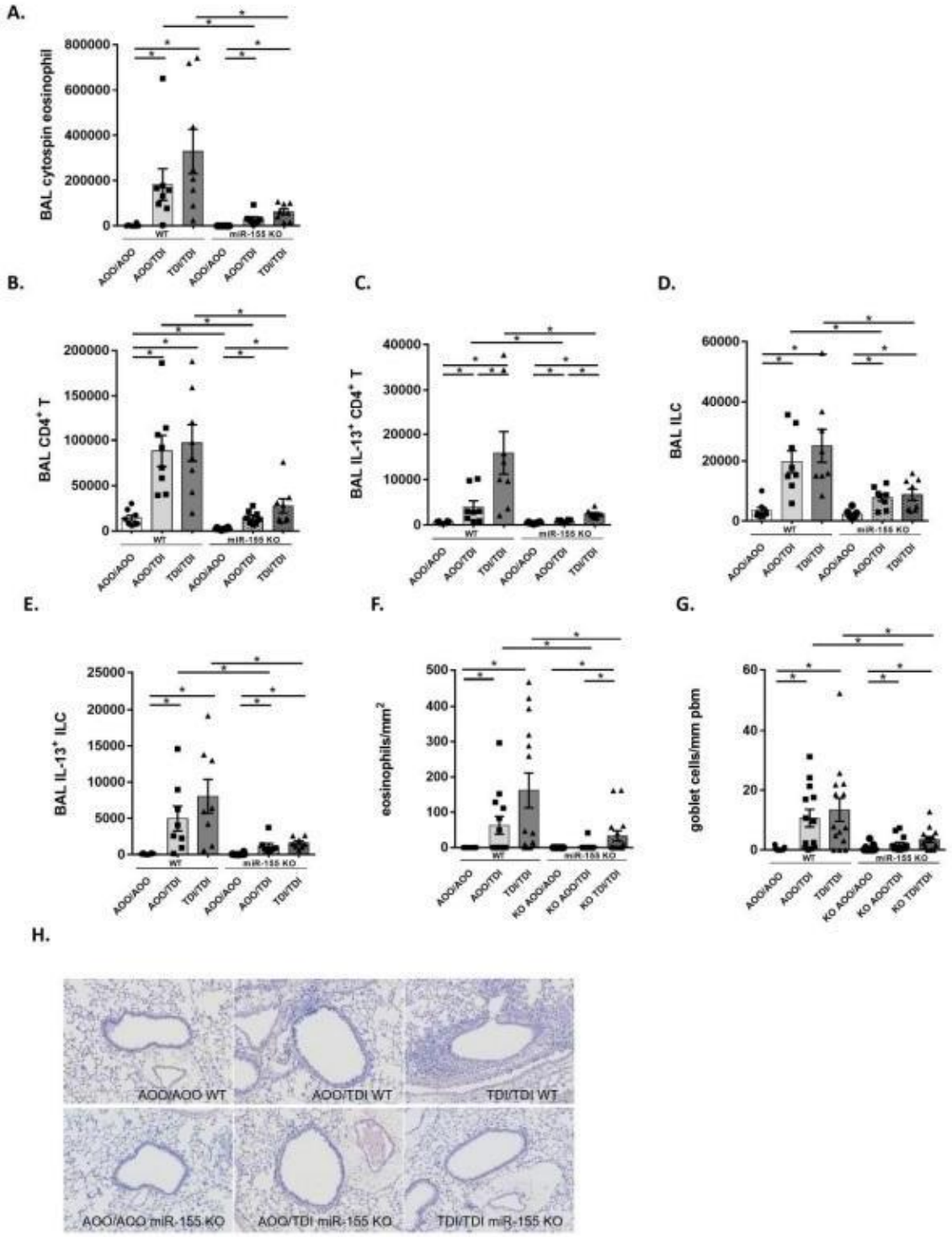

*Figure 16: Absence of miR-155 attenuates TDI-induced inflammation. WT and miR-155 KO mice were exposed to AOO/AOO (white bars), AOO/TDI (light gray bars) or TDI/TDI (dark gray bars). Total eosinophils (A) were determined on cytospin. BAL cells were stimulated for 4 hours with phorbol 12-myristate 13- acetate/ionomycin + protein transport inhibitors, intracellular labeled and analyzed using flow cytometry (B-E). CD4+ T cells (CD45+ , CD11c- , CD11b- , CD45R- , CD5+ , TCRβ + , CD4+) (B), IL13+ CD4+ T cells (C), ILCs (CD45+ , Lin- (CD11c- , CD11b- , CD45R- , CD5- , TCRβ -), CD3- , CD4- , CD90+) (D), IL-13+ ILC (E). Data are representative of at least two independent experiments and expressed as mean ± SD. N =8. * P <0.05. Quantification of congo-red stained peribronchial*

*eosinophils (F) periodic acid–Schiff–stained mucus-producing goblet cells (G) and photomicrographs of Haematoxylin & Eosin-stained lung tissue (H). Data are combined of two independent experiments and represented as mean ± SD. N =13- 18. * P <0.05*

TDI-induced pulmonary expression of IL-33 and IL-1, but not CCL5, is miR-155 dependent

To unravel the mechanisms underlying the attenuated inflammatory responses in the TDI- exposed miR-155 KO mice, we investigated the expression of the type 2-promoting cytokine IL-33, IL-1 as well as the T-cell and eosinophil-attracting chemokine CCL5 (139, 140). The TDI-induced IL-33 protein levels in lung homogenate of miR-155 KO mice were significantly decreased compared to the corresponding TDI-exposed WT mice after combined dermal sensitization and airway challenge (TDI/TDI), but not after sole airway challenge (AOO/TDI) (Figure 17A). IL-1 protein levels also increased upon TDI exposure compared to vehicle. Notably, IL-1 expression was miR-155 dependent after AOO/TDI exposure, but not after TDI/TDI exposure (Figure 17B). TDI-exposure also resulted in a strong increase of CCL5 expression compared to the vehicle group, which was however similar in WT and miR-155 KO mice (Figure 17C).

Role of miR-155 in TDI-induced type 2 responses in lymph nodes and in serum IgE

To investigate type 2 responses in the lymph nodes, auricular lymph nodes of WT and miR-155 KO mice were isolated and stimulated with concanavalin A; next IL-13 protein levels were measured in the cell culture supernatant. The auricular lymph nodes from TDI/TDI-exposed mice showed a significant increase in IL-13, with no difference between the genotypes (Figure 17D). TDI exposure led to elevated total IgE levels in serum in WT mice, which was absent in the miR-155 KO mice (Figure 17E).

Effect of miR-155 deficiency on isocyanate-induced airway hyperresponsiveness

To investigate the involvement of miR-155 in TDI-induced airway hyperresponsiveness, the peak resistance in response to increasing concentrations of carbachol was measured in WT and miR-155 KO mice. In the TDI/TDI group, there was a nominal increase in the peak resistance in WT mice, compared to the vehicle-exposed control group and the AOO/TDI group. TDI exposure in the miR-155 KO mice did not increase airway responsiveness (Figure 17F).

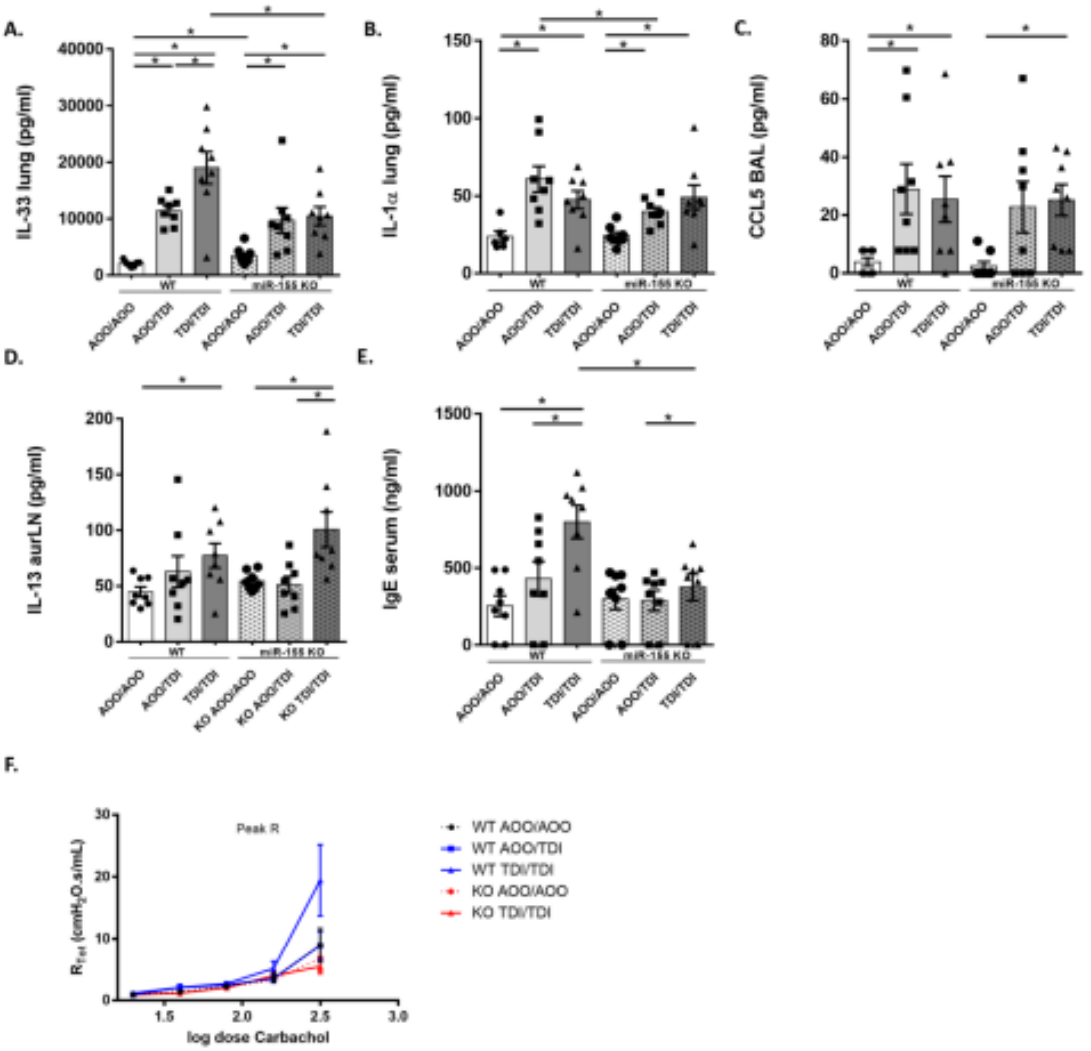

*Figure 17: Role of miR-155 in TDI-induced type 2 responses and airway hyperresponsiveness. Murine IL-33 protein levels in whole lung homogenate (A), IL-1α protein levels in whole lung homogenate (B), CCL5 protein levels in BAL (C). IL-13 protein levels in the supernatants of concanavaline A stimulated auricular LNs (D) and IgE titers in serum (E) were determined by ELISA. Airway resistance (R) of WT mice (black & blue lines) or miR-155 KO mice (red lines) exposed to TDI or vehicle (AOO/AOO) was measured in response to increasing doses of carbachol (F). No R-measurement in AOO/TDI miR-155 KO group due to limited availability of miR155 KO mice. Data of A-D are expressed as mean ± SD, data of (E) are expressed as mean ± SEM, N =8-10. * P <0.05.*

STUDY V

Innate lymphoid cells 2 (ILC2) in the transition from pediatric wheezing to asthma

Bonato M, Bazzan M, Maes T, Brusselle G, Turato G, Padrin Y, Turrin M, Cosio MG, Barbato A, Saetta M, and Baraldo S. Thematic poster at European Respiratory Society Conference 5th-9th of September 2022.

Partnership: Laboratory for Translational Research of Obstructive Pulmonary Diseases, Ghent University (BE)

BACKGROUND

Asthma is a heterogeneous disease. Multiple phenotypes, characterized by different clinical presentations and functional patterns have been described with different implications about prognosis and treatment response (105). Despite increasing advances in these fields, biological mechanisms (endotypes) underlying phenotypes are still not fully understood. Type-2 inflammation, characterized by eosinophilic airway inflammation, is detectable in over 80% of asthmatics (141) and in the majority of cases, it is driven by Th2 lymphocytes, especially in atopic asthma phenotype (99,142). Data from large cohort studies on severe asthma suggest that evidence of eosinophilic inflammation can occur also without an allergic background (5, 143, 144). Recent data support a role for type 2 innate lymphoid cells (ILC2s) as an important determinant of airway eosinophilia in adult asthma as an important source of IL-5 and IL-13 (143, 144) indeed we demonstrated that ILC-2 has a crucial role in pathogenesis of TDI related asthma, a model of T2-high non-atopic asthma (STUDY IV). Although ILC2s have also been identified in humans, studies on the role of human ILC2s in asthma are very limited, especially in children.

AIM OF THE STUDY

As for adult asthmatics, we hypothesize also in wheezing children, the existence of several endotypes: T2-high atopic, T2-high non-atopic and T2-low and their related clinical-functional phenotypes. Moreover, we assume that in T2-high non-atopic endotype ILC-2 can be the major driver of type-2 inflammation.

METHODS AND MATERIALS

Study population

One hundred and nine children were recruited at the Department of Women's and Children's Health, University of Padova, Italy, from 2002 to 2014. All of the children underwent bronchoscopy for the appropriate clinical indications according to European Respiratory Society guidelines (117,145). Fiberoptic bronchoscopy was well tolerated by all of the children. Written consent was obtained from the children's parents. The study was performed according to the Declaration of Helsinki and was approved by the Ethics Committee of the Padova City Hospital.

Clinical-functional evaluation of patients at baseline

Clinical-functional evaluation protocol at baseline, including: questionnaire for symptoms and disease assessment, clinical records, spirometry and blood examination was the same in Study II.

Bronchoscopy and bronchial biopsies processing

Full details on the bronchoscopy, bronchial biopsy, and BAL procedures have been previously described in Study II. Inflammatory cells (eosinophils, neutrophils, mast cells, macrophages, CD4+ T lymphocytes) were quantified in the area extending 50 μm beneath the BM and were expressed as the number of positive cells per square millimeter of subepithelium. Basement membrane thickening and percentage of destroyed epithelium has been measured as in Study II. IL4+ and IL5+ cells have been also quantified as for other inflammatory cells. Innate lymphoid cells type 2 (ILC2) have been quantified in bronchial biopsies as in Study IV: ILC2 were detected with specific antibodies anti-CD3 and anti-GATA3, ILC2 were defined as GATA-3+ and CD3- cells (Figure 6).

Clinical-functional evaluation of patients at follow-up

Clinical-functional evaluation protocol at follow-up, including: questionnaire for symptoms and disease assessment, clinical records, spirometry and blood examination was the same in Study II.

Definitions

Definitions of wheezing and atopy were the same applied in Study II as for definition of asthma at follow-up. Based on published data a threshold of 23 eosinophils/mm^2 in bronchial biopsies was used to discriminate between T2-high and T2-low endotypes in wheezing children (127).

Statistical analysis

All analyses were performed using SPSS (v26, IBM Armonk, NY, USA) (level of significance $p<0.05$). For continuous variables, normal distributions were tested using the Shapiro-Wilk test. Comparisons among groups were evaluated with either Student's t test or Mann-Whitney-U or Kruskal-Wallis test as appropriate. Distributions of categorical variables were compared using the Chi-square test or Fisher's exact test when the sample size was small ($n<5$).

RESULTS

One hundred and nine children were recruited. Among them, 61 (55.9%) reported current wheezing at the time of bronchoscopy, while 48 (44.1%) did not report wheezing (NW). Among wheezing children, 21 (34.4%) had T2-high atopic wheezing (T2H-A), 24 (39.3%) had T2-high non-atopic wheezing (T2H-nA) and 16 (26.3%) had T2-low (T2L) wheezing.

Clinical and functional characteristics of the three endotypes are reported in Table 11.

Table 11. Clinical and functional characteristics of three endotypes and non wheezing children

	T2H-A	T2H-nA	T2L	NW	p-value
Subjects, n (%)	21 (34.4)	24 (39.3)	16 (26.3)	48 (44.1)	-
Male, n (%)	11 (52.3)	7 (29.1)^	12 (75)	26 (54.1)	.032
Age, y	7.1 ± 3.5*	4.9 ± 1.9	4.3 ± 1.9	4.9 ± 2.5	.018
Age of symptoms onset, y	2.7 ± 3.3	2.0 ± 1.9	1.5 ± 1.3	n.a.	n.s.
Symptoms frequency [0-6]	3.5 (3-5)	3 (2.5-4)	3 (3-4.25)	n.a.	n.s.
OCS treated, n (%)	1 (4.7)	1 (4.2)	0 (0)	5 (10.4)	n.s.
ICS treated, n (%)	8 (38.1)	9 (37.5)	4 (25)	8 (16.6)	n.s.
FEV1, %pred.	87 ± 13	95 ± 14	87 ± 15	104 ± 11§	.018
Blood eosinophils, cell/mcl	468 ± 479#	251 ± 154	215 ± 101	246 ± 207	.047

*Data are expressed as mean ± standard deviation, frequency absolute (percentage), median (interquartile range). P-values have been calculated using Kruskal-Wallis test. OCS=oral corticosteroids, ICS=inhaled corticosteroids, FEV1=forced expired volume in the first second of expiration ^significantly lower than NW (p=0.038) and T2L (p=0.003); *significantly higher than T2H-nA (p=0.018), T2L (p=0.009) and NW (p=0.008); § lower than T2H-A (p=0.013), T2H-nA (p=0.069), T2L (p=0.033); #significantly higher than T2H-nA (p=0.041), T2L (p=0.045) and NW (p=0.008)*

We observed that T2H-A were significantly older and had more blood eosinophils than T2H-nA, T2L and NW, while the prevalence of females was higher in the T2H-nA endotype. Age of symptoms onset and frequency did not differ between the three endotypes. All three groups showed a lower respiratory function impaired than non wheezing children (although in the limit of normality). Finally, the prevalence of children on treatment with oral and inhaled corticosteroids did not differ between the three groups and non wheezers.

Table 12. Pathological characteristics of three endotypes and non wheezing children

	T2H-A	T2H-nA	T2L	NW	p-value
Subjects, n (%)	21 (34.4)	24 (39.3)	16 (26.3)	48 (44.1)	-
BM thickness, µm	5.1 (3.9-5.8)	4.8 (3.9-5.9)	4.6 (3.6-5.8)	3.8 (3-4.9)^	<.001
Destroyed epithelium, %	71 (47.7-83.5)	66 (63-96)	62 (41-93)	40 (14-77)*	.007
Eosinophils, cells/mm²	134 (78.7-194.8)§	74 (36.6-141.6)‡	7.1 (0-13)	11.5 (3.5-47.8)	<.001
Neutrophils, cells/mm²	123.9 (71.5-426.7)	225 (69.9-365.4)	141 (51.8-301.9)	132.7 (69-188)	n.s.
Macrophages, cells/mm²	125.6 (77.9-288.2)	76.8 (0-194.5)	84.5 (0-125.7)	82 (36-198.9)	n.s.
Mast cells, cells/mm²	103.3 (15.5-330.8)	124.4 (89.4-444.5)	139.1 (78.3-412.5)	91 (56-225)	n.s.
Lymphocytes, cells/mm²	197.2 (62.3-529.5)	327.2 (116.9-755.3)	137 (63.3-210.3)	276.1 (152-386.5)	n.s.
IL-4+ cells, cells/mm²	151.4 (140.8-259)£	98.3 (51.2-128.5)	87.8 (55.5-100.2)	42.1 (28-61.5)	.001
IL-5+ cells, cell/mm²	364.3 (300.5-415.5)†	404 (342.5-498.5)+	309 (154.2-330.2)	303 (184-338)	<.001
ILC-2, cells/mm²	1.7 (0-9.5)	10.9 (7.6-33)$	1.5 (0-11.8)	0 (0-6.2)	.002

*Data are expressed as frequency absolute (percentage), median (interquartile range). P-values have been calculated using Kruskal-Wallis test. ^significantly lower than T2H-A (p<0.001), T2H-nA (p<0.001) and T2L (p=0.001); *significantly lower than T2H-A (p=0.009), T2H-nA (p=0.003) and T2L (p=0.028); ç significantly higher than T2L (p<0.001) and NW (p<0.001); ‡significantly higher than T2L and NW (p=0.003 for both); $ significantly higher than T2H-A (p=0.01), T2L (p=0.005) and NW (p=0.045); £ significantly higher than T2H-nA (p=0.049), T2L (p=0.027) and NW (p=0.001); †significantly higher than T2L (p=0.047) and NW (p=0.047); +significantly higher than T2L (p=0.047) and NW (p=0.052).*

Pathological findings

Table 12, resume pathological characteristics (both structural and inflammatory) of bronchial biopsies among the three endotypes and non-wheezing children. All wheezing children, independently from their endotype, showed structural alterations typical of asthma: a thickened basement membrane and epithelial shedding (Figure 18A and B). By definition, T2H-A and T2H-nA showed more eosinophils in bronchial biopsies than T2L but importantly we did not observe significant quantitative differences in eosinophilic infiltrate among T2H-A and T2H-nA. Regarding type 2 interleukins, IL4+ expression was significantly higher in T2H-A in comparison to other endotypes and NW (Figure 19A) while IL5+ expression was higher in T2H endotypes independently from atopy presence (Figure 19B). Finally, innate lymphoid type 2 cells were significantly increased in T2H-nA in comparison to T2H-A, T2L and NW (Figure 19C).

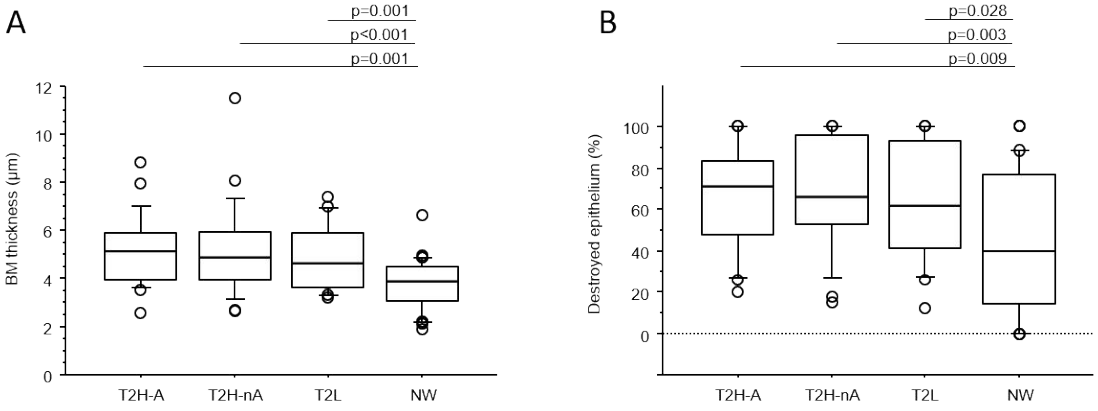

Figure 18. Boxplots reporting structural features typical of asthma basement membrane thickness (A), destroyed epithelium (B)in children with T2-high atopic (T2H-A), T2 high non-atopic asthma (T2H-nA), T2-low asthma (T2L) and non-wheezing children (NW). Bottom and top of the box-plot: 25th and 75th percentiles; solid line: median; brackets: 10th and 90th percentiles.

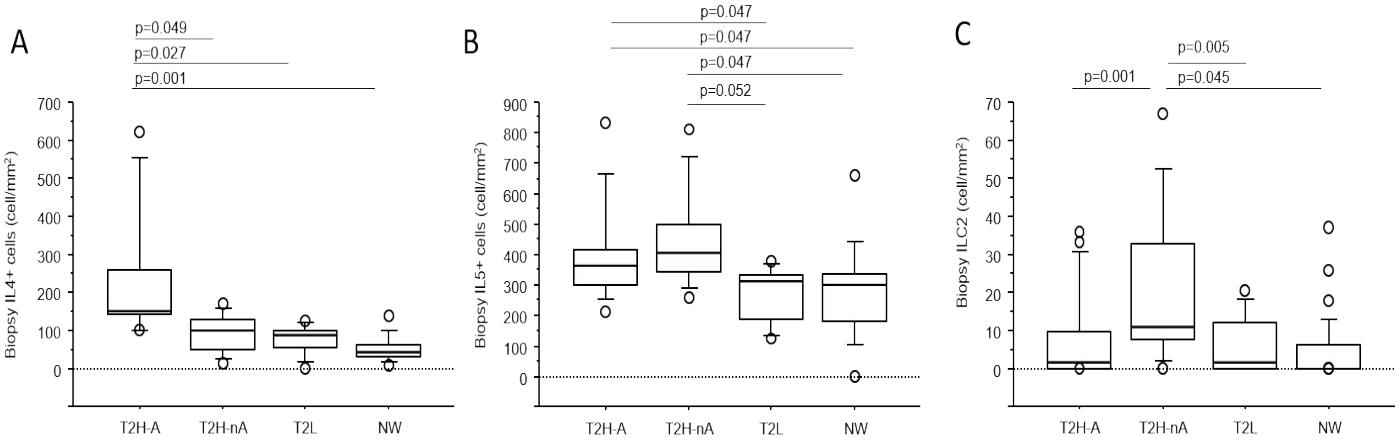

Figure 19. Boxplots reporting values of IL-4 expression (A), IL-5 expression (B) and ILC2 counts (C) in children with T2-high atopic (T2H-A), T2 high non-atopic asthma (T2H-nA), T2-low asthma (T2L) and non-wheezing children (NW). Bottom and top of the box-plot: 25th and 75th percentiles; solid line: median; brackets: 10th and 90th percentiles.

Relation between inflammatory parameters

We observed in the whole cohort, that the number of eosinophils in bronchial biopsies was directly related to ILC2 (p=0.014; r=0.282; figure 20A), IL-5+ cells (p<0.001; r=0.595; figure 20B) and IL-4+ cells (p<0.001; r=0.510; figure 20C), while ILC2 number was directly related to IL-5+ cells (p=0.002; r=0.494; figure 20D) but not to IL-4+ cells (figure 20E). Moreover, IL4+ cells were directly related to serum total IgE (p=0.012; r=0.373; figure 20F). When we assessed these analyses stratifying for the single groups we did not observe any significant correlation.

Clinical evolution of endotypes at follow-up

Considering wheezing as a transitory condition, we performed a follow-up (median 5.5 years) of our cohort to confirm the persistence of symptoms across growth. At follow-up, data of 93 children out of 109 were available. Sixteen children refused to participate at follow-up for geographical distance. Among 93 children, 38 (40.8%) received asthma diagnosis and among them 30 (32.2%) had allergic asthma (AA) while 8 (8.6%) had non-allergic asthma (nAA). Conversely, 55 children (59.2%) were non asthmatics (NA). Figure 21A shows the evolution of our cohort from baseline to follow-up in relation to the three endotypes object of the study. The highest incidence of symptoms remission from baseline to follow-up has been observed in the T2L group (57.3%) while both T2 high endotypes showed a similar percentage of remission (31.9% and 40.9%). Interesting, while all T2H-A who did not lose symptoms at follow-up remain atopic, in T2H-nA we observed a transition from non-atopic to atopic endotype (p=.091). Finally, in T2H-nA endotype, we observed that baseline ILC2 were higher in those who developed atopic asthma at follow-up (p=0.04; figure 21B).

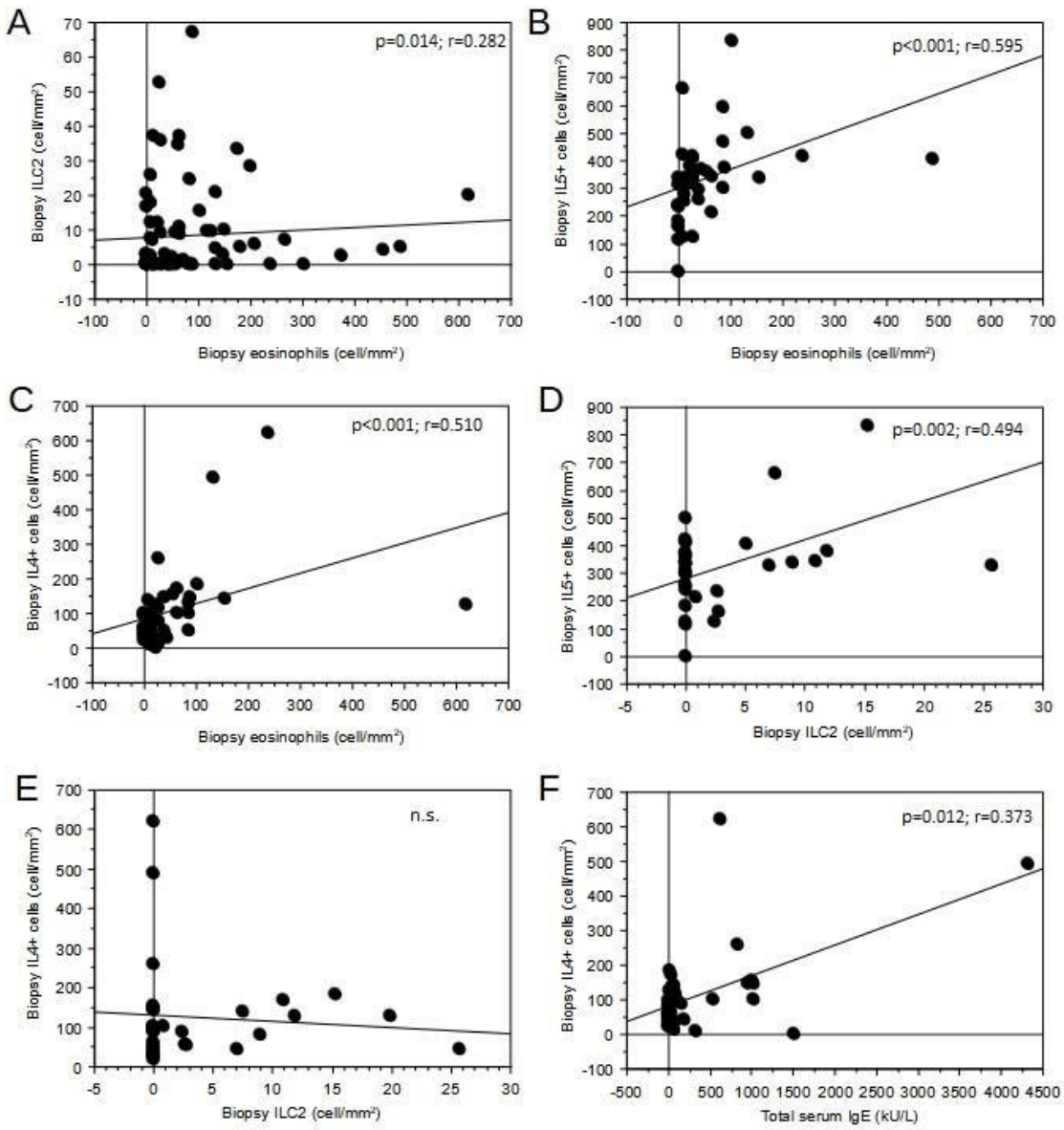

Figure 20. Relationship between eosinophils in bronchial biopsies and ILC2 (A), IL-5+ cells (B), IL4+ cells (C), between ILC2 and IL5+ cells (D), IL4+ cells (E), relationship between IL4+ cells and total IgE (F). A significant, but modest, positive correlation emerged in figure A, B, C, D and F.

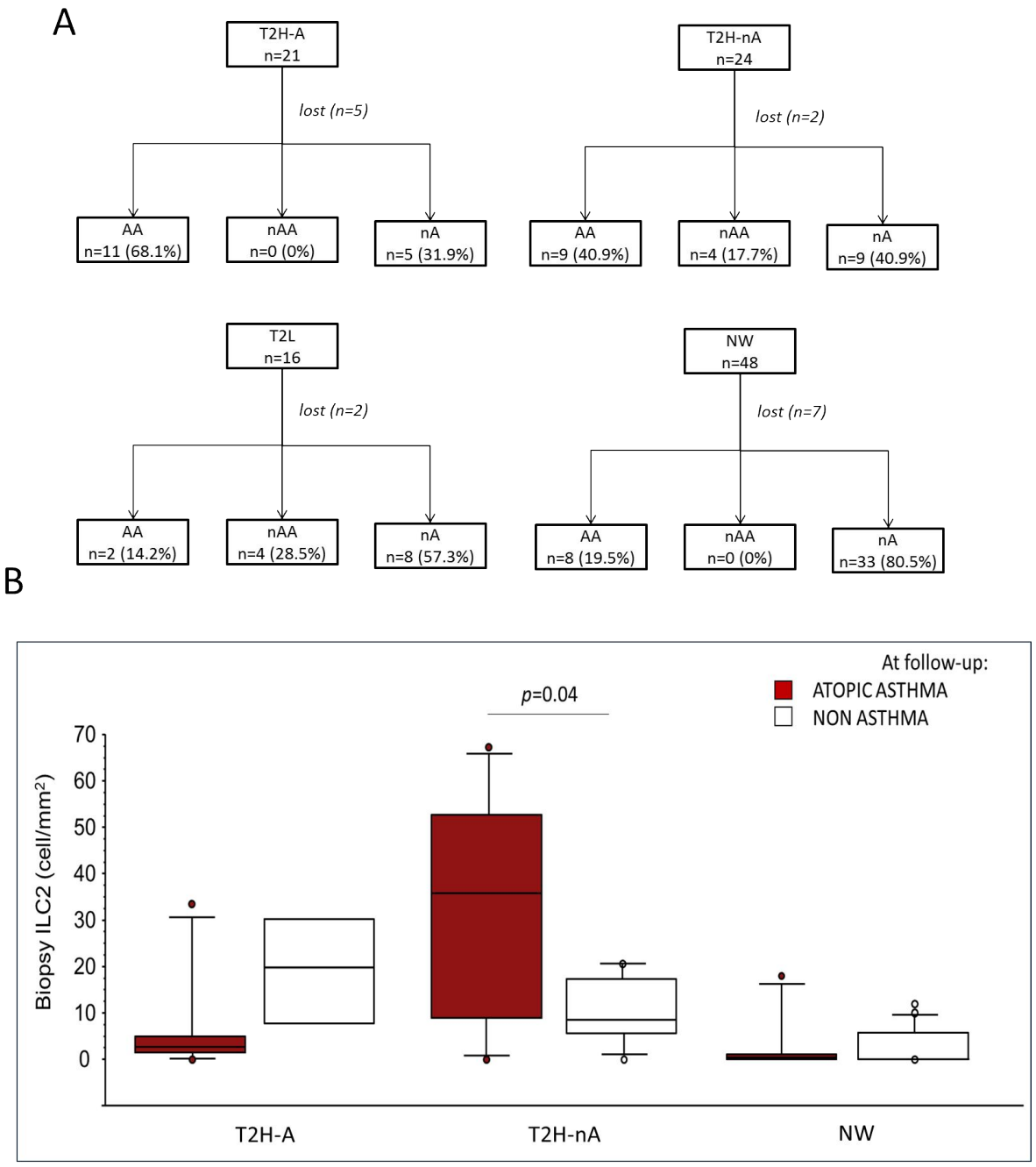

Figure 21. Flow diagram showing clinical evolution from baseline to follow-up of the children included in our study stratified according to baseline endotype (A) Boxplots reporting the comparison of baseline values of ILC2 in T2H-A and T2H-nA wheezing endotypes accordingly to the development of atopic asthma in comparison to loss of symptoms (B).

STUDY VI

Deficient Immune Response to Viral Infections in Children Predicts Later Asthma Persistence.

Baraldo S, Contoli M, Bonato M, Snijders D, Biondini D, Bazzan E, Cosio MG, Barbato A, Papi A, Saetta M. Am J Respir Crit Care Med. 2018 Mar 1;197(5):673-675. doi: 10.1164/rccm.201706-1249LE. PMID: 28862881.

Partnership: University of Ferrara, Dpt. of Translational Medicine

BACKGROUND

Rhinovirus infection is the most frequent cause of asthma exacerbations in adults and children (146). Furthermore, data from longitudinal studies suggest that wheezing episodes associated with viral infections early in life are a major risk factor for the development of asthma later in life (147). Moreover, longitudinal studies showed that children who were sensitized to aeroallergens (even without respiratory symptoms) had a greater risk of having viral wheeze (148), suggesting that allergic sensitization per se could influence susceptibility to viral infections. Impaired immune response to viral infections has been proposed as a mechanism for increased susceptibility to infections in asthmatic patients (149). The relationship between viral infections, innate immunity, and asthma and atopy is complex, particularly in childhood. Whether an abnormal immune response is present in the airways of young children or whether it develops later as a consequence of long-term immune deregulation still needs to be investigated.

AIM OF THE STUDY

For these purposes, we investigated *ex-vivo* rhinovirus-induced interferon production in bronchial epithelial cells from wheezing children. We also investigated the immunopathologic profile in airway biopsy specimens of these children and correlated these profiles with the *ex-vivo* innate responses to rhinovirus. Finally we followed-up prospectively these children to investigate whether the impaired rhinovirus-induced interferon production could be a risk factor for the development of asthma later in life. Results of this study have been already partially published previously (150).

METHODS AND MATERIALS

Study population

The study population included 47 children who underwent bronchoscopy for appropriate clinical indications at the Department of Pediatrics of the University of Padova, Padua, Italy. Children were recruited from March 2007 to May 2008. Written consent was obtained from children's parents after they were informed that bronchial brushings and biopsy specimens would be taken for research purposes (117,145). The study was performed according to the Declaration of Helsinki and was approved by the Ethics Committee of the Padova City Hospital.

Clinical-functional evaluation of patients at baseline

Clinical-functional evaluation protocol at baseline, including questionnaire for symptoms and disease assessment, clinical records, spirometry and blood examination were the same in Study II. Definitions of wheezing and atopy were the same applied in Study II.

Clinical-functional evaluation of patients at follow-up

Clinical-functional evaluation protocol at follow-up, including questionnaire for symptoms and disease assessment, clinical records, spirometry, FeNO measurement, and blood examination were the same in Study II. Definition of asthma at follow-up was the same applied in Study II.

Bronchoscopy and bronchial biopsies processing

Full details on the bronchoscopy, bronchial biopsy, and BAL procedures have been previously described in Study II. Inflammatory cells (eosinophils, neutrophils, mast cells, macrophages, CD4+ T lymphocytes, IL4+ cells and IL5+ cells) were quantified in the area extending 50 μm beneath the BM and were expressed as the number of positive cells per square millimeter of subepithelium. Basement membrane thickening and percentage of destroyed epithelium has been measured as in Study II.

Viral stocks

Rhinovirus type 16 (RV16; a major group rhinovirus) was obtained from the Health Protection Agency Culture Collections, Health Protection Agency Microbiology Services, Salisbury, United Kingdom, and used for all experimental conditions described. The virus was used at a multiplicity of infection of 5 for all experiments (151).

Primary bronchial epithelial cell cultures and cell processing

Human bronchial epithelial cells were harvested during bronchoscopy by means of brushing and were frozen as described in other studies (152,153). Bronchial epithelial cells were exposed to rhinovirus at room temperature for 1 hour, and then washed with phosphate buffered saline (PBS) to eliminate unbound virus and incubated for different time point (0, 8 hr and 48 hr if available). At each time point (0, 8 and 48 hr) experiments were performed in duplicate. We prioritized 0 and 8 hr cultures and then, if enough cells were available, we also set up plates for the 48 hr time point. Total mRNA levels and supernatants were obtained at the appropriate time points.

Quantitative TaqMan real-time RT-PCR

Quantitative real-time PCR was carried out with specific primers and probes for rhinovirus, IFN-β, IFN-λ (specific for IFN-λ19), IL-8 (Qiagen), and 18S rRNA (a housekeeping gene). Interferon, IL-8 mRNA, and viral RNA (vRNA) expression were

normalized to 18S rRNA levels, compared with standard curves, and expressed as copy numbers per microgram of RNA.

ELISA

ELISAs for detection of IFN-β, IFN-λ, and IL-8 levels in cell-culture supernatants were performed according to the manufacturers' instructions. Plates were read on a Labsystems plate reader and analyzed with SoftMax Pro software (Molecular Devices, Sunnyvale, Calif). The ELISA kit for IFN-λ1 used in the study shows ~90% cross reactivity with recombinant human IFN-λ2 and around ~35% cross reactivity with recombinant human IFN-λ3.

Statistical analysis

All cases were coded and the measurements were made without knowledge of clinical data to avoid observer bias. Comparisons among groups were evaluated with either ANOVA or the Kruskal-Wallis test, followed, when results were significant, by using Student t tests or Mann-Whitney U tests, as appropriate. Correlation coefficients were calculated by using the nonparametric Spearman rank method. P values of .05 or less were considered to indicate statistical significance.

RESULTS

Thirteen of the 47 primary cultures did not produce significant growth. Therefore clinical and experimental data refer to the 34 of 47 children whose primary bronchial epithelial cells were successfully cultured (17 with wheezing and 17 without wheezing). Clinical characteristics at baseline are reported in Table 13.

Age and sex were not significantly different among the 2 groups of children. Lung function values were obtained in all children able to cooperate with the test and were normal in all children. No children were chronically treated with oral or high-dose inhaled corticosteroids. Eleven of 17 wheezing children received inhaled salbutamol as needed, whereas the remaining 6 patients were taking low doses of inhaled corticosteroids (equivalent daily doses of beclomethasone ranging from 200-400 mg). No differences were observed between wheezing children treated with inhaled steroids and those who were not. Finally, we observed that wheezing children had higher levels of total serum IgE and blood eosinophils.

Table 13. Clinical-functional Characteristics of the cohort at Baseline in Relation to Wheezing condition at baseline

	GROUPS AT BASELINE			
	Whole Cohort	**Wheezing**	**Non-wheezing**	***P* Value**
Subjects, *n* (%)	34	17 (50%)	17 (50%)	
Sex, male (%)	18 (53%)	11 (64%)	7 (41%)	n.s.
Age, yr	5.4 ± 2.6	5.3 ± 2.7	5.4 ± 2.5	n.s.
Age at wheezing onset, yr	2.1 ± 2.7	2.1 ± 2.7	-	n.a.
Wheezing frequency (0–6)	3 (0–6)	3 (0–6)	-	n.a.
FEV1, % predicted*	91 ± 11.5	88.7 ± 16.2	93 ± 6.7	n.s.
FEV1/FVC, %*	95 ± 4.2	94 ± 6.8	95 ± 2.1	n.s.
Total serum IgE, kU/L	50 (3–3111)	95 (9–3111)	38 (3–2188)	.068
Blood eosinophils, cell/mcl	386 (0-1760)	535 (90-1760)	247 (0-660)	.013

Definition of abbreviations: FEV1 = forced expiratory volume in 1 second; FVC = forced vital capacity; n.s. = not significant. Data are expressed as counts (percentages); mean ± SD or median (min-max). P values refer to the comparison between children with asthma and those with no asthma at follow-up.

Ex vivo interferon induction and rhinovirus replication in bronchial epithelial cells

RV16 vRNA was undetectable in all primary cell cultures included in the study before the experimental infection. Similarly, IFN-λ and IFN-β mRNA levels were very low (close to the lowest limit of detection) in uninfected cells from all groups.

Eight hours after RV16 infection, a significant induction in IFN-λ and IFN-β mRNA levels was observed in all subject groups (approximately 3-fold log10 increase, p< .001). However, the induction of IFN-λ and IFN-β (Figure 22A and B) in wheezing children was significantly lower than that seen in non-wheezing children, conversely rhinovirus replication was significantly higher (p=0.001). Among non-wheezing children, atopics showed a similar impairment in interferon induction after RV16 (Figure 22C and D) and enhanced rhinovirus replication (p=0.012).

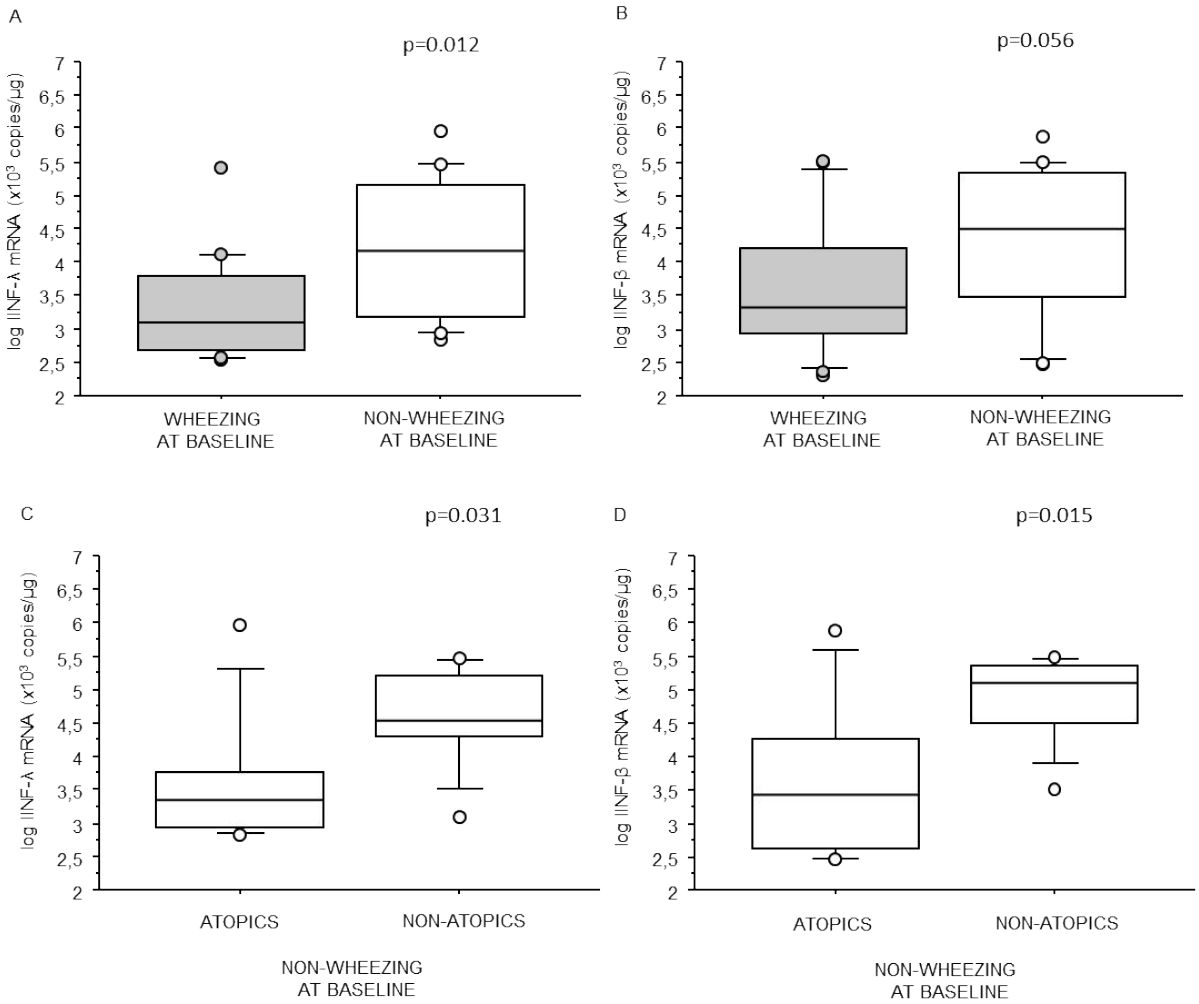

Figure 22. Boxplots reporting the IFN-λ and IFN-β mRNA levels expressed as logarithmic scale according to the presence of wheezing at baseline (A and B) and the presence of atopy in the non-wheezing group (n= 17, C and D). Solid line represents the median; bottom and top of the boxes are the 25th and 75th percentiles; brackets correspond to the 10th and the 90th percentiles. P-values refers to Mann-Whitney-U test.

Twenty-one cell-culture supernatants were available to analyze the IFN-β and IFN-λ protein levels 48 hours after infection. RV16 infection resulted in significantly increased IFN-β protein production in all groups, but the levels were significantly lower wheezing than non-wheezing children (67.8±23.8 vs 120.3±53.1 pg/ml; p=0.011). IFN-λ protein levels were detectable in samples from 9 subjects only, too few for a meaningful analysis.

Correlations linking *ex vivo* immune responses to immunopathological parameters

We then explored the interplay between the type 2 microenvironment and the structural changes in the airways and the *ex vivo* immune responses and viral replication. *Ex vivo* RV16-induced IFN-β mRNA levels at 8 hours were inversely correlated with airway eosinophil counts (p= 0.016; r=−0.58), similarly a non-significant trend was observed for IFN-λ (p=0.058; r=-0.41). Furthermore, ex vivo

RV16-induced IFN-β and IFN-λ mRNA levels at 8 hours were inversely related to blood eosinophil counts (respectively, p=.071 and r=−0.31 and p=0.048 and r=-0.32), to total serum IgE (respectively, p=.042 and r=−0.35 and p=0.003 and r=-0.53). Finally ex vivo RV16-induced IFN-β protein levels at 48 hours were inversely related to the expression of IL-4 in airway biopsy specimens (respectively, p=.027 and r=−0.57) and the degree of epithelial damage (respectively, p=.029 and r=−0.56).

<u>Ex vivo interferon induction and rhinovirus replication in relation to asthma status at follow-up</u>

After a mean of 7.6 ± 0.7 years we performed a clinical-functional follow-up of children. Follow-up data were available for 27 children: 62% of children with wheezing at baseline developed asthma at follow-up, whereas 21% among non-wheezing children. Children with asthma at follow-up, when compared with children without asthma, had lower IFNλ and IFNβ mRNA expression and increased viral replication at 8 hours (Figure 23).

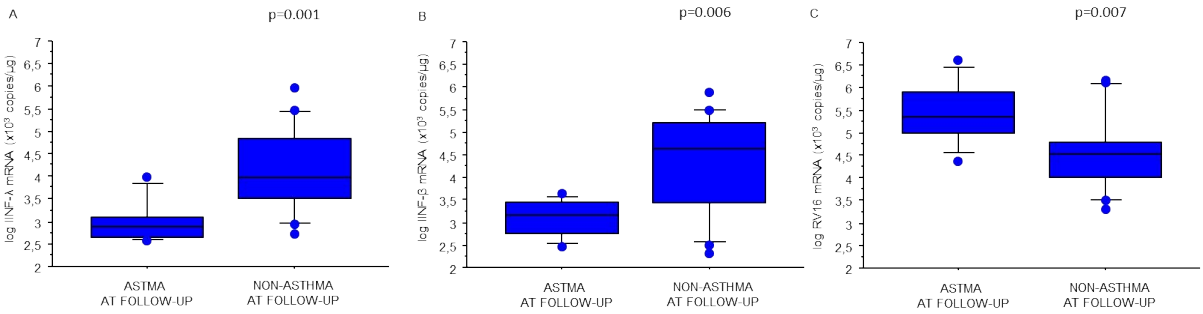

Figure 23. Boxplots reporting the IFN-λ (A), IFN-β (B), and RV16 (C) mRNA levels expressed as logarithmic scale according to the presence of asthma status at follow-up. Solid line represents the median; bottom and top of the boxes are the 25th and 75th percentiles; brackets correspond to the 10th and the 90th percentiles. P-values refers to Mann-Whitney-U test.

STUDY VII

Air Pollution Relates to Airway Pathology in Children with Wheezing.

Bonato M, Gallo E, Bazzan E, Marson G, Zagolin L, Cosio MG, Barbato A, Saetta M, Gregori D, Baraldo S. Ann Am Thorac Soc. 2021 Dec;18(12):2033-2040. doi: 10.1513/AnnalsATS.202010-1321OC. PMID: 34004126;

Partnership: Unit of biostatistics, University of Padova; Environment protection and prevention agency of Veneto Region

BACKGROUND

The pathological hallmarks of the disease encompass both chronic airway inflammation, usually eosinophilic, and airway remodeling. This includes thickening of the subepithelial basement membrane (BM), shedding of the epithelial layer, an increased smooth muscle area, increased mucus production, and neoangiogenesis. Our group has previously demonstrated that some of these features, particularly BM thickening, are early events in the natural history of the disease, being already present in early infancy, even in children with the mildest forms of asthma and without atopy (119, 154, 155).

Despite the growing burden of asthma, causes and pathophysiological mechanisms underlying the disease remain to be established. Asthma is a multifactorial disorder caused by the complex interaction between genetic and environmental factors (156, 157).

Several epidemiological studies demonstrated that outdoor air pollution is an important contributing factor to both asthma exacerbations and, possibly, new-onset cases (104, 158).

A recent statement from the American Thoracic Society concluded that there is now enough epidemiological evidence to indicate a causal link between long-term exposure to outdoor air pollution and new cases of incident asthma in children (101). Of note, Khreis and colleagues (159) have recently estimated the incidence of asthma related to air pollution by using a validated and harmonized European land-use regression model: they suggested that matching nitric dioxide, particulate matter, and black carbon minimum concentrations may prevent as much as 33% of the incident cases of asthma in children. Of note, outdoor air pollution is associated with impaired lung function, with increased hyperreactivity and airway inflammation being measured indirectly through fractional exhaled nitric oxide (160, 161). These observations highlight the urgent need to reduce children’s exposure to outdoor air pollution.

Despite such evidence, the pathophysiological mechanisms linking air pollution and asthma development have not been fully understood. In particular, no study has

addressed the influence of long-term outdoor air pollution exposure on inflammatory and structural changes in the airways of children with wheezing.

AIM OF THE STUDY

In this study, we sought to examine the relationship between previous exposure to nitrogen dioxide (NO2), particulate matter less than 10 μm in aerodynamic diameter (PM10), and sulfur dioxide (SO2) levels and the pathological traits typical of asthma in a cohort of children living in Northeast Italy, one of the most polluted regions in Europe.

METHODS AND MATERIALS

Study Population

Children were recruited at the Department of Woman and Child Health, University of Padova, Padova, Italy, from 2002 to 2014. The study was performed according to the Declaration of Helsinki and was approved by the local ethics committee. Written consent was obtained from the children's parents. All children underwent bronchoscopy (with bronchoalveolar lavage [BAL] and bronchial biopsy) on the basis of appropriate clinical indications according to the European Respiratory Society and American Thoracic Society guidelines (117, 145). An additional endobronchial biopsy for research purposes was performed with approval of the ethics committee and consent from the parents. Fiberoptic bronchoscopy was well tolerated by all children.

Clinical-functional evaluation of patients

Before bronchoscopy, all patients were evaluated by a respiratory pediatrician. The pediatrician collected a detailed clinical history, examined the child, and administered parental interviews as in Study II. The presence of wheezing with a pattern suggestive of asthma was based on reporting of repeated episodes of wheezing in the previous year, which was often associated with cough and dyspnea, particularly at night or in the early morning. Furthermore, wheezing had to be present even apart from colds (multitrigger) and had to be responsive to prescribed bronchodilators. Wheezing frequency and severity was defined as in Study II. At baseline, all children underwent routine blood tests, including a complete white blood cell count (total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, and basophils) and testing for total/specific IgE. The presence of atopy was defined as in Study II.

Air pollution exposure evaluation

Clinical and pathological data were combined with information collected from the regional air pollution monitoring system and supplied by Regional Agency for Environmental Protection and Prevention of Veneto Region (ARPAV).

Data from 2002 onward on daily concentrations of PM10, NO2, and SO2, together with meteorological data on temperature, relative humidity, and atmospheric pressure, were retrieved from the monitoring stations of the Environmental Protection and Prevention Agency of the Veneto Region. Children were linked to the

data of the monitoring station nearest to their residence (with a maximum distance set at 20 km) (162). The distribution of pollutants in the Veneto region is highly homogeneous because of its particular morphological condition. The Po valley, of which Veneto occupies the eastern part, is a vast flat area in northern Italy surrounded by mountains and a shallow sea. This particular morphological structure leads to a very low wind speed and facilitates air pollutants' stagnation.

Methods and technologies used to measure air pollutant concentrations were those designated by national and international consensus (163). Moreover, air pollution concentrations were compared with the 2005 World Health Organization air quality guidelines cutoffs (164) of 50 μg/m^3 for PM10 and 40 μg/m3 for NO2. None of the children changed residential addresses during the study.

Bronchoscopy and bronchial biopsies processing

Full details on the bronchoscopy, bronchial biopsy, and BAL procedures have been previously described in Study II. Inflammatory cells (eosinophils, neutrophils, mast cells, macrophages, and CD4+ T lymphocytes) were quantified in the area extending 50 μm beneath the BM and were expressed as the number of positive cells per square millimeter of subepithelium.

Statistical Analysis

Statistical Analysis and statistical models have been developed by E.G. (Unit of Biostatistics, Epidemiology and Public Health, Department of Cardiac, Thoracic, Vascular Sciences and Public Health). Children's characteristics were expressed by using the median and interquartile range (IQR) for continuous variables and counts and percentages for categorical variables. Comparisons among groups were evaluated with either the Student's t test or the Mann-Whitney U test as appropriate. Distributions of categorical variables were compared by using the $\chi 2$ test or Fisher's exact test when the sample size was small ($n < 5$). We performed analysis by using a generalized additive model with a Gamma in extenso family (165) to evaluate the association between air pollution and both inflammation and structural parameters measured in the biopsy specimen. We also analyzed the impact of outdoor air pollution on relevant clinical parameters (i.e., wheezing frequency, RTI frequency).

We used penalized cubic regression splines to account for the nonlinear relationship between the pollutant and the outcome. The possible delayed effect in time was analyzed by using the average concentrations of pollutants from 0 to 90 days before the bronchoscopy. Therefore, "lag 0–90" indicates the average concentration of the pollutant in the 90 days preceding the bronchoscopy, "lag 0–89" indicates the average concentration of the 89 days before, and so on. The model structure was chosen according to the lowest Akaike information criterion (166). All models were adjusted for temperature, atmospheric pressure, relative humidity, and wheezing. To assess the potential influence of crucial confounding factors on our study, such as atopy, secondhand smoke exposure, ICS treatment, and age of children at

bronchoscopy, we included them in the model as possible covariates. However, these covariates did not change the fit of the models. A second analysis was performed separately on the two subpopulations of children with and without wheezing. Results reported as rate ratios (RRs) and relative 95% confidence intervals (CI) were calculated for an increase in the IQR of the pollutant. The lag with the highest RR in absolute terms for each outcome is labeled as the “best RR.” Missing environmental data (4%) were imputed by using multiple imputation with a expectation-maximization with bootstrapping algorithm (167). All analyses were performed by using R statistical software and the Amelia ad mgcv R package (R Foundation for Statistical Computing) (168,169).

RESULTS

Clinical and Demographic Characteristics

Initially, 121 children were enrolled, clinical data were recorded, and bronchial biopsy specimens were examined for all children. However, data on outdoor pollution exposure were available for 98 out of 120 children (81%). The clinical and pathological features of children excluded because of a lack of pollution data did not differ from those of children included in the study.

At the time of bronchoscopy, 53 children (54%) had a history of repeated wheezing episodes even apart from colds and were responsive to bronchodilators, whereas 45 (46%) did not have wheezing reported in their clinical history. Clinical characteristics of the cohort are summarized in Table 1. The mean age at the onset of wheezing was 4.5 years, and children with wheezing had an age and sex distribution similar to those of children without wheezing. Children with wheezing showed higher levels of serum IgE ($p=0.042$). ICS treatment tended to be more frequent in children with wheezing than in children without wheezing; only a minority of children were receiving oral corticosteroids in both groups. The two groups of children had comparable histories of RTIs and secondhand smoke exposure. Finally, no differences were observed between children with wheezing and children without wheezing in the blood total and differential cell counts, including those for blood eosinophils (Table 14).

Table 14. Clinical, blood cell counts and pathological features in the whole cohort and in children with and without wheezing

	Whole Cohort	With Wheezing	Without Wheezing	*P*
Subjects	98 (100%)	53 (54%)	45 (46%)	—
Male	53 (54%)	27 (50%)	26 (57%)	n.s.
Age, yr	4.5 (4–6)	5 (3–6.5)	4 (4–5.5)	n.s.
Symptom onset, yr	1.5 (0.5–3)	1.5 (0.5–3)	N/A	—
Symptom frequency (scale of 0–6)	0 (0–3)	3 (2–4)	0	—
RTI during previous yr	1 (0.5–1)	0.5 (0.5–1)	1 (0.5–1.5)	n.s.
ICS therapy at bronchoscopy	23 (23%)	16 (30%)	7 (15%)	n.s.
OCS therapy at bronchoscopy	6 (6%)	2 (3%)	4 (6%)	n.s.
Secondhand smoke	29 (41%) (*n* = 70)	19 (48%) (*n* = 40)	10 (33%) (*n* = 30)	n.s.
Atopy	42 (43%)	26 (50%)	16 (34%)	n.s.
Rhinitis prevalence	17 (22%) (*n* = 74)	12 (28%) (*n* = 42)	5 (15%) (*n* = 32)	n.s.
Dermatitis prevalence	29 (30%) (*n* = 94)	18 (35%) (*n* = 51)	11 (25%) (*n* = 43)	n.s.
BLOOD CELL COUNT				
Leukocytes, cells/μl (*n* = 78/98)	7,350 (5,765–9,040)	6,880 (5,640–8,617)	7,919 (6,205–9,340)	n.s.
Neutrophils cells/μl (*n* = 78/98)	2,855 (1,845–4,152)	2,420 (1,810–3,750)	3,215 (2,500–4,690)	n.s.
Lymphocytes, cells/μl (*n* = 78/98)	3,135 (2,365–4,332)	3,140 (2,365–3,997)	3,050 (2,430–4,495)	n.s.
Monocytes, cells/μl (*n* = 78/98)	560 (420–675)	530 (427–660)	580 (400–745)	n.s.
Eosinophils, cells/μl (*n* = 98/98)	190 (120–310)	220 (119–323)	170 (117–300)	n.s.
Basophils, cells/μl (*n* = 78/98)	30 (20–50)	30 (20–50)	30 (25–45)	n.s.
Serum IgE, kU/L	57 (23–170)	72 (32–526)	50 (11–82)	.042
BRONCHIAL BIOPSY				
Basement membrane, μm (*n* = 98/98)	4.2 (3.4–5.1)	4.8 (3.8–6)	3.8 (3.1–4.4)	<.0001
Destroyed epithelium, % (*n* = 93/98)	60 (35–88)	67 (46–91)	44 (18–85)	0.008
Eosinophils, cells/mm^2 (*n* = 85/98)	28 (8–86)	62 (23–146)	9 (0–35)	.0002
Neutrophils, cells/mm^2 (*n* = 85/98)	135 (72–275)	133 (65–342)	137 (77–209)	n.s.
Mast cells, cells/mm^2 (*n* = 80/98)	128 (62–370)	220 (78–450)	100 (53–238)	.012
Lymphocytes, cells/mm^2 (*n* = 70/98)	315 (152–560)	321 (84–690)	314 (175–524)	n.s.
Macrophages, cells/mm^2 (*n* = 81/98)	101 (41–206)	113 (44–215)	82 (42–176)	n.s.
BRONCHOALVEOLAR LAVAGE				
Total cellularity, cells × 10^3/ml (*n* = 88/98)	200 (100–457)	173 (75–376)	315 (117–580)	n.s.
Eosinophils, cells × 10^3/ml (*n* = 86/98)	0 (0–1.3)	0 (0–2)	0 (0–0)	n.s.
Neutrophils, cells × 10^3/ml (*n* = 86/98)	29 (3.4–164)	22 (4–76)	46 (3.1–263)	n.s.
Lymphocytes, cells × 10^3/ml (*n* = 85/98)	13 (4–25)	13 (5–24)	13 (3.7–28.5)	n.s.
Macrophages, cells × 10^3/ml (*n* = 85/98)	142 (72–251)	139 (61–382)	149 (88–261)	n.s.

Definition of abbreviations: ICS = inhaled corticosteroid; IgE = immunoglobulin E; kU/L = 2.5 ng/ml; OCS = oral corticosteroid; N/A = not applicable; NS = not significant; TI = respiratory tract infection. Data are expressed as n (%) or the median (interquartile range). The P values refer to the Mann-Whitney U test, Student's t test, or chi-square test comparison between children with wheezing and children without wheezing.

As reported in (STUDY II), the pathological features characteristic of adult asthma were already present in children with wheezing. Children with wheezing had an increased reticular BM thickness ($p<0.0001$) and epithelial shedding ($p=0.008$) compared with children without wheezing. Inflammatory infiltrates also differed between the two groups, with children with wheezing displaying more eosinophils ($p=0.0002$) and more mast cells ($p=0.01$) than children without wheezing. No differences were observed with regard to neutrophil, macrophage, and CD4+ T lymphocyte counts in biopsy specimens. BAL fluid inflammatory cell counts were similar in the two groups.

Outdoor Air Pollution Exposure

Data on average levels of outdoor air pollutants during the 90 days before bronchoscopy are summarized in Table 15. Of note, SO2 levels measured during the observation period were negligible across the whole region, so we did not consider this pollutant for further analyses. As shown in Table 15, all children in our cohort were exposed to high levels of pollutants. Exposure to NO2 exceeded the World Health Organization threshold for half of the days, whereas PM10 exceeded the threshold for 38% of the days. Of note, the levels of exposure to PM10 and NO2 did not differ among different districts or between children with wheezing and children without wheezing.

Table 15. Pollutant exposure during the 90 days before bronchoscopy stratified for children's district of residence for the whole cohort, children with wheezing, and children without wheezing

	n (%)	Whole Cohort		With Wheezing		Without Wheezing	
		PM10 (*μg/m3*)	NO2 (*μg/m3*)	PM10 (*μg/m3*)	NO2 (*μg/m3*)	PM10 (*μg/m3*)	NO2 (*μg/m3*)
VENETO	98 (100)	40 (25–66)	40 (27–53)	37 (24–60)	37 (25–50)	45 (27–75)	43 (30–57)
Padova	35 (35)	58 (41–68)	45 (39–54)	43 (35–66)	42 (39–53)	63 (58–74)	49 (42–56)
Venezia	34 (34)	43 (32–54)	32 (25–41)	40 (31–54)	31 (25–41)	45 (37–54)	39 (28–45)
Vicenza	13 (14)	45 (35–59)	38 (32–47)	42 (33–51)	35 (31–39)	54 (42–64)	45 (38–50)
Treviso	16 (17)	36 (29–60)	37 (32–50)	31 (29–50)	33 (30–44)	46 (31–61)	41 (33–51)

Definition of abbreviations: NO_2 = nitric dioxide; PM_{10} = particulate matter less than 10 μm in aerodynamic diameter. Data are expressed as the median (interquartile range).

As shown in Figure 24A, in the whole cohort, BM thickness significantly increased with prolonged exposure to PM10. For each IQR increase in the PM10 concentration, we observed a significant enlargement of the BM of up to 30%, particularly from lag 0–15 to lag 0–90 (best RR at lag 0–63, 1.29; 95% CI, 1.09–1.52).

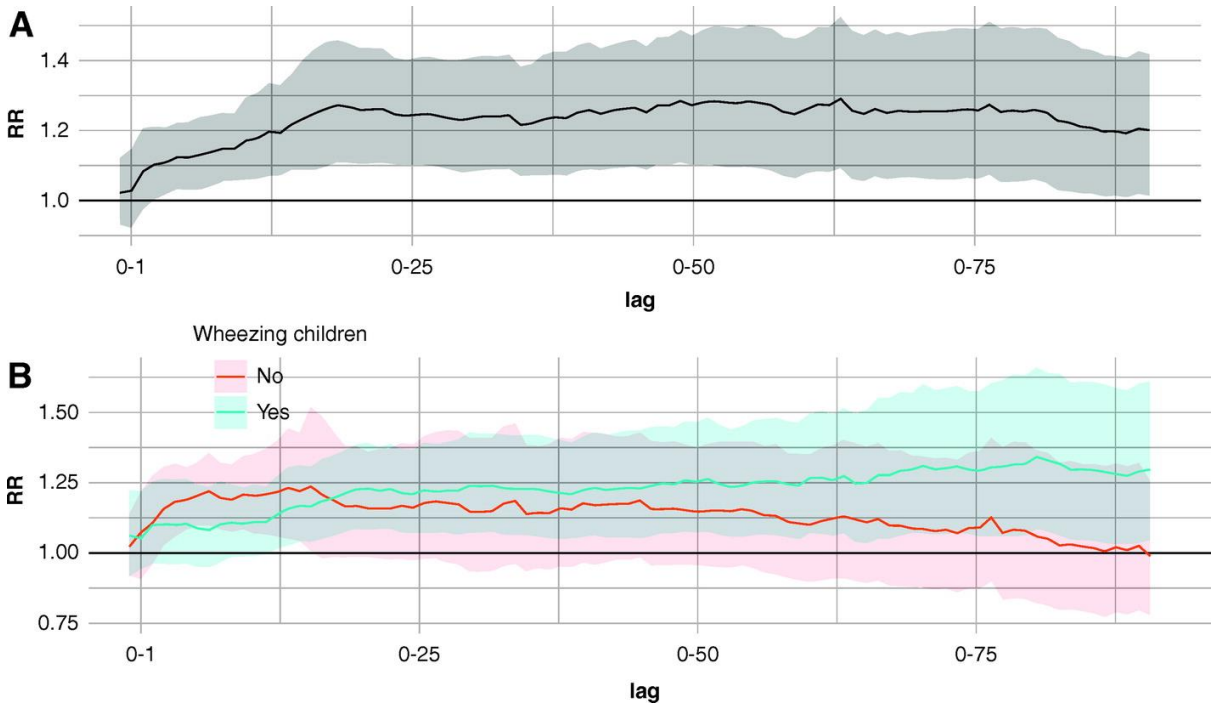

Figure 24. The strength of the association between basement membrane thickness and exposure to particulate matter less than 10 μm in aerodynamic diameter. The membrane thickness rate ratio (RR) for each interquartile range increase of the moving average concentration of particulate matter less than 10 μm in aerodynamic diameter (PM10) from 0 to 90 days before bronchoscopy is shown. (A) Data for the whole cohort. (B) Data for children with wheezing (blue) and children without wheezing (red) shown separately. The line indicates the RR and its variation in each lag duration, and the shaded area indicates the confidence interval (CI). When the lowest (or the highest) limit of the CI (shaded area) does not include 1, the association is statistically significant.

When stratifying our study population by the presence of wheezing (Figure 24B), a positive association was found in children with wheezing for an exposure to PM10 longer than 13 days (best RR at lag 0–80, 1.34; 95% CI, 1.09–1.66), whereas an association at earlier time points was detected in children without wheezing (best RR at lag 0–7, 1.23; 95% CI, 1.1–1.36). In our cohort, we did not observe any influence of NO2 on BM thickness or of PM10 or NO2 on epithelial integrity.

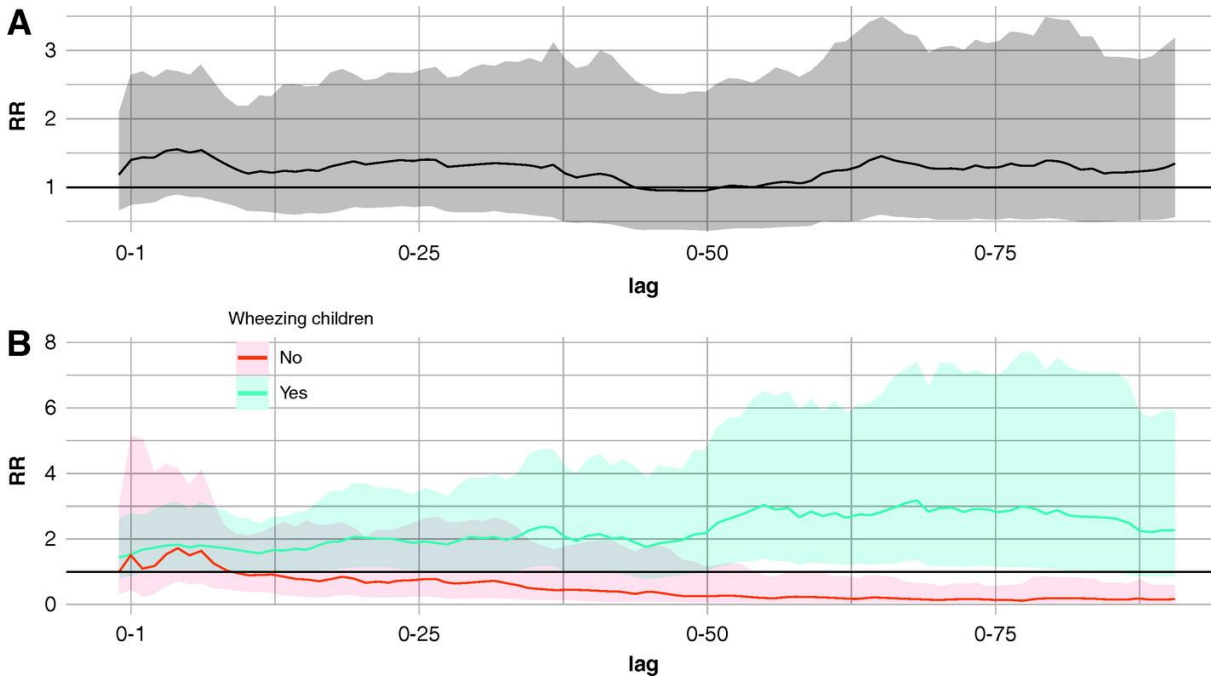

Figure 25. The strength of the association between eosinophils in bronchial biopsy specimens and exposure to particulate matter less than 10 µm in aerodynamic diameter (PM10). The eosinophil rate ratio (RR) for each interquartile range increase of the moving average concentration of particulate matter less than 10 µm in aerodynamic diameter from 0 to 90 days before bronchoscopy is shown. (A) Data for the whole cohort. (B) Data for children with wheezing (blue) and children without wheezing (red) shown separately. The line indicates the RR and its variation during each lag, and the shaded area indicates the confidence interval (CI). When the lowest (or the highest) limit of the CI (shaded area) does not include 1, the association is statistically significant.

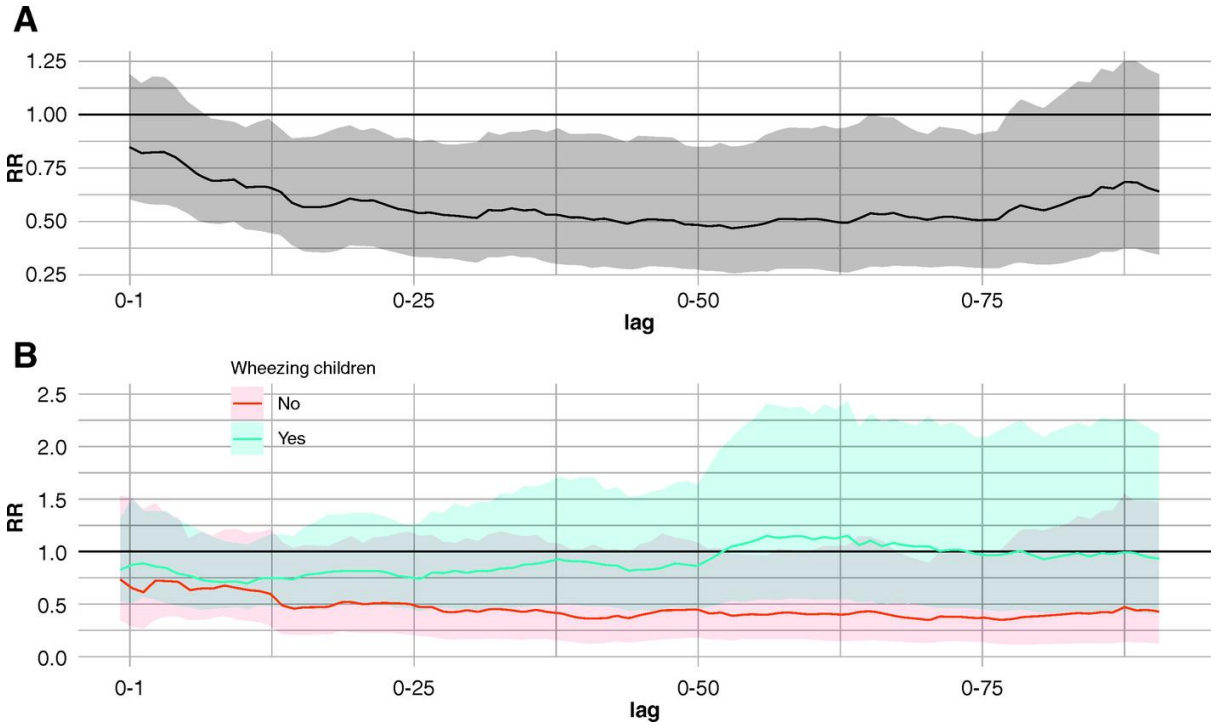

Figure 26. The strength of the association between neutrophils in bronchial biopsy specimens and exposure to particulate matter less than 10 µm in aerodynamic diameter. The neutrophil rate ratio (RR) for each interquartile range increase of the moving average concentration of particulate matter less than 10 µm in aerodynamic diameter (PM10) from 0 to 90 days before bronchoscopy is shown.

(A) Data for the whole cohort. (B) Data for children with wheezing (blue) and children without wheezing (red) shown separately. The line indicates the RR and its variation during each lag, and the shaded area indicates the confidence interval (CI). When the lowest (or the highest) limit of the CI (shaded area) does not include 1, the association is statistically significant.

With regard to the association between air pollution and inflammatory features (Figures 25 and 26), we observed different patterns in children with wheezing and in children without wheezing. In children with wheezing, there was a positive association between prolonged exposure to PM10 and tissue eosinophilic inflammation, as shown in Figure 25B. This association progressively increased with prolonged exposure, reaching its maximum value at lag 0–68, when the number of eosinophils tripled for each IQR increase in the PM10 concentration (RR, 3.16; 95% CI, 1.35–7.39). Conversely, in children without wheezing, prolonged exposure to PM10 was associated with reduced eosinophil numbers in bronchial biopsy specimens (Figure 25B) (best RR at lag 0–77, 0.12; 95% CI, 0.02–0.6). In children without wheezing, prolonged exposure to PM10 also reduced the number of neutrophils (best RR at lag 0–70, 0.36; 95% CI, 0.14–0.89; Figure 26B), although this association only trended toward statistical significance. No influence of PM10 exposure on neutrophils was found in children with wheezing.

Regarding NO2 exposure, we observed weak negative associations during longer lags with eosinophils (best RR at lag 0–55, 0.33; 95% CI, 0.14–0.77) and during shorter lags with neutrophils (best RR at lag 0–14, 0.57; 95% CI, 0.34–0.93) in the whole cohort. In our cohort, other inflammatory cell subtypes (lymphocytes, macrophages, mast cells) in bronchial biopsy specimens were not influenced by air pollution. Although exposure to PM10 and NO2 influences airway tissue inflammation, we did not observe any significant relationship between air pollution and BAL fluid or blood inflammatory cell counts (including blood eosinophils). Furthermore, there was no link with clinical parameters, such as the frequency of wheezing symptoms and frequency of RTIs.

STUDY VIII

Air Pollution Exposure Impairs Airway Epithelium IFN-β Expression in Pre-School Children.

Bonato M, Gallo E, Turrin M, Bazzan E, Baraldi F, Saetta M, Gregori D, Papi A, Contoli M, Baraldo S. Front Immunol. 2021 Oct 18;12:731968. doi: 10.3389/fimmu.2021.731968. PMID: 34733277; PMCID: PMC8558551.

Partnership: Unit of biostatistics, University of Padova; Environment protection and prevention agency of Veneto Region

BACKGROUND

Air pollution is increasingly recognized as an important cause of asthma exacerbations and, possibly, as a cofactor for asthma origins (158). Many studies have shown that the exposure to pollutants is associated to increased risk of respiratory viral infections, i.e. the most frequent triggers of asthma exacerbations, including rhinovirus, influenza and respiratory syncytial virus. In asthma patients there is evidence that the exposure to pollutants in the week preceding a respiratory viral infection increases the severity of the associated asthma exacerbations (170). The mechanisms by which air pollution may increase susceptibility to infections and favor viral-induced exacerbations in asthma patients are still largely unknown.

Previous studies- though not unanimously- have identified impaired antiviral immune response as a possible mechanism for increased susceptibility to infections in asthmatic patients. In particular, in STUDY V we reported, impaired type I (IFN-β) and type III interferon (IFN-λ) production following rhinovirus-16 (RV16) infection in bronchial epithelial cells of pre-school asthmatic and/or atopic children compared to non-asthmatic non-atopic children and that such impaired IFN response was associated to persistence of asthma symptoms up to school-age. To the best of our knowledge, no study has ever investigated the association between viral-induced IFN responses in bronchial epithelial cells and exposure to air pollution in children at risk for asthma.

AIM OF THE STUDY

In this study we sought to investigate the relationship between exposure to air pollutants (nitrogen dioxide-NO2, particulate matter less than 10 μm in aerodynamic diameter-PM10, sulfur dioxide-SO2) and RV16-induced IFN-β and λ expression in primary bronchial epithelial cells obtained from a cohort of pre-school children. Aim of this study is to evaluate whether RV16 induced IFN-β and λ mRNA levels were affected by the ambient air concentration of: i) nitrogen dioxide-(NO2), ii) particulate matter less than 10 μm in aerodynamic diameter (PM10) and iii) sulfur dioxide (SO2).

METHODS AND MATERIALS

Study Population

The study design and the population of the children cohort considered in this study has been previously described in detail in STUDY VI. The study was performed according to the Declaration of Helsinki and was approved by the Ethics Committee of the Padova City Hospital. Children underwent bronchoscopy, with bronchoalveolar lavage, for appropriate clinical indications according to current guidelines (117, 145).

Air Pollution Exposure Evaluation

Daily levels of PM10, NO2 and SO2 were retrieved from the monitoring stations of the Environmental Protection and Prevention Agency of Veneto Region (ARPAV). Outdoor pollution exposure and pollutant cut-off considered have been described in STUDY VII.

Primary Bronchial Epithelial Cell Cultures for Detection of IFN-β, IFN-λ, and Viral RNA

Bronchial epithelial cells were harvested by brushing, cultured and experimentally infected with Rhinovirus Type 16 (RV16). RV16-induced IFN-β and λ expression was measured by quantitative real time PCR, as was RV16vRNA. Detailed methodology have been explained in STUDY VI.

Statistical Analysis

Children's characteristics were expressed as median and IQR for continuous variables, and counts and percentages for categorical variables. The difference in explanatory variables was assessed using a Chi-squared test or Fisher test for dichotomous and categorical variables, or Mann–Whitney U test for continuous variables. Statistical model used to verify the relationship between outdoor air pollution retrospective exposure and RV16 vRNA copies, IFN-β and IFN-λ mRNA copies was described in STUDY VII.

In view of the limited sample size and the high degree of skewness in the covariates, to evaluate stability of the results, a sensitivity analysis was conducted by replacing the variable indicating presence of asthma with the one representing the presence of atopy (166,167). All analyses were performed using R statistical software (168,169).

RESULTS

Patients' characteristics were previously detailed in STUDY VI. The bronchoscopic procedure was well tolerated by all children, and no complications were encountered. Clinical indications for bronchoscopy did not differ significantly between wheezing and non-wheezing children. Wheezing and non-wheezing children had a similar age and gender distribution (Table 16). No children were chronically treated with oral or high-dose inhaled corticosteroids. Wheezing children showed higher levels of peripheral blood eosinophils ($p=0.02$), BAL eosinophils were not different in the two groups. The two groups of children had a comparable history of respiratory

tract infections (RTI). Though wheezing and non-wheezing children were matched for atopic status (as per design of the original study), a non-significant trend for increased levels of total serum IgE was observed in wheezing children compared to non-wheezing (p=0.074).

Table 16. Comparison between wheezing and non-wheezing children.

	WHOLE COHORT		WHEEZING CHILDREN	NON-WHEEZING CHILDREN	p
Subjects	34 (100%)		17 (50%)	17 (50%)	-
Male (n; %)	18 (52.9%)		11 (64.7%)	7 (41.2%)	0.169
Age (y)	5 [4-6]		5 [3.5-6.5]	5 [4-5.5]	0.973
Symptoms onset (y)	1 [0.5-3.5]		1 [0.5-3.5]	n.a.	-
RTI (ep/month)	1 [0-2]		2 [0.5-2.5]	2 [1-2]	0.786
ICS (n; %)	11 (32%)		10 (58.8%)	1 (5.9%)	0.001
Air pollution exposure **PM10 (μg/m^3)** **NO_2 (μg/m^3)**	N=30 40.5 [23.0-53.0] 39.8 [28.9-47.5]		N=14 40.5[27.5-59.1] 40.7[32.2-50.4]	N=16 34.5[22.6-46.2] 36.0[24.0-46.9]	 0.381 0.270
Serum IgE (kU/L)	47.5 [13.9-221.5]		89 [29-504]	32 [12.2-136]	0.079
Blood Eosinophils (cell/μL)	269 [142-539]		443 [220-710]	260 [74.5-361]	0.024
BAL Eosinophils (%)	0 [0-2.25]		0 [0-1.5]	0 [0-3.5]	0.483
Biopsy Eosinophils (cell/mm^2)	14 [6.7-126.5]		63 [13-161]	12 [0-81]	0.217
Basement membrane thickness (μm)	4.66 [3.83-5.19]		5.19 [4.36-5.96]	4.05 [3.49-4.79]	0.032
IFN-β mRNA (x10^3 copies/μg)	3.7 [1.0-77.6]		2.1 [0.8-16.8]	32.2 [2.9-213.3]	0.049
IFN-l mRNA (x10^3 copies/μg)	3.8 [0.8-21.3]		1.2 [0.4-6.5]	14.2 [1.4-142.5]	0.011
RV16 vRNA (x10^3 copies/μg)	44.4 [12.3-542.1]		224.3 [35.5-772.6]	25.5 [9.8-47.5]	0.009

The p-value is referred to the Mann-Whitney-U test or Chi-square/Fisher's exact comparison between asthmatic and non-asthmatic children. Data are expressed as median [interquartile range]. RTI: respiratory tract infections during previous year ICS: inhaled corticosteroid therapy at bronchoscopy n.a.: not applicable

RV16-induced IFN-β induction in asthmatic children was significantly reduced compared to non-asthmatic children (p=0.049); a similar reduction was also observed for IFN-λ (p=0.011). The reduction in IFNs levels was mirrored by a significant increase in RV16 vRNA levels in asthmatic children compared to non-asthmatics (p=0.009) as previously described STUDY VI.

Data on air pollution were available for 30 out of 34 children. Data on median levels of outdoor air pollution on lag01 are summarized in Table 1. SO2 levels were virtually undetectable across the whole region so we did not consider this pollutant for further analyses. As shown in the table children in our cohort were exposed to high levels of air pollutants. The median exposure values for NO2 and PM10 were very close to the corresponding WHO recommended highest limit. Of note, we did not observe significant different levels of exposure to PM10 and NO2 among districts, nor between asthmatic and non-asthmatic children.

When considering the whole cohort of preschool children, a negative association between acute (lag01) exposure to PM10 or NO2 and the levels of RV16-induced IFNβ mRNA in HBEC was observed (Figure 27 and Table 17). For each increase of 1 ug/m3 of NO2 we found a significant decrease of 2.3 x103 in RV16-induced IFN-β mRNA copy number. For each increase of 1ug/m3 of PM10 a significant decrease of 1 x103 in RV16-induced IFN-β mRNA copies was observed (Figures 27A, B). No significant associations were detected between RV16-induced IFN-λ mRNA and NO2 nor PM10. Exposure to increasing levels of NO2 (but not PM10) was positively associated with increased RV16 replication in HBEC (Table 17).

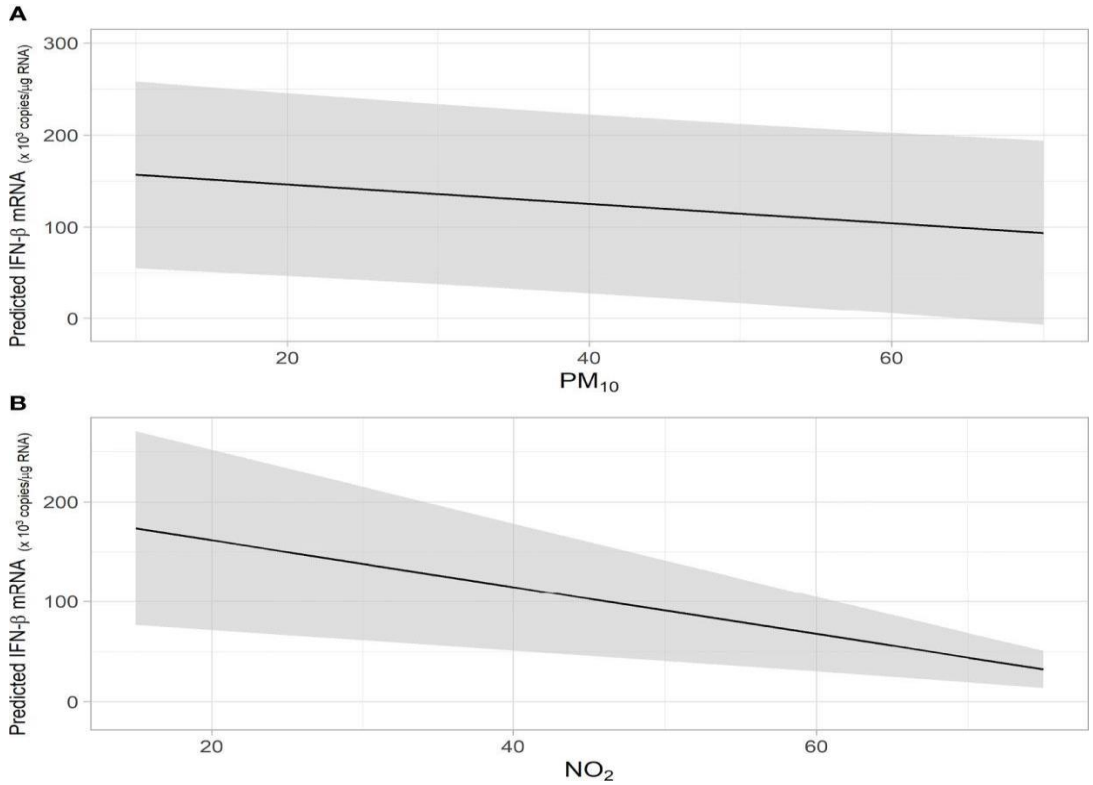

Figure 27. The figures show the predicted values of IFN- β mRNA in association with short-term exposure to PM10 (A) and NO2 (B). Shaded area indicates the confidence interval (CI). Predicted values of IFN- β mRNA were obtained from Generalized Linear Model (GLM).

Since we have previously shown that both asthma and atopy were associated with reduced IFN expression by epithelial cells, we performed a sensitivity analysis in which the presence of asthma was replaced with the presence of atopy.

Consistently, the analyses confirmed the significant decrease of IFN-β mRNA copies in association with both PM10 and NO2. However, the significance of the association between RV16 vRNA copies and NO2 was lost in this sensitivity analysis. We also confirmed null associations between RV16 replication and PM10, or between IFN-λ mRNA copies and PM10/NO2 (data not shown).

Table 17. β coefficients and relative 95% Confidence Interval (CI) of the multivariable models.

Outcome	Pollutant	β	95% CI
IFN- β mRNA (x10^3 copies/µg)	PM10	-1.05	-1.97; -0.14
	NO2	-2.36	-3.70; -1.01
IFN-λ mRNA (x10^3 copies/µg)	PM10	1.04	-0.71; 2.79
	NO2	0.80	-0.99; 2.58
RV16 vRNA (x10^3 copies/µg)	PM10	8.06	-13.10; 29.21
	NO2	31.73	6.59; 56.88

Studies on COPD

STUDY IX

White blood cell counts as indicators of disease severity in COPD: a "real life study"

Study in progress

BACKGROUND

Chronic Obstructive Pulmonary Disease (COPD) is a major cause of morbidity and mortality throughout the world. It is characterized by airflow limitation that is not fully reversible and is associated with an abnormal inflammatory reaction of the lungs, mainly in the small airways and the lung parenchyma (10). Our group in past years, already described this disease as characterized by an abnormal innate and adaptive immune inflammation including neutrophils, eosinophils, monocytes, and T and B lymphocytes (61, 83, 171) and all of them were directly related to progressive airflow limitation, respiratory failure and exacerbation risks. Recently, many authors proposed the utilization of blood white cell counts for clinical purposes with a hypothetical prognostic value and as a treatable trait as a proxy of airways inflammation for clinical purposes with. However, the difficult differential diagnosis between asthma and COPD in older age is a major bias in these studies. Recently, blood eosinophils have been introduced in the GOLD document as a predictor of exacerbation risk and more than 100 eosinophils/mcl is now considered a cut-off for introducing steroid inhalation treatment (10). Moreover, we observed in a previous study that low blood lymphocytes (<1800 cell/mcl) and their decline have been shown to be related to worse outcomes in smokers with and without COPD (172,173). Moreover, an association between the ratio between blood neutrophils/blood lymphocytes and disease severity, exacerbations, and higher hospitalization rate has been reported (174).

AIM OF THE STUDY

We aim to investigate the relationship between the blood leukocyte count (lymphocytes, neutrophils, eosinophils and monocytes) and clinical-functional features of a large real-life cohort of COPD patients at purpose of assessing the utility of blood white cell count as a biomarker for disease severity and comorbidities association.

METHODS AND MATERIALS

Study population

We conducted a cross-sectional real-life study recruiting a convenience sample of adult COPD patients attending the outpatient COPD clinic of our institutions (Respiratory Disease Clinic, Padova University Hospital and Treviso City Hospital) from January 2012 to January 2020. The study included all patients aged at least 18 years with a diagnosis of COPD made according to Global strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease (10). All patients attended our clinic for routine health care assessment and were not specifically selected for this study. Exclusion criteria were limited to a lack of

informed consent, previous history of asthma, lack of blood cell count in previous 12 months.

Patient evaluation and outcomes measurement

At the visit, we recorded the clinical history with particular focus on comorbidities, results of the physical examination, pulmonary function tests, weight and height, treatment and exacerbations during the previous 12 months, previously thoracic radiological exams. COPD Assessment Test (CAT) and Modified British Medical Research Council Questionnaire (mMRC) have been proposed to patients as part of the clinical evaluation. Results of the most recent blood leukogram during the previous 12 months have been collected. Only blood samples taken in a period of clinical stability have been considered. We defined the presence of emphysema or bronchiectasis by the presence of these comorbidities at emphysema at CT scan; chronic bronchitis by the presence of chronic cough and sputum production > 3 months/years (175).

Statistical analysis

All analyses were performed using SPSS software (version 25.0.0.1 for Windows, IBM, Armonk NY, USA). Patients' characteristics are expressed as means ± SDs or median (interquartile range) for continuous variables and counts and percentages for categorical variables. For continuous variables, normal distributions were tested using the Shapiro-Wilk test. Comparisons among groups were evaluated with either Student t test or Mann-Whitney U test, as appropriate. Distributions of categorical variables were compared with Chi-square test or Fisher exact test when the sample size was small (n < 5). Correlation coefficients were calculated using nonparametric Spearman's rank method.

RESULTS

Four hundred patients have been recruited. All the characteristics of the cohort are reported in Table 18. The majority of patients were males (71.5%), former smokers (70%) with a median age of 77.6 years. Our patients have a mean of 0.79±1.07 acute exacerbations during the year previous the visit, of them 0.43±0.89 were mild-moderate while 0.36±0.66 were severe. The median airflow obstruction, measured as FEV1 %pred. and FEF25-75 %pred. were 67% and 30% respectively, median CO transfer was also moderately reduced (71%). The majority of patients were in GOLD 2 stage (40.7%, n=163), followed by GOLD 1 (36.5%, n=146), GOLD 3 (13.8%, n=55) and GOLD 4 (9%, n=36). Two hundred and seventy out of 400 underwent CT scan for clinical purposes: 59.2% had emphysema and 14% had bronchiectasis. Among comorbidities, arterial hypertension was largely the most frequent (65.7%), followed by chronic heart failure (33.2%), peripheral artery disease (26.7%) and cancer (26.25%). Other respiratory comorbidities affected about 10% of subjects.

Table 18. Clinical, functional characteristics and blood cell counts of patients included in the study.

Subjects, n	400
Gender, male, n (%)	286 (71.5)
Age, years	77.6 [72.8-82.9]
Smoke habit, n (%) Never Former Current	 12 (3) 281 (70) 107 (27)
Previous year acute exacerbations, n mild-moderate, n severe, n	0.79±1.07 0.43±0.89 0.36±0.66
FEV1, %pred.	67 [51-86]
FEF 25-75, %pred.	30 [18-44]
DLCO, %	71 [48-84]
mMRC scale	2 [1-2]
CAT score	12 [6-18]
Inhalatory treatment, n (%) ● No treatment ● LAMA or LABA alone ● LAMA/LABA ● LABA/ICS ● LAMA + LABA/ICS ● LAMA/LABA/ICS	 22 (5.5) 92 (23) 92 (23) 47 (11.5) 129 (32.5) 18 (4.5)
COMORBIDITIES	
Arterial hypertension, n (%)	263 (65.7)
Emphysema on CT scan, n (%)*	160 (59.2)
Chronic bronchitis, n (%)	158 (39)
Chronic heart failure, n (%)	133 (33.2)
Peripheral artery disease, n (%)	107 (26.7)
All Cancer, n (%) Lung cancer, n (%) Other epithelial malignancies, n (%) Other non-epithelial malignancy, n (%)	105 (26.25) 29 (7.2) 70 (17.5) 6 (1.5)

Dyslipidemia, n (%)	99 (24.8)
Type 2 diabetes, n (%)	79 (19.7)
Atrial fibrillation, n (%)	70 (17.5)
Gastro-esophageus reflux disease, n (%)	67 (16.7)
Ischemic cardiopathy, n (%)	63 (15.7)
Obesity, n (%)	62 (15.5)
Obstructive sleep apnea, (%)	43 (10.7)
Bronchiectasis, n (%)*	36 (14)
Osteoporosis, n (%)	36 (9)
Chronic liver failure, n (%)	11 (2.7)
Chronic kidney failure, n (%)	7 (1.7)
BLOOD CELL COUNT	
Leukocytes, cell/mcl	7736±2298
Neutrophils, cell/mcl	4700±1782
Lymphocytes, cell/mcl	1964±747
Monocytes, cell/mcl	561±201
Eosinophils, cell/mcl	199±161

*Data are expressed as median [IQR] or frequency absolute (relative) as appropriate. *Data available for 270 out 400 patients. LAMA=long acting muscarinic antagonist; LABA=long acting beta2 agonist; ICS=inhaled corticosteroids; CT=computer tomography; FEV1= forced expiratory volume in the first minute; FEF25-75=forced expiratory flow between the 25 and the 75 percentiles of expiration.*

Blood white cells count

Median values of blood leukocytes count were: 7520 cell/mcl total leukocytes of whom 4400 cell/mcl neutrophils, 1845 cell/mcl lymphocytes, 540 cell/mcl monocytes and 170 cell/mcl eosinophils. Male patients showed significantly lower blood lymphocytes in comparison to females (1888±682 vs 21670±868; p=0.002). We observed a weak although significant inverse relation between age and blood neutrophils (p=0.005; p=-0.123) and lymphocytes (p=0.005; r=-0.153). To assess if white blood cells counts of our COPD patients, although within the normal range, significantly differ from general population values we considered as a comparison median values of blood white cell counts with a group of 1747 healthy subjects with

similar ethnological and demographical characteristics extracted by the study of Troussard et. al. (176). We observed that our cohort showed significative higher levels of total leukocytes (7736±2298 vs 6600±2990; p<0.001), neutrophils (4700±1782 vs 3600±2189; p<0.001) and monocytes (561±201 vs 430±251; p<0.001) while significantly lower levels of lymphocytes (1964±747 vs 2300±1212; p<0.001). We did not observe a significant difference for eosinophils (199±161 vs 210±267; p=n.s.).

Neutrophils

We observed that blood neutrophils were weakly but significatively directly related to all severity markers including symptoms scales as mMRC (p=0.012; r=0.182) and CAT (p=0.012; r=0.192), the number of acute exacerbation in the previous year (p=0.019; r=0.192) both severe (p< 0.001; r=0.293) and mild-moderate (p=0.016; r=0.181), and inversely related to pulmonary function included FEV1 (p=0.001; r= -0.189) and DLCO (p=0.052; r=-0.192). In particular, as shown in figure 28A, patients in GOLD3 and 4 stages had significantly higher blood neutrophils in comparison to GOLD 1 and 2. Moreover, they were significantly higher in current smokers in comparison to past smokers (5069±1719 vs 4587±1772; p=0.034). Finally, we observed that patients with bronchiectasis and chronic bronchitis had significantly higher blood neutrophils than patients without these comorbidities (5785±2182 vs 4594±1700; p=0.006; figure 28B and 4992±1949 vs 4489±1612; p=0.014).

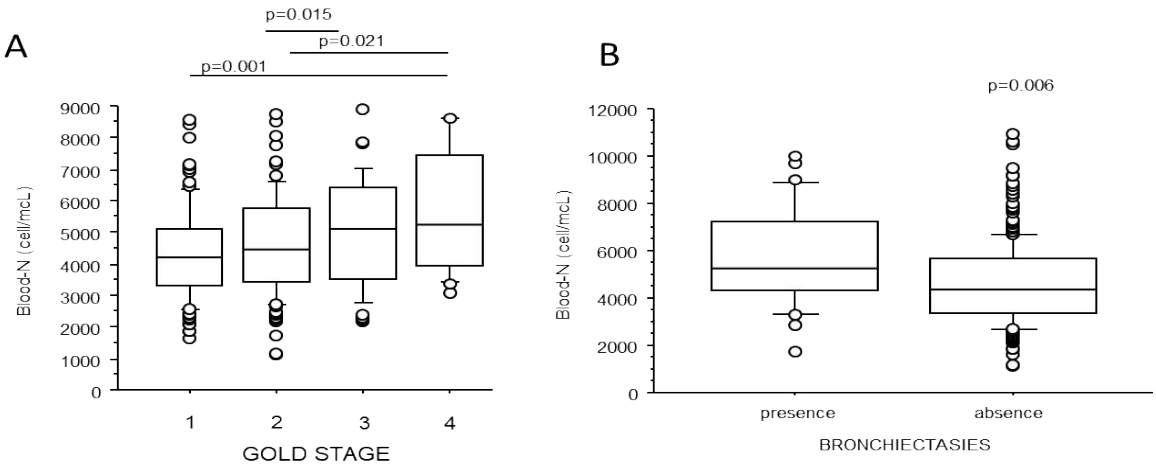

Figure 28. Boxplots illustrating the levels of blood neutrophils in relation to GOLD stage (A) and the presence of bronchiectasis (B). Solid line represents the median; bottom and top of the boxes are the 25th and 75th percentiles; brackets correspond to the 10th and the 90th percentiles.

Lymphocytes

We observed that also blood lymphocytes were weakly but significatively directly related to pulmonary function including FEV1 %pred. (p=0.001; r=+0.179) and FEF25-75 %pred. (p=0.001; r=+0.142). In particular, as shown in figure 29A, patients in GOLD4 stage had significantly lower blood lymphocytes level in comparison to

GOLD 1, 2 and 3 (p<0.001 for all three). We did not observe a significant relation between blood lymphocytes to DLCO and the number of acute exacerbations in the previous year. Among comorbidities, we observed that patients with emphysema at CT scan had lower levels of blood lymphocytes (1833±674 vs 2088±887; p=0.029; figure 29B).

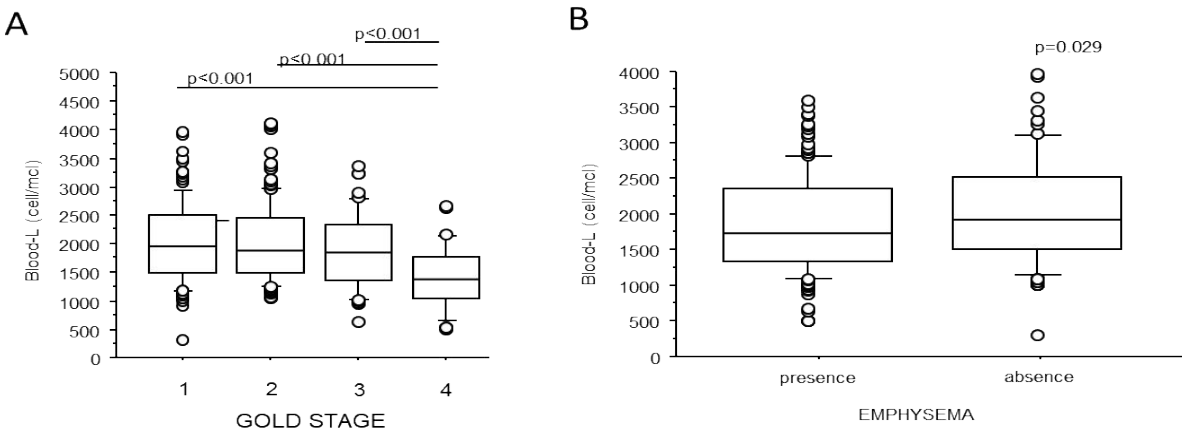

Figure 29. Boxplots illustrating the levels of blood lymphocytes in relation to GOLD stage (A) and the presence of emphysema (B). Solid line represents the median; bottom and top of the boxes are the 25th and 75th percentiles; brackets correspond to the 10th and the 90th percentiles.

Neutrophils to Lymphocytes Ratio

When we considered the neutrophils to lymphocytes ratio (NLR), we observed a significant significant direct relation to symptom scales, including mMRC (p<0.001; r=0.230) and CAT (p=0.021; r=0.217), a significant direct relation to the number of acute exacerbations during the previous year (p=0.020; r=0.133) both severe (p< 0.001; r=0.313) and mild-moderate (p=0.004; r=0.156), and an inverse relation to pulmonary function including FEV1 %pred. (p<0.001; r=-0.257), FEF25-75 %pred. (p=0.027; r=-0.153) and DLCO (p=0.021; r=-0.230).

Monocytes

Monocytes were directly related to exacerbations but not to respiratory function impairment or respiratory symptoms. In particular, monocytes were directly related to the number of total exacerbations during the previous year (p=0.018; r=0.217), both severe (p=0.001; r=0.296) and mild-moderate (p=0.007; r=0.248). Among comorbidities, we observed that patients with chronic bronchitis had significantly higher levels of blood monocytes (628±198 vs 522±193; p=0.005).

Eosinophils

Finally, we did not observe any significant relationship between blood eosinophils and disease severity nor any association to a specific comorbidity.

STUDY X

Deranged immune response to viral infections occurs in COPD patients and correlate with the disease severity

Study in progress

Partnership: University of Ferrara, Dpt. of Translational Medicine

BACKGROUND

Chronic Obstructive Pulmonary Disease (COPD) is a major cause of morbidity and mortality throughout the world. It is characterized by airflow limitation that is not fully reversible and is associated with an abnormal inflammatory reaction of the lungs, mainly in the small airways and the lung parenchyma (10). Exacerbations, acute events characterized by worsening in lung functions and symptoms requiring the use of antibiotics and/or systemic corticosteroids, are the clinical core of the disease. Indeed, exacerbations are directly related to disease progression, mortality and disease-related costs (177). Infections of the tracheobronchial tree are the most common causes of COPD exacerbations. Although bacterial infections have been considered in the past the main infective cause of exacerbations, recent evidence suggests that common respiratory viruses (in particular rhinovirus) are responsible for a high proportion of exacerbations, and may even trigger subsequent bacterial infections (178, 179). Thus, the identification of molecular mechanisms that pave the way to exacerbation can provide novel targets of potential pharmacological intervention able to change the natural history of the disease. We have previously reported (Study III) that a defective innate immune response to rhinovirus (with impaired IFN production) is a characteristic of asthma, which is present from the early stages of the disease and is linked to chronic type 2 inflammation of the airways. Accumulating evidence from experimental work with epithelial cells *in vitro* but also some preliminary data coming from *in vivo* models of COPD exacerbation support also the presence of impaired immune response (180,181). Whether disturbance of the immune host response and in particular of IFNs signaling pathway may occur in vivo in peripheral lungs of COPD patients and whether it is related to the clinical presentation of the disease is yet unknown.

AIM OF THE STUDY

We hypothesize that a deranged immune response to viral infections may occur in COPD patients and may correlate with the extent of inflammation in the peripheral lung. To this aim we evaluated the activation of the type 1 IFN pathway (i.e expression of the receptors IFNAR1/IFNAR2 and signaling mediators MDA-5 and IRF3) in peripheral samples of patients with COPD compared to appropriate smoking and nonsmoking controls. In parallel, we examined the infiltration of lung tissue by NK cells and eosinophils, which were correlated with IFN mediators and with clinical/functional traits.

METHODS AND MATERIALS

Subject Characteristics and Definitions

We collected peripheral lung tissue from 58 subjects undergoing surgery for appropriate clinical indications: lung resection for solitary peripheral nodules or lung volume reduction surgery for severe emphysema. The subjects were categorized into the following three groups: COPD patients (smokers with persistent airflow obstruction; n=22); smokers with normal lung function (SC; n=21) and non-smokers controls with normal lung function (nSC n = 15). Diagnosis of COPD has been done according to the Global Initiative for chronic obstructive lung disease (10). Smokers have been defined as less or more than 100 cigarettes during their entire life, cumulative smoke exposure has been expressed by packs/years. Diagnosis of CB has been confirmed when patients reported a productive cough (producing mucus) that lasts at least three months and happens multiple times over the course of at least two years. Each patient underwent clinical and functional pre-surgical evaluation including: anamnesis, physical examination, electrocardiography, blood tests, and pulmonary function tests, these data have been utilized for this study. Smokers with normal lung function and nonsmokers did not receive antiinflammatory therapy (e.g., oral or inhaled corticosteroids) or antibiotics within the month preceding surgery. The study conformed to the Declaration of Helsinki and was approved by the local ethics committee. Informed written consent was obtained for each subject undergoing surgery.

Immunohistochemistry and Morphometric Analysis

Lung tissue preparation and immunohistochemistry were performed with standard methods. Briefly, sections were treated for 60 minutes with primary antibodies rabbit anti-human IFNAR1 and IFNAR2 (GTX54322 and GTX54289 from GeneTex, Irvine CA, USA), MDA-5 (700360 from Invitrogen, Carlsbad CA, USA) and murine anti-human IRF-3 (sc-376455 from Santa Cruz Dallas TX, USA). Biotinylated secondary antibodies, combined with streptavidin-HRP in the ABC (Avidin Biotin Complex, Vectastain Elite ABC kit; Vector Laboratories, Burlingame, USA) and DAB Chromogen were used to reveal the signal. Sections were counterstained in hematoxylin, dehydrated and mounted. The expression of IFN receptors and signaling mediators was quantified in alveolar macrophages, alveolar walls and bronchiolar epithelium. To quantify expression in alveolar macrophages at least 20 nonconsecutive high-power fields and at least 100 macrophages inside the alveolar spaces were evaluated for each subject. Digital images from the stained sections were obtained with a light microscope (Leica DM 2000) connected to a video recorder and a computerized image analysis system (LAS, Leica Application Suite). To standardize the results, cell counts were expressed as percentage of positive macrophages over total macrophages examined. Expression of the mediators was also evaluated in alveolar walls and bronchiolar epithelium using a semiquantitative score (0: no staining; 1: weak staining; 2: moderate staining; 3: strong staining) and expressed as percentage of maximal staining.

For detection of NK cells Human NKp46/NCR1 MAb from Bio-techne (Minneapolis, MN; USA) was used and signal revealed with streptavidin-HRP Complex and DAB as chromogen. For detection of eosinophils the mouse monoclonal anti-Eosinophil Cationic Protein (Diagnostic Developments, Uppsala, Sweden) was used, upon antigen retrieval (0.1% trypsin in 0.1% calcium chloride (pH 7.8) at 37° C for 15 minutes). Staining was detected with the EXPOSE Mouse and Rabbit Specific AP (red) Detection IHC Kit (AbCam, Cambridge UK). Positively stained cells were expressed as the number of cells per mm^2 of the examined tissue area. Negative controls for nonspecific binding were processed, either omitting the primary antibody or using isotype IgG, and revealed no signal.

Statistical Analysis

All cases were coded and the measurements were made without knowledge of clinical data. All analyses were performed using SPSS (v26, IBM Armonk, NY, USA) (level of significance $p<0.05$). For continuous variables, normal distributions were tested using the Shapiro-Wilk test. Group data were expressed as median and interquartile range or frequency (absolute number and percentage) when appropriate. Differences between groups were analyzed using the following tests for multiple comparisons: the analysis of variance and the unpaired Student's t test for clinical data and molecular findings, and the Kruskal-Wallis test and the Mann-Whitney U test for morphologic data, Chi square or Fisher's exact test for nominal variables. Correlation coefficients were calculated using Spearman's rank method.

RESULTS

Clinical Characteristics of Study Subjects

The clinical characteristics of the subjects examined are shown in Table 19. Demographic analysis revealed that age was similar in the three groups of subjects. Moreover, smokers with COPD and control smokers had a similar cumulative exposure to cigarette smoking and prevalence of active smoking. COPD and smokers showed a significantly higher prevalence of male gender in comparison to nSC ($p=0.005$). As expected from the selection criteria, subjects with COPD had significantly lower values of FEV1 % predicted and FEV1/FVC % as compared with control smokers and non-smokers ($p<0.001$). Moreover, values of FEV1/FVC were significantly decreased also in smoker controls in comparison to non-smokers ($p=0.044$). The distribution according to GOLD stages was: 4 patients with very severe COPD (GOLD stage IV), 2 with severe COPD (GOLD stage III), 8 with moderate (GOLD stage II) and 8 with mild disease (GOLD stage I). Chronic bronchitis was present in 50% of COPD patients in our study (11 out of 22) and only in one patient among smoking controls ($p<0.001$).

Table 19. Clinical e demographic characteristics of the study groups.

	COPD	Smoker controls (SC)	Non-smoker controls (nSC)	p-value
Subjects, n	22	21	15	
Age (yrs)	70 [65-76]	68 [60-64]	69 [65-72]	0.592
Male/Females, n (%)	20 (90) / 2 (10)	17 (81) / 4 (19)	5 (33) / 10 (67)	0.005
Current/Former smokers, n (%)	11 (50) / 11 (50)	7 (33) / 14 (67)	---	0.278
Smoking exposure (p-yrs)	42 [35-50]	40 [23-50]	---	0.451
Dyspnoea (mMRC)	1 [1-2]°	0 [0-1]	0 [0-0]	0.025
Acute exacerbations (n)	1 [0-2]	n.a.	n.a.	---
FEV1/FVC (%)	61 [47-65]*	75 [73-81]#	81 [79-83]	<0.001
FEV1 (%)	67 [49-84]$	102 [91-109]	104 [93-118]	<0.001
deltaFEV1 postBD (L)	0.06 [-0.02 – 0.1]	n.a.	n.a.	---
Chronic bronchitis, n (%)	11 (50) /11 (50)	1 (4) / 20 (96)	—	<0.001

*Data are expressed as median [interquartile range] or frequency absolute number (percentage) as appropriate. P-value is referred to by the comparison between the three/two groups performed by Kruskal-Wallis/Mann-Whitney or Chi-square as appropriate. *p<0.001 vs SC and nSC; #p=0.044 vs nSC; $p<0.001 vs SC and nSC. ° data available for 13 patients out of 22.*

Immunohistochemical findings

In our cohort, immunoreactivity for IFN receptors (IFNAR1 and 2) and mediators (IRF-3 and MDA-5) in peripheral lung was most prominent in alveolar macrophages. Distinct immunoreactivity was also observed in the peripheral airways (epithelial cells) and in the alveolar walls (either on pneumocytes and interstitial infiltrating cells). When detailed quantitative analysis was performed on alveolar macrophages, we observed that the percentage of IRF-3$^+$ alveolar macrophages was increased in COPD and in control smokers, compared with non-smoker controls (p=0.013 and p=0.032, respectively, Table 20, figure 30A). The percentage of MDA-5$^+$ macrophages was extremely variable, with no significant difference between smokers with COPD, smokers with normal lung function and nonsmoking controls (Table 2). The expression of both receptors IFNAR1 and IFNAR2 also did not differ significantly among the three groups of subjects. When we graded by means of a semiquantitative score the immunoreactivity for IFN receptors and mediators in the alveolar walls and bronchiolar epithelium, we also did not see any difference among the study groups (Table 20). We then quantified the infiltration of the peripheral lung by NK cells (Table 20, figure 30B). We observed increased numbers of NK cells in COPD compared to both control smokers with normal lung function and nonsmokers (p<0.0001 for both comparisons). Smoking controls (SC) showed tendentially higher levels of NK cells in comparison to non-smoking controls (p=0.062). Conversely,

when we quantified the infiltration of eosinophils in the lung parenchyma, there was no difference among the three groups of subjects examined (Table 20).

Table 20. Immunohistochemical findings of the study groups

	COPD	Smoker controls (SC)	Non-smoker controls (nSC)	p-value
IRF3$^+$- macrophages (%)	6.5 [1-35]*	5 [0-17]#	0 [0-1.7]	0.023
IRF3$^+$- alveolar wall (%)	0 [0-20]	0 [0-33]	0 [0-0]	0.577
IRF3$^+$- epithelium (%)	0 [0-50]	0 [0-35]	0 [0-10]	0.965
MDA5$^+$- macrophages (%)	56 [37-79]	74 [34-86]	53 [34-71]	0.365
MDA5$^+$- alveolar wall (%)	20 [0-41]	41 [21-62]	10 [0-20]	0.211
MDA5$^+$- epithelium (%)	0 [0-50]	0 [0-50]	0 [0-18]	0.444
IFNAR1$^+$- macrophages (%)	10 [0-32]	6 [0-13]	-	0.393
IFNAR1$^+$- alveolar wall (%)	3 [0-33]	0 [0-20]	-	0.842
IFNAR1$^+$- epithelium (%)	7.5 [0-50]	16.5 [0-37.5]	-	0.952
IFNAR2$^+$- macrophages (%)	1.5 [0-18]	6 [0-15]	-	0.889
IFNAR2$^+$- alveolar wall (%)	0 [0-5]	0 [0-13]	-	0.905
IFNAR2$^+$- epithelium (%)	34 [0-50]	33 [0-58]	-	0.462
Eosinophils (cell/mm^2)	0.3 [0-1.3]	0.7 [0.3-3.7]	0.2 [0-0.9]	0.094
NK lymphocytes (cell/mm^2)	10.5 [9-17]$	5 [3-9]£	4 [1-4]	<0.001

*Data are expressed as median [interquartile range]. P-value reported the comparison between the groups performed by Kruskal-Wallis or Mann-Whitney-U test. *p=0.013 vs NS; #p=0.032 vs NS; $ p<0.001 vs SC and NS; £ p=0.062 vs NS calculated by Mann-Whitney-U test.*

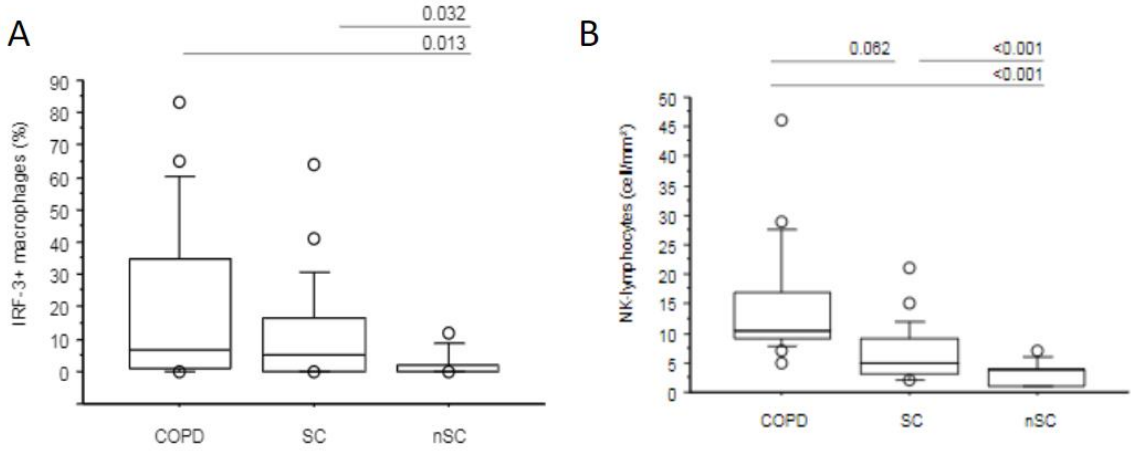

Figure 30. Boxplots illustrating the percentage of IRF3+-macrophages (A) and NK lymphocytes (B) in patients with COPD, smoker controls (SC) and non-smoker controls (nSC). Solid line represents the median; bottom and top of the boxes are the 25th and 75th percentiles; brackets correspond to the 10th and the 90th percentiles.

Relation between innate mediators/inflammatory parameters

In the whole cohort, we observed a positive correlation between the expression of IRF3 and that of MDA-5 in alveolar macrophages (p=0.01; r=0.34). Of importance, in all subjects macrophages expressing IRF-3 and MDA-5 were positively related to the numbers of eosinophils infiltrating the lung (p=0.011; r=0.34 and p=0.004; r=0.38). The expression of IRF-3 in macrophages was also positively related to the numbers of NK cells in lung tissue (p=0.016; r=0.31). These correlations remained significant when analysis was limited to smokers (COPD + SC) but not when we restricted the analysis to COPD group alone.

Relation between innate mediators/clinical and functional parameters

To gain insight into the functional significance of the observed difference we correlated the expression of innate immunity mediators to functional parameters and clinical presentation (presence of chronic bronchitis, MMRC Dyspnea scale, number of exacerbations in the previous year). Among COPD patients, the expression of IRF-3 was directly related to pulmonary function, expressed as FEV1% pred (p=0.016; r=0.525; figure 31A), while no relation has been observed with mMRC scale. When stratified by the presence of chronic bronchitis, a non-significant trend was observed in the expression of IRF-3^{+} in COPD patients with chronic bronchitis (p=0.063). Of interest, IRF3 expression was indirectly related to the number of acute exacerbations during the previous year (p=0.018; r=-0.653; figure 31B). We then combined the information on bronchitis and FEV1 and correlated with that of IRF3 expression. As shown in figure 32A, we observed that patients with low lung functionv(FEV1%pred. <25th percentile) and concomitant chronic bronchitis had also lower expression of IRF-3. We did not observe any significant correlation in COPD patients between NK levels and the clinical variables considered (FEV1, mMRC, acute exacerbations and chronic bronchitis). However, as shown in figure 32B we observed that patients with low lung function and concomitant chronic bronchitis had lower levels of NK.

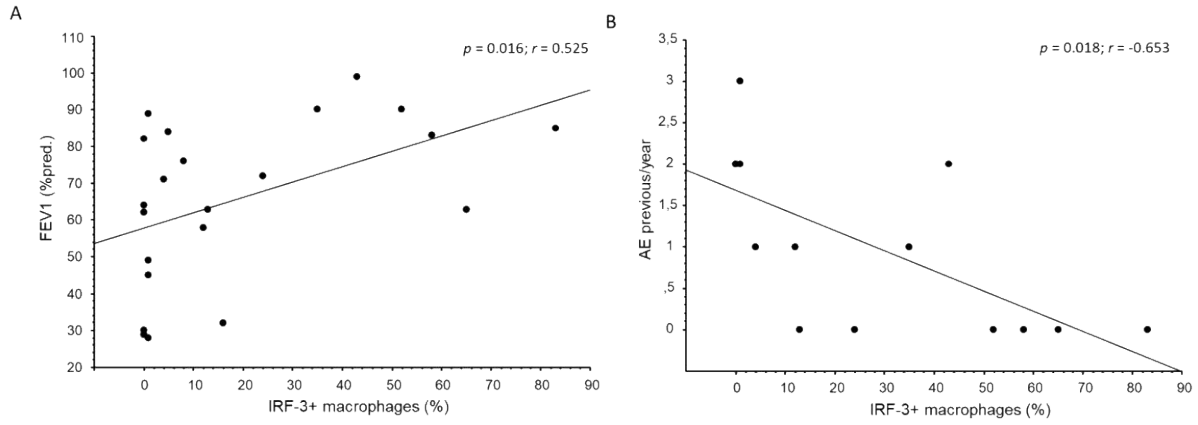

Figure 31. Bivariate scatterplots reporting in the COPD group the relationship between the percentage of IRF3+macrophages (x axes) and FEV1 (%pred.) (A) and the number of acute exacerbations in the previous year (B). Regression line is represented.

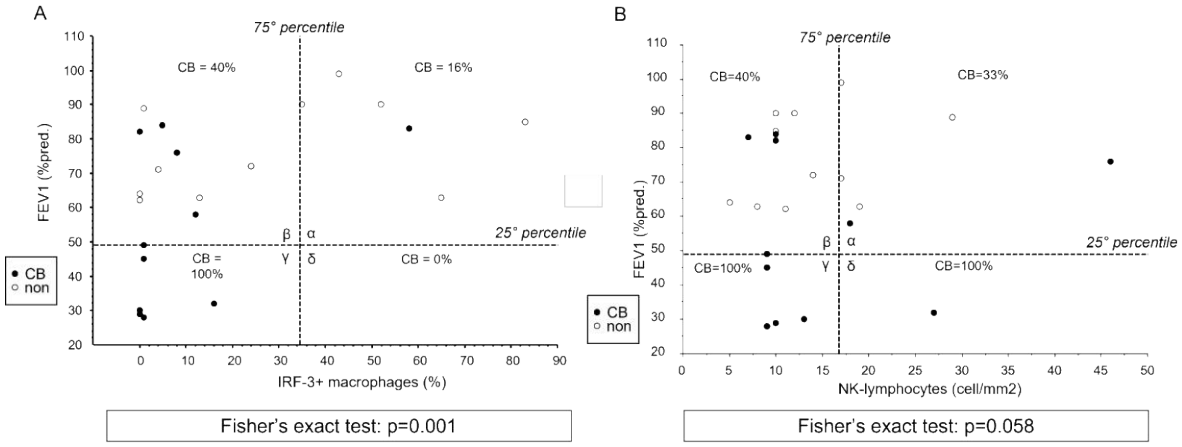

Figure 32. Bivariate scattergram reporting in COPD patients the relationship between FEV1 (%pred.) and the percentage of IRF3+ macrophages (A) or lymphocytes NK (B) in lung biopsies stratified for the presence (black dots) or the absence (white dots) of chronic bronchitis (CB). Fisher analysis showed a significant association between CB presence, airflow impairment and low IRF3 expression. P-value have been calculated by Fisher's exact test 4x2 contingency table.

STUDY XI

Quantification and role of innate lymphoid cell subsets in Chronic Obstructive Pulmonary Disease.

Blomme EE, Provoost S, De Smet EG, De Grove KC, Van Eeckhoutte HP, De Volder J, Hansbro PM, Bonato M, Saetta M, Wijnant SR, Verhamme F, Joos GF, Bracke KR, Brusselle GG, Maes T. Clin Transl Immunology. 2021 Jun 5;10(6):e1287

Partnership: Laboratory for Translational Research of Obstructive Pulmonary Diseases, Ghent University (BE)

BACKGROUND

Innate lymphoid cells (ILCs) are relatively rare, tissue-resident cells that make up the first line of defense at the mucosal barrier surface of the lung. ILCs are activated by epithelial and myeloid cell-derived cytokines and alarmins, leading to the production of effector cytokines that modulate immune responses. ILCs do not express rearranged antigen-specific receptors, which make fast expansion and activation possible (182). Three non-cytotoxic subsets can be distinguished, which parallel T-helper cells regarding their transcription factor and cytokine secretion. ILC1 expresses T-BET and secretes IFN-γ. ILC2 expresses GATA3 and produces both IL-5 and IL-13. ILC3 expresses RORγt and secretes both IL-17 and IL-22 (183). These cytokines have been associated with the pathogenesis of chronic obstructive pulmonary disease (COPD), which is characterized by abnormal chronic inflammatory responses to inhaled noxious particles and gasses (184). IFN-γ contributes to the activation of alveolar macrophages, and IFN-γ+CD8+ cells positively correlate with disease severity (STUDY X). High levels of IL-13 are found in serum of patients suffering from an acute exacerbation of COPD (185). IL-17A is involved in the recruitment of neutrophils and the formation of lymphoid follicles in COPD (186)). IL-22 mRNA and protein levels are increased in COPD patients, and IL-22-deficient (−/−) mice have reduced numbers of CS-induced pulmonary neutrophils and do not show CS-induced alveolar enlargement and airway remodeling (187)

Others have demonstrated increases in ILC1 frequency in the lungs of severe COPD patients compared to healthy subjects or those with mild-to-moderate COPD (188), and increased circulating IFN-γ-producing ILC1 in subjects with severe COPD (189). To what extent specific ILC subsets in the human lung are associated with clinical parameters of COPD has not been investigated. Moreover, localisation of all three ILC subsets by immunohistochemistry within the same human lung has not been previously assessed.

AIM OF THE STUDY

We assessed human pulmonary ILC subsets by flow cytometry and immunohistochemistry in lung tissue from well-characterised COPD patients and control subjects. We also investigated whether pulmonary ILC numbers and activation are altered in our COPD mouse model and whether they contribute to CS-induced innate inflammatory responses.

METHODS AND MATERIALS

Analysis of human samples

Study population

Subject characteristics are summarised in Table 21. Lung resection tissue was obtained from subjects who underwent lobectomy for solitary pulmonary tumors. Tissue at maximum distance of the pathologic lung lesion was collected and declared free of signs of pneumonia or tumor invasion. Subjects receiving chemo- or radiotherapy or suffering from recent COPD exacerbations or infections were excluded. Lung tissue from 9 control subjects (3 never smokers, 3 ex-smokers and 3 smokers, all without airflow limitation) and 15 COPD patients (4 GOLD I, 9 GOLD II and 2 GOLD III) was used for flow cytometric analysis. Two COPD GOLD II smoking patients, 2 smoking controls and 2 never smokers were selected for IHC analysis. Ex-smokers were defined as subjects who refrained from smoking for ≤1 year. A COPD Assessment Test was performed prior to surgery. Smoking habits and medication use were evaluated by interview, while COPD diagnosis was made based on preoperative spirometry. Written informed consent was obtained from all donors, according to the protocol approved by the Medical Ethical Committee of Ghent University Hospital (2016/0132).

Sample processing and flow cytometry

Samples of fresh lung resection tissue were fixed in paraformaldehyde 4% (Sigma, Bornem) for 20 h and paraffin-embedded for later immunohistochemical/immunofluorescent (IHC) analysis. Remaining fresh lung tissue was processed into cell suspensions as described in (190). Cell counting was performed with the Coulter Counter (Beckman Coulter, Inc., Fullerton, CA), and cells were stained for flow cytometry. Non-specific binding was reduced by pre-incubating the samples with human IgG. The LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Thermo Fisher, Waltham, MA) was used to exclude dead cells. To exclude lineage markers, the following human monoclonal antibodies were used: peridinin chlorophyll protein–cyanine 5.5 (PerCP)-conjugated anti-CD1a (HI49), anti-CD3 (OKT3), anti-CD14 (63D3), anti-CD19 (HIB19), anti-CD11c (3.9), anti-CD11b (M1/70), anti-CD123 (6H6), anti-CD34 (581), anti-TCRα/β (IP26), anti-TCRγ/δ (B1), anti-BDCA2 (201A), anti-FCεR1 (AER-37) and anti-CD235A (HI264).To distinguish ILC subsets based on surface staining, the following antibodies were used: fluorescein isothiocyanate (FITC)-conjugated anti-CD45 (HI30), allophycocyanin (APC)-conjugated anti-NKp44 (P44-8), phycoerythrin (PE)/indotricarbocyanine (Cy7)-conjugated anti-CD117 (104D2), Brilliant Violet 421TM-conjugated anti-CD127 (A019D5), Brilliant Violet

605TM-conjugated anti-CD56 (HCD56) (all from BioLegend, San Diego, CA), PE-conjugated CD161 (HP3610, eBioscience) and biotinylated anti-CRTH2 (BM19; Miltenyi Biotec, Bergisch Gladbach, Germany) in combination with streptavidin-APC-Cy7 (BD Biosciences, San Jose, CA). To identify ILC subsets based on cytokine staining, the same PerCP-conjugated lineage antibodies were used, except for CD3, which was replaced by APC-Cy7-conjugated anti-CD3 (SK7). In addition, FITC-conjugated anti-CD45 (HI30), BV605-conjugated anti-CD56, APC-conjugated anti-CD161 (HP-3G10, all from BioLegend), PE-TR-conjugated anti-IFN-γ (EB 45.B3), PE-conjugated anti-IL-13 (EB 85BRD) and PECy7-conjugated anti-IL17 (EB eBio64DEC17, all from eBioscience, San Diego, CA) were used.

Immunohistochemical/immunofluorescence analysis

Innate lymphoid cell was visualised using specific antibodies against CD3 (Bio-Rad, Hercules, CA), CD20 (Invitrogen, Rockford, IL), CD68 (Invitrogen), CD56 (Novus Biologicals, Abingdon) and mast cell tryptase (Abcam, Cambridge) in combination with T-BET (Santa Cruz, Dallas, TX), GATA3 (Biocare Medical, Pacheco, CA) or RORγt (Millipore, County Cork). ILC populations were defined as CD3−T-BET+(ILC1), CD3−GATA3+(ILC2) and CD3−RORγt+(ILC3), and negative for CD20, CD68, CD56 and mast cell tryptase (deep space black chromogen exclusion).

Statistical analysis

Statistical analysis was performed with SPSS 24.0 and 26.0 (SPSS Inc. Chicago, IL). Non-parametric tests (Kruskal–Wallis and Mann–Whitney U) were used to compare independent groups, according to standard statistical criteria. Values were reported as median ± IQR. P-values < 0.05 (*) were considered statistically significant. Linear regression analyses were performed for the association between CAT scores or lung function and ILC1. GraphPad Prism (GraphPad Software Inc., San Diego, CA) was used to analyse data.

Murine experiments

Mice

Male C57BL/6J mice (8–9 weeks old) were purchased from Charles River (Ecully). To investigate the functional role of ILCs, C57BL/6NTac, Rag2 −/− and Rag2/Il2rg −/−mice were purchased from Taconic (Germantown, NY). Experimental protocols were approved by the Animal Ethical Committee of the Faculty of Medicine and Health Sciences (Ghent University, ECD 14/76 and 16/68) and were carried out in accordance with institutional guidelines for animal care.

Exposure protocol

Mice were exposed to mainstream tobacco smoke from 5 cigarettes (Standard Research Cigarette 3R4F, University of Kentucky, without filters), 4 times daily using a whole-body exposure system as we have previously performed extensively (136, 137, 191-193). There were 30-minute smoke-free intervals between each exposure.

In the smoking chambers, an optimal smoke–air ratio of 1/6 was set. Control groups were exposed to air. This procedure was followed for 5 consecutive days/week for 1, 4 or 24 weeks. Analysis was performed the day after the last smoke exposure, when mice were sacrificed with pentobarbital overdose (Vétoquinol NV/sa, Aartselaar). Results of the 4-week experiments are representative of ≥ 2 independent experiments.

Lung histology and bronchoalveolar lavage

Haematoxylin/eosin staining and CD3/B220 staining of lung sections, bronchoalveolar lavage, cell counts and RT-PCR were performed as described previously (191, 137). Neutrophils on lung tissue were visualized with anti-mouse myeloperoxidase (R&D Systems, Abingdon, UK) and counterstained with haematoxylin. Protein measurements in BAL were determined by ELISA (CCL2 and CXCL1, R&D Systems, Abingdon, UK) or using the High-Sensitivity BD Cytometric Bead Array (BD Biosciences, San Jose, CA) according to the manufacturers' instructions.

Flow cytometry

Cells were pre-incubated with FcR blocking antibody to reduce non-specific binding (anti-CD16/CD32, Clone 2.4G2). All staining procedures were performed in PBS without Ca2+ and Mg2+ but containing 5 mM EDTA and 1% BSA. Cells were stained with a combination of fluorochrome-conjugated monoclonal antibodies (surface markers) similar to 137 CD3 (145-2C11), CD5 (53-7.3), TCR-β (H57-597), CD4 (GK1.5), CD45 (30-F11), CD11b (M1/70), CD11c (N418), CD45R (RA3-6B2), CD90.2 (30-H12), Ly6G (1A8), Siglec-F (E50-2440), NK1.1 (PK136), Ly6C (BD AL-21), MHCII (BL M5/114.15.2) and biotinylated anti-T1/ST2 (MD Bioproducts, St. Paul, MN) in combination with streptavidin–phycoerythrin (SAV-PE) (BD Biosciences). For cytokine staining, cells were stimulated for 4 h at 37°C and 5% CO2 with a cocktail of PMA and ionomycin, supplemented with brefeldin A and monensin (eBioscience). Cells were fixed with the Foxp3 transcription factor fixation/permeabilisation set and stained with antibodies against IFN-γ (XMG1.2), IL-13 (eBio13A) and IL-17A (17B7) for cytokine staining and with T-BET (EBio4B10), GATA3 (EB TWAJ) and RORγt (EB B2D) for nuclear staining (all eBioscience). Flow cytometric analysis was performed using LSRFortessa, and data were assessed using FlowJo Software.

RESULTS

Pulmonary ILC1 are elevated in COPD patients

To investigate the involvement of ILCs in COPD, human pulmonary ILC frequencies were analysed using flow cytometry of single-cell suspensions of freshly isolated lung tissue from COPD patients (n= 15) and control subjects (n = 9) (gating strategy Figure 33A). Subject characteristics are shown in Table 21. The frequency of CD161+CD127+ILCs was similar in COPD and control subjects (Figure 33B). However, discrimination of the different ILC subsets within the CD161+CD127+ILC

population based on surface marker expression showed that ILC1 (CRTH2−CD117−) was the dominant subset in COPD. Moreover, ILC1 frequency differed significantly among the groups (Kruskal–Wallis $P < 0.05$), and the median+IQR showed a numerical increase between never-smoking controls, smoking controls (ex+current) and COPD patients (Figure 33C). In the COPD group, no apparent differences in the distribution of ILC subsets in ex- or current smokers were detected. Notably, in this cohort, ILC1 frequencies from COPD GOLD I subjects were relatively high, which coincided with a high number of pack-years. The frequencies of CRTH2+ILC2 and CRTH2−CD117+ (both NKp44+ and NKp44−) ILC3 did not differ significantly within the CD161+CD127+ILC population (Figure 33C).

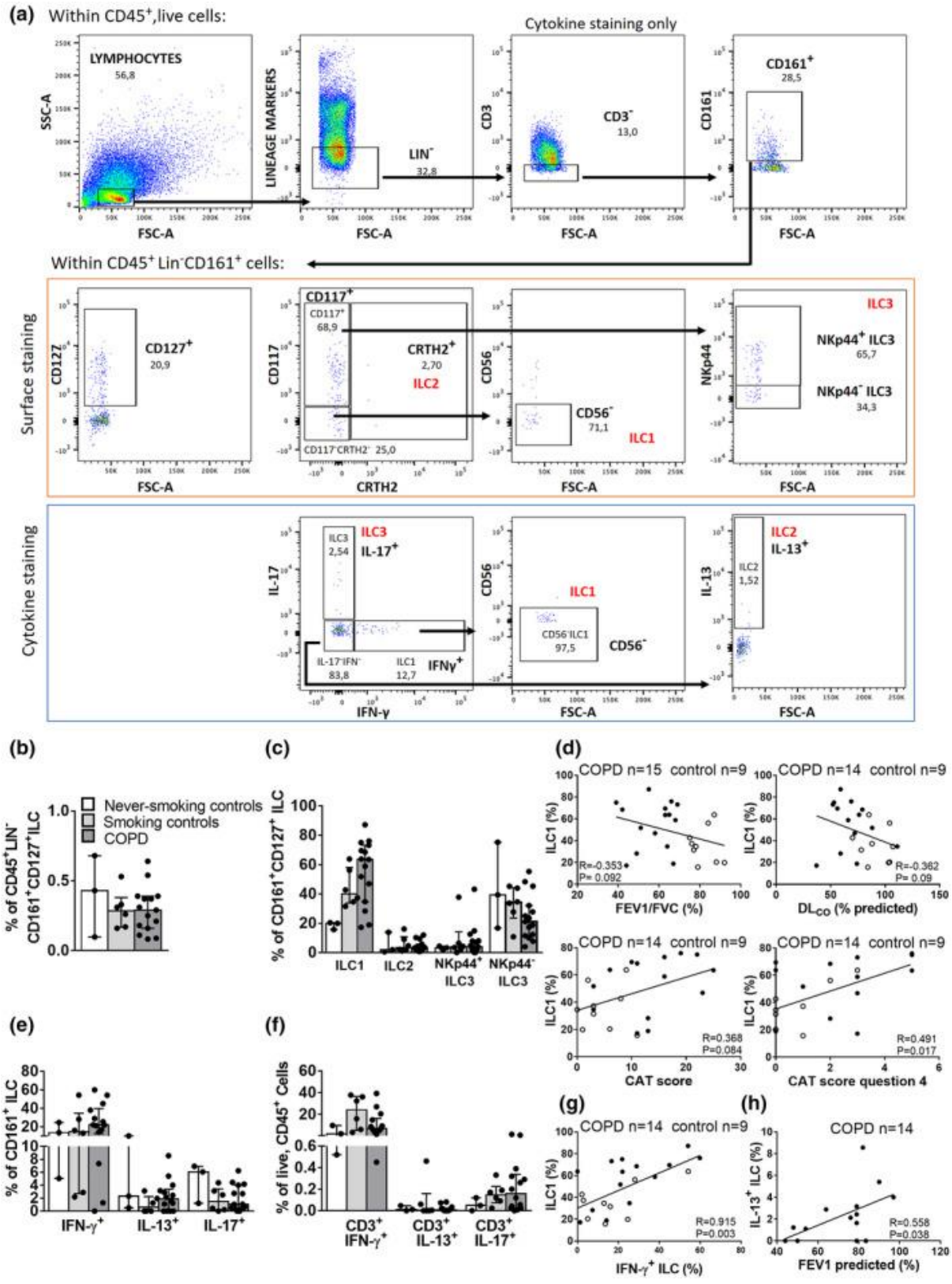

Figure 33. Quantification of pulmonary ILC in COPD patients and correlation with clinical parameters. For flow cytometric analysis, ILCs were surface-stained and gated as live, CD45+ lymphocytes that are Lin−(CD3, CD19, CD11c, CD11b, CD1a, CD14, CD34, CD123, TCRαβ, TCRγδ, BDCA2, CD235A and FcεR1) CD161+CD127+. ILC subsets were further defined as CD117−CRTH2−CD56− ILC1, CRTH2+ILC2 and CD117+CRTH2−NKp44+/−ILC3 (a, upper panel). For ILC labelling following 4h of

stimulation: ILCs were gated as live, CD45+ lymphocytes that were Lin−(CD19, CD11c, CD11b, CD1a, CD14, CD34, CD123, TCRαβ, TCRγδ, BDCA2, CD235A and FcεR1) CD3−CD161+, followed by defining subsets as IFN-γ+IL-17−CD56−ILC1, IFN-γ−IL-17−IL-13+ILC2 and IFN-γ−IL-17+ILC3 (a, lower panel). Percentage of CD161+CD127+ ILC of CD45+ live cells (b) and ILC subsets based on surface staining, expressed as % of CD161+CD127+ILC (c). Bar graphs on (b), (c), (e) and (f) represent the median values per group; error bars represent the interquartile range (IQR). Spearman's correlation between % of ILC1 and FEV1/FVC%, %DLCO, total CAT score and score on CAT question 4 (grade of breathlessness when walking up hill or a flight of stairs) (d). Percentages of ILC subsets based on cytokine staining, expressed as the % of CD161+ ILC (e). Percentage of Lin+CD56−CD3+ lymphocyte subsets based on cytokine staining, expressed as the % of CD45+, live cells: IFN-γ+IL-17− T, IFN-γ−IL-17−IL-13+ T and IFN-γ−IL-17+ T (f). Spearman's correlation between % of ILC1 and % of IFN-γ+ILC (g) and % of IL-13+ILC and FEV1% predicted (h) in control subjects (clear dots) and COPD patients (black dots). Group sizes: GOLD I = 3 or 4; GOLD II = 8 or 9; GOLD III = 2, never-smoking controls = 3, ex-smoking controls = 3, smoking controls = 3.

Table 21. Subjects characteristics

Characteristics	Controls (n=9)	COPD (n=15)
Gender (male/female)	3/6	10/5
Age, years	62 [57-76]	66 [59-60]
Body mass index	25 [57-76]	66 [59-69]
Smoking status (never/current/former)	3/3/3	0/8/7
GOLD stage 1/2/3/4	n.a.	4/9/2/0
FEV1% predicted	124 [114-126]	79 [59-80.5]
FVC% predicted	121 [103-136]	99 [92-108]
FEV1/FVC	70 [77-87]	63 [50-65]
CAT score	3 [2-8]	13 [10-18]
DLCO	87.9 [84-104]	67.5 [56.8-75.5]

Data are expressed as median [interquartile range]. n.a.: not applicable; FEV1: forced expiratory volume in 1s; FVC: forced vital capacity; DLCO: diffusing capacity of the lungs for carbon monixide.

To investigate whether any of all of the ILC subsets were associated with disease severity, a Spearman correlation analysis was performed between ILC subsets and clinical and functional markers of COPD severity. The frequency of ILC1 tended to inversely correlate with FEV1/FVC% (as marker of airflow limitation) and with diffusing lung capacity of carbon monoxide (DLCO, a marker of emphysema and gas exchange) (Figure 33D). The frequency of ILC1 positively correlated with the total score in the COPD Assessment Test (CAT); the higher the CAT score, the larger the impact of COPD on a patient's health status. In addition, regression analysis

demonstrated that ILC1 frequency significantly correlated with an indicator of dyspnoea (breathlessness) in the CAT test (Figure 33D), even when adjusted for smoking status. Using lung single-cell suspensions from the same cohort, we evaluated intracellular cytokine production within ILCs (gating strategy Figure 33A) and CD3+T cells by stimulating the cells with phorbol myristate acetate (PMA)/ionomycin, followed by flow cytometric analysis. Similar to the data from the surface marker staining, the IFN-γ+ ILCs represented the largest ILC subset, in both COPD and control subjects (Figure 33E). The CD3+IFN-γ+ T-cell frequencies were similar in never-smoking controls and COPD but tended to increase in smoking controls (Figure 33F). ILC1 frequency identified with surface marker staining strongly correlated with the frequency of IFN-γ+ILCs (Figure 33G). In COPD patients, IL-13+ILC frequency is positively associated with FEV1% predicted (post-bronchodilator) (Figure 33H). No significant associations between the frequencies of the other ILC subsets and these clinical parameters were found (data not shown).

Pulmonary ILC localize near lymphoid aggregates in COPD

Next, we determined the presence and localisation of all three ILC subsets, in lung tissue samples of COPD patients and controls using immunohistochemical analysis. ILCs were identified as CD3− cells, positive for the transcription factor T-BET (ILC1), GATA3 (ILC2) or RORγt (ILC3). B cells, NK cells, macrophages and mast cells were excluded by positivity for CD20, CD56, CD68 and mast cell tryptase, respectively. In assays using isotype controls for the antibody combinations, no staining was observed. Despite being rare cells, all three ILC subsets could be identified in the same lung tissue. In never-smoking control lung tissue, ILCs localized in the parenchyma. In lung tissue from smoking controls and COPD patients (GOLD stage II), they localized in and near lymphoid aggregates (Figure 34). Also, the three T-helper subsets (Th1, Th2 and Th17) could be identified. Using immunofluorescence, which allows visualization of antibody staining in different channels separately, the presence of the three ILC subsets in the lung was confirmed (Figure 35A-C).

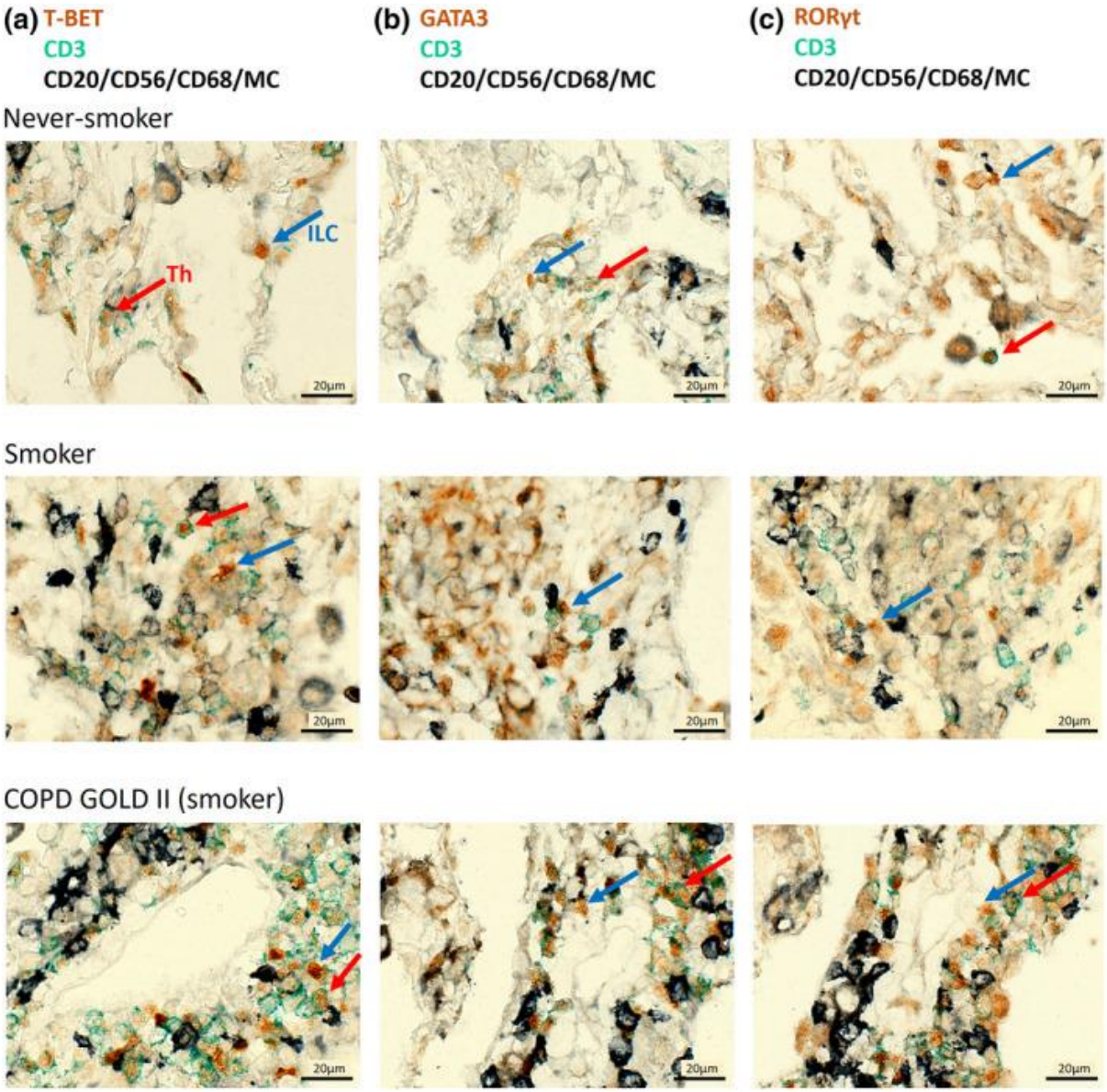

*Figure 34. Immunohistochemical detection of pulmonary ILC and Th cells in COPD patients and controls. T-BET/GATA3/RORγt in brown, CD3 in green and antibodies against CD20, CD56, CD68 and mast cell tryptase (exclusion cocktail) in black. Staining was performed in COPD GOLD II patients and smoking and never-smoking controls. T-BET+CD3−ILC1 (in brown, blue arrow) and T-BET+CD3+Th1 cells (in brown with green border, red arrow) negative for exclusion markers **(a)**. GATA3+CD3−ILC2 (blue arrow) and GATA3+CD3+Th2 cells (red arrow) negative for exclusion markers **(b)**. RORγt+CD3−ILC3 (blue arrow) and RORγt +CD3+Th17 cells (red arrow) negative for exclusion markers **(c)**. Scale bar: 20 µm.*

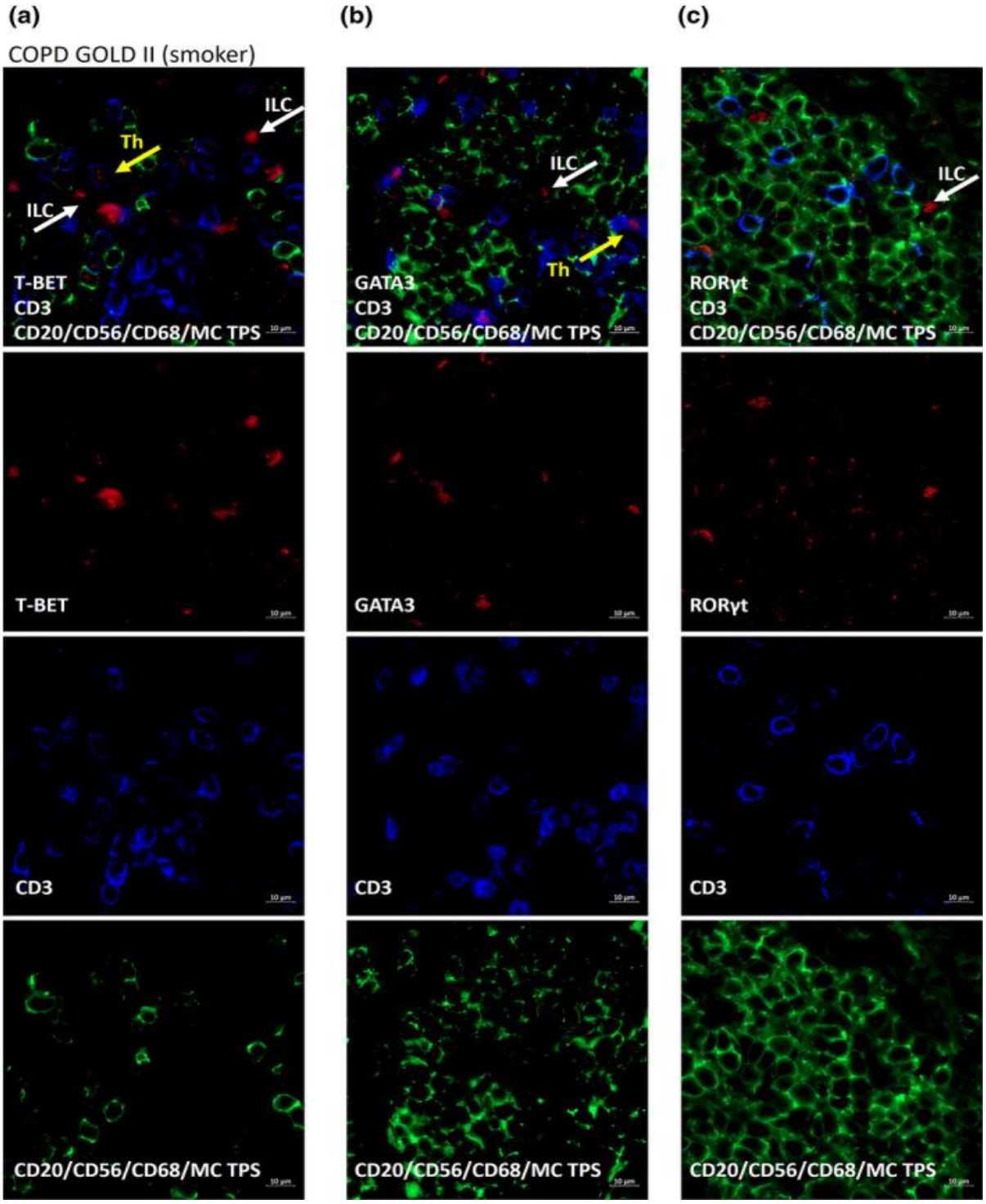

Figure 35. Immunofluorescent detection of pulmonary ILC and Th cells in COPD patients. T-BET/GATA3/RORγt (red), CD3 (blue), antibodies against CD20, CD56, CD68 and mast cell tryptase (MC TPS) (exclusion cocktail, green). Staining was performed on a COPD GOLD II patient (smoker). T-BET+CD3−ILC1 (red, white arrow), negative for exclusion markers and T-BET+CD3+Th1 (red with blue border, yellow arrow) (a). GATA3+CD3−ILC2 (white arrow) and GATA3+CD3+Th2 cell (yellow arrow) negative for exclusion markers (b). RORγt+CD3−ILC3 (white arrow) negative for exclusion markers (c).

CS induces increases in ILCs in a mouse model of COPD

Given that ILCs are an important source of cytokines associated with COPD pathogenesis, and mechanistic studies with human samples have not yet been performed, we investigated whether the total number of ILCs and the different ILC subsets were altered in a COPD mouse model. C57BL/6 mice were exposed to air or CS for 1, 4 or 24 weeks, followed by the evaluation of ILCs in the bronchoalveolar lavage (BAL) fluid and lung tissue. ILC subsets were identified according to the expression of surface markers and specific transcription factors: T-BET+ILC, T-BET−RORγt−ILC and RORγt+ILC represent ILC1, ILC2 and ILC3, respectively. Notably, CS exposure induced significant increases in the total lineage− CD90+ILCs, as well as all ILC subsets in BAL, at all time points (Figure 36B,C). Also, the % of CD90+ILCs increased within the CD45+ population. Within CD90+ILCs, the % ILC2 reduced upon CS exposure, whereas the % of ILC1 increased, after 4 and 24 weeks (Figure 36D). CS exposure also increased the numbers of ST2+ILC2 in BAL, but to a much lower level. In (lavaged) digested lung tissue, data on ILC frequencies upon CS exposure were inconclusive (not shown).

Signature cytokine expression upon CS exposure was evaluated in ILC (gating strategy Figure 37A) and in Th cells in BAL fluid. All cytokine-producing ILC subsets and corresponding Th subsets were increased in BAL following 4 and 24 weeks of CS exposure (Figure 37B-E). Within the CD90+ILC population, there was a predominant increase in the % of IFN-γ+ILCs both after 4 and 24 weeks. Also, other inflammatory cells were evaluated in the COPD model. As reported previously, a significant increase in the total numbers of inflammatory cells in BAL was observed in CS-exposed mice after 4 and 24 weeks. At all time points, neutrophils, monocytes and dendritic cells were increased in BAL following CS exposure. Lymphoid aggregates were observed after 24 weeks.

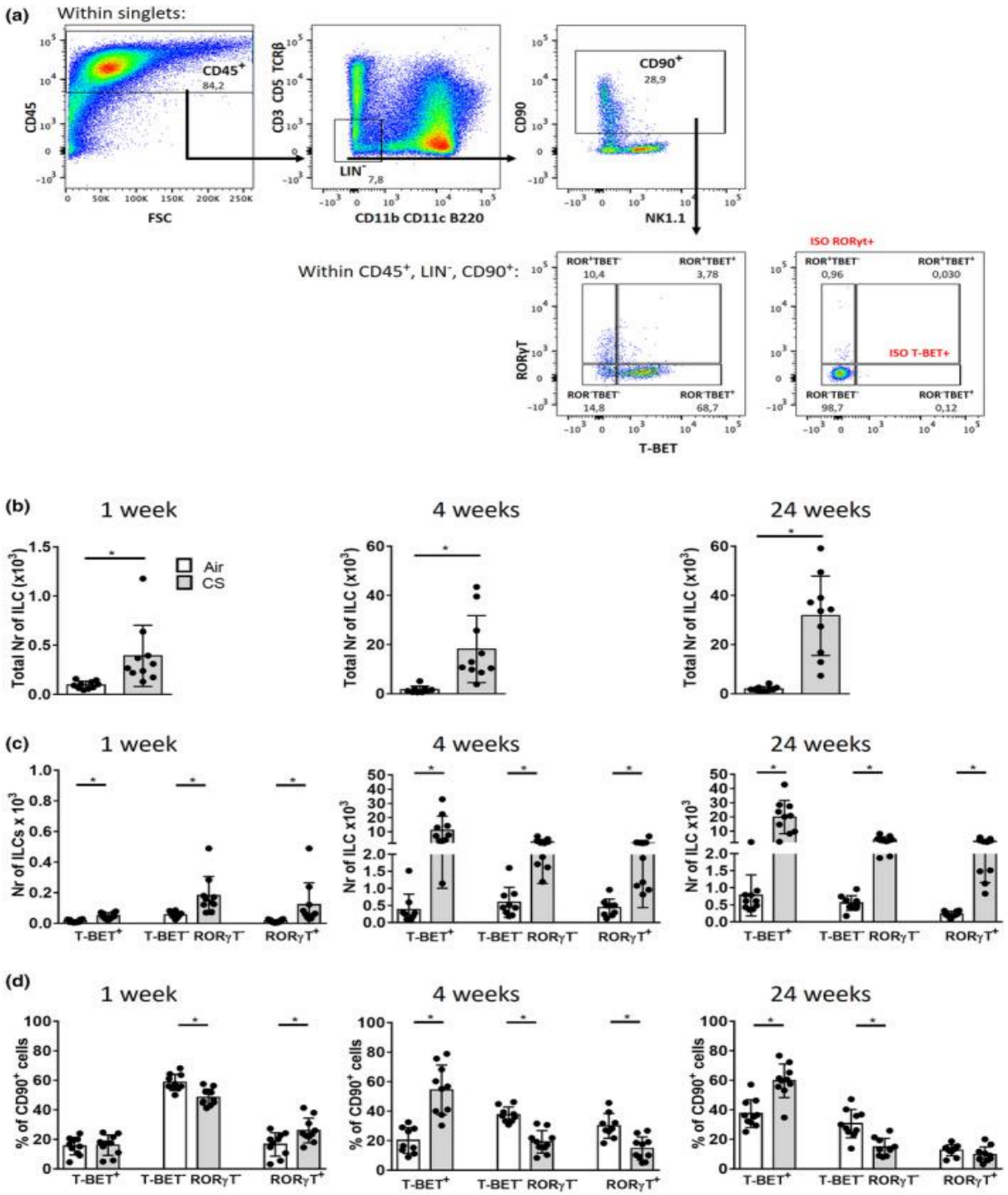

Figure 36. All pulmonary ILC subsets are increased following cigarette smoke exposure in a mouse model. Total ILCs were gated as CD45+Lin− (CD3−CD5−TCR-β−) Lin−(CD11b−CD11c−B220−) CD90+. ILC subsets were subsequently defined as RORγt−T-BET+ ILC1, RORγt−T-BET− ILC2 and RORγt+ T-BET− ILC3 ***(a)****. Total number of ILC* ***(b)****, ILC subsets* ***(c)*** *and % of ILC within CD45+Lin−Lin− CD90+ cells in mice exposed to CS (grey bars) or air (white bars) for 5 days or 4 or 24 weeks. Results are shown as mean values; error bars represent the standard deviation. N = 10 mice per group. *P < 0.05. Data from the 4-week experiments are representative of 2 experiments.*

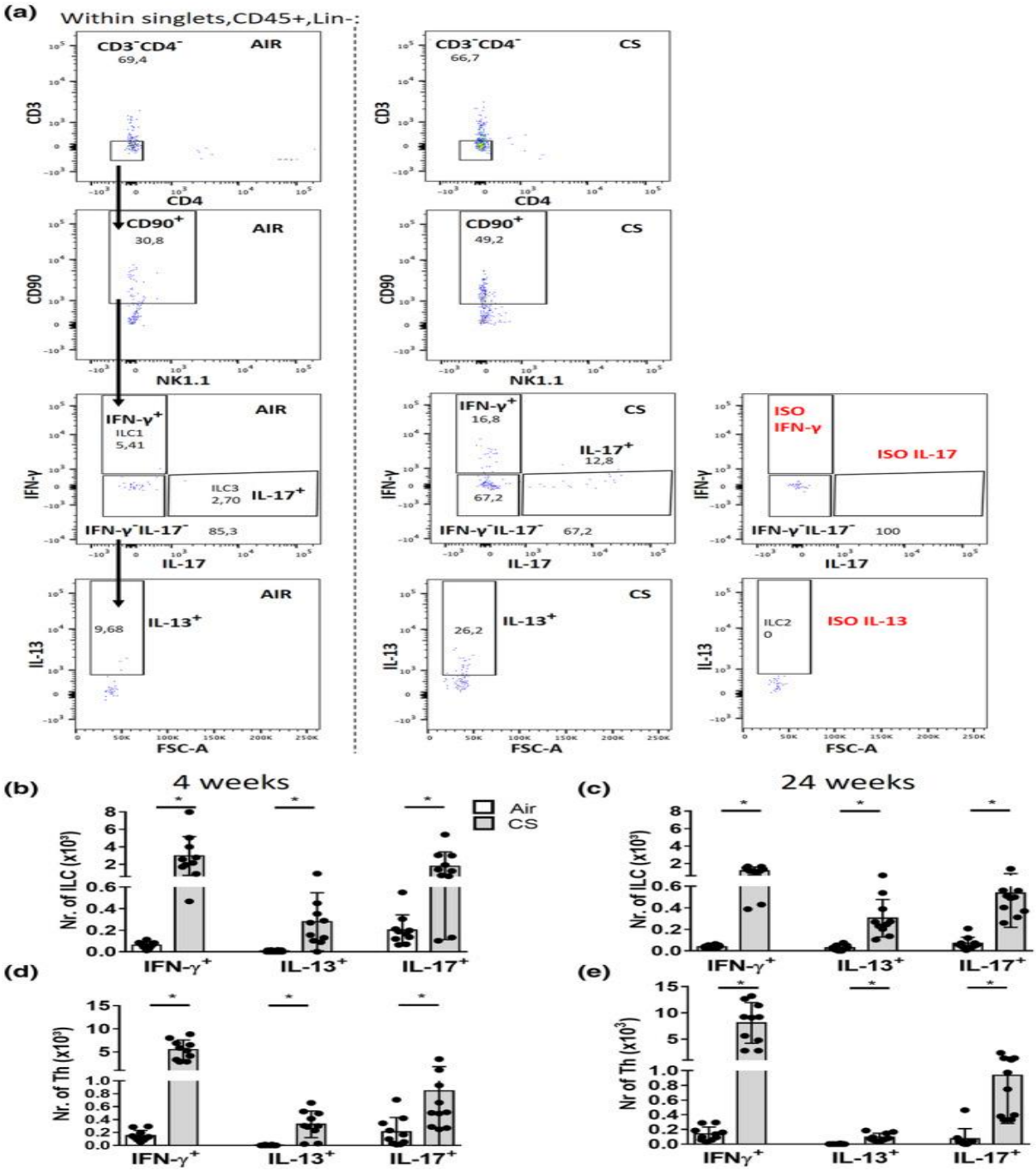

Figure 37. Signature cytokine expression in ILC and Th cells following cigarette smoke exposure in a mouse model. ILC gating following 4 h of stimulation: CD45+Lin−(CD5−TCR-β−)Lin−(CD11b−CD11c−B220−)CD3−CD4−CD90+ ILCs were gated, followed by defining IFN-γ+IL-17−ILC1, IFN-γ−IL-17−IL-13+ILC2 and IFN-γ−IL-17+ILC3 ***(a)****. Total numbers of cytokine-producing ILC* ***(b, c)*** *and Th subsets (CD45+, CD11b−CD11c−B220−, CD5+TCR-β+, CD3+CD4+)* ***(d, e)*** *in mice exposed to CS (grey bars) or air (white bars) for 4 or 24 weeks. Results are shown as mean values; error bars represent the standard deviation. N = 10 mice per group. *P < 0.05. Data of the 4-week experiments are representative of 2 experiments.*

ILC deficiency in *Rag2* −/− mice does not prevent CS-induced innate inflammation

The total numbers of activated ILCs increased in the COPD model (Figure 38), and we previously demonstrated that CS-induced innate inflammation is still present in *scid* mice that lack B- and T-lymphocytes but still have ILC. Thus, we hypothesized that ILC, as an important source of IFN-γ, IL-13 or IL-17, could drive CS-induced inflammatory responses. To evaluate how the additional absence of ILCs would affect CS-induced inflammation, we exposed WT (C57BL/6NTac background), *Rag2* −/− and *Rag2*/*Il2rg* −/− mice to air or CS for 4 weeks. Whereas ILCs are present in BAL fluid of *Rag2* −/− mice that lack an adaptive immune system, they are absent in *Rag2*/*Il2rg* −/− mice (Figure 38A). In air-exposed *Rag2* −/− mice, there were no Th cells, but the numbers of ILCs, monocytes and dendritic cells were higher than in air-exposed WT mice (Figure 38A-E). CS exposure of *Rag2* −/− mice increased the numbers of total BAL cells, ILCs, dendritic cells and neutrophils (Figure 38A,C,E, F). In *Rag2*/*Il2rg* −/− mice, CS-induced increases in total BAL cells, monocytes, dendritic cells and neutrophils were also present (Figure 38C-F). Hence, ILC deficiency in the absence of a functional adaptive immune system does not prevent CS-induced innate inflammation. The measurement of pro-inflammatory cytokines and chemokines in BAL fluid demonstrated that CS exposure significantly induced CCL2 (MCP-1), CXCL1 (KC), IL-6 and TNF-α protein levels in WT mice, and *Rag2* −/− and *Rag2*/*Il2rg* −/− mice compared to air exposure (Figure 6g, h–j). CS-induced IL-6, TNF-α and CXCL1 levels were significantly higher in Rag2−/− mice than in *Rag2*/*Il2rg* −/− mice. CS exposure also increased IFN-γ levels in *Rag2* −/− mice, whereas IFN-γ levels were below the detection limit in both WT- and *Rag2*/*Il2rg* −/−-deficient mice (Figure 38K). Thus, although CS-induced pro-inflammatory mediators were produced by the three mouse strains, the levels of several cytokines in *Rag2* −/− mice were reduced with ILC deficiency.In lung tissue, the numbers of neutrophils close to the airways were elevated upon CS exposure in all three mouse strains (Figure 39A,B). To evaluate the potential impact of the absence of ILCs on tissue destruction, MMP-12 mRNA levels in lung tissue were evaluated. CS exposure induced increases in MMP-12 mRNA levels in the three strains (Figure 39C). MMP-12 expression was higher in the CS-exposed *Rag2* −/− mice than in the CS-exposed WT mice. MMP-12 mRNA levels in the *Rag2*/*Il2rg* −/− mice tended to attenuate compared to *Rag2* −/− mice.

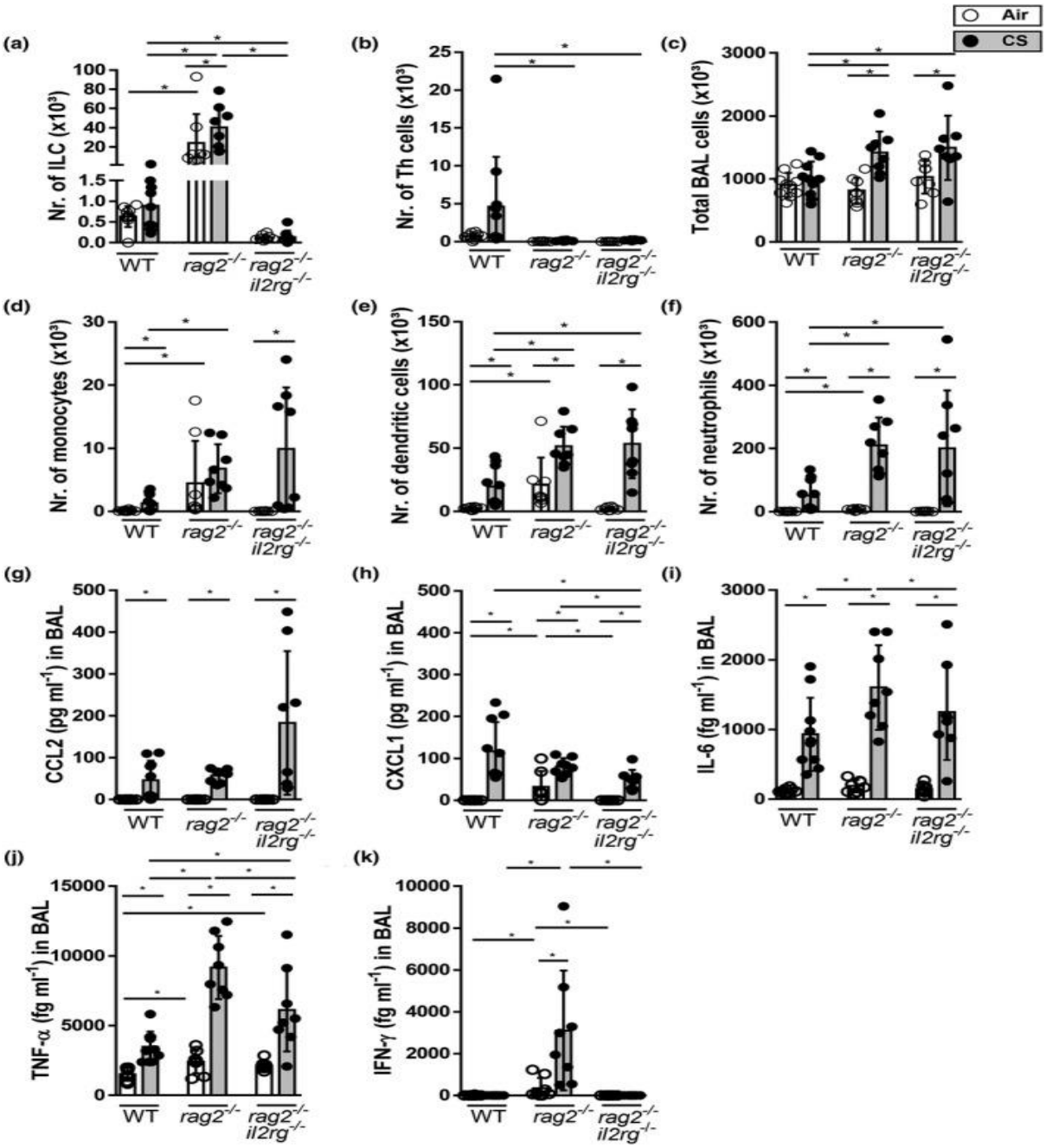

*Figure 38. In the absence of adaptive immunity, ILC deficiency does not prevent CS-induced airway inflammation in a mouse model. C57BL/6NTac, Rag2 −/− and Rag2/Il2rg −/− mice were exposed to CS (grey bars) or air (white bars) for 4 weeks. Total numbers of ILCs (a) and Th cells (b). Total number of BAL cells (c) monocytes, (d) dendritic cells (e) and neutrophils (f). Protein levels of CCL2 (g), CXCL1 (h), IL-6 (i), TNF-α (j) and IFN-γ (k) were determined in BAL fluid. Results are expressed as mean ± SD. N = 8–10 mice per group. *P < 0.05.*

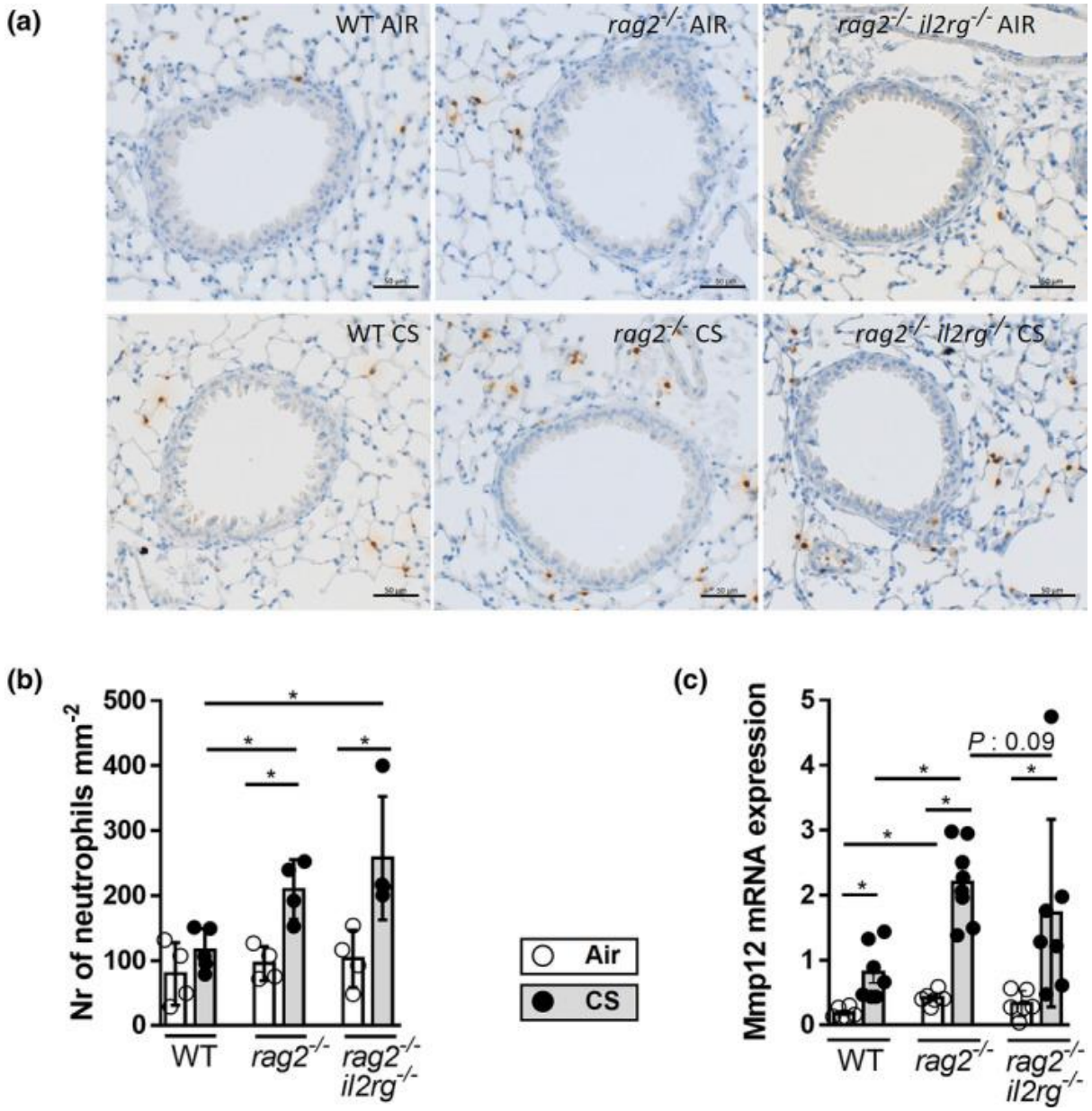

*Figure 39. FIn the absence of adaptive immunity, ILC deficiency does not prevent CS-induced neutrophilic inflammation or MMP-12 expression in murine lung tissue. C57BL/6NTac, Rag2 −/− and Rag2/Il2rg −/− mice were exposed to CS (grey bars) or air (white bars) for 4 weeks. Myeloperoxidase (MPO)-positive neutrophils in lung tissue are stained in brown **(a)** and quantified (numbers mm−2). N = 4 or 5 mice per group **(b)**. MMP-12 mRNA expression in lung tissue **(c)**. Expression was determined by RT-PCR and normalised to HPRT and GADPH. N = 6 or 7 mice per group. Results are expressed as mean ± SD. *P < 0.05. Scale bar: 50 µm.*

STUDY XII

Risk Factors for Development and Severity of COVID-19 in COPD Patients. *Bonato M, Semenzato U, Tinè M, Bazzan E, Damin M, Biondini D, Casara A, Romagnoli M, Turato G, Cosio MG, Saetta M, Baraldo S. Front Med (Lausanne). 2021 Aug 9;8:714570.*

BACKGROUND

Coronavirus disease 2019 (COVID-19), caused by the SARS-CoV-2, emerged in late 2019 in China and subsequently became a pandemic. SARS-CoV-2 may lead to pneumonia and respiratory failure. In Chronic Obstructive Pulmonary Disease (COPD) the underlying lung abnormalities, along with impairment of the immune responses to respiratory infections (194, 195), might make these patients more susceptible to the development and severity of COVID-19.

The incidence of COPD in COVID-19 cohorts has been reported to range from 0 to 10% in China and 5.6–9.2% in Italy (196), although how the diagnosis of COPD was made was often unclear (197). However, the risk of patients with known COPD for the development of COVID-19 and its morbid consequences have not been clearly investigated. Underlying COPD may lead to respiratory failure, need for Intensive Care Unit (ICU) admission, mechanical ventilation or death (197).

This study was designed to: 1) evaluate the incidence of COVID-19 in a cohort of known COPD subjects followed longitudinally; 2) study the possible risk factors for the development of COVID-19; 3) identify the pathophysiological factors influencing the clinical outcome.

METHODS AND MATERIALS

The cohort included 370 subjects with COPD, diagnosed according to last version of GOLD document (10), all routinely followed in the COPD clinics at Padova University Hospital and Treviso City Hospital, Italy. All subjects provided written consent. The mean follow-up duration was 5.3 ± 2.7 years (date of first inclusion October 11th, 2012; date of last inclusion February 12th, 2019). Subjects with asthma or history of asthma were excluded. Subjects underwent yearly clinical evaluation including pulmonary function tests and blood cell count. High-resolution computed tomography (HRCT) was done at diagnosis. Annual frequency of exacerbations was recorded (10). We considered as COVID-19 cases all patients of the cohort who presented at the two public hospitals for symptomatic COVID-19 from February 21st until November 14th, 2020, whose SARS-CoV-2 infection was confirmed by real-time reverse transcription polymerase chain reaction from a nasopharyngeal swab/tracheal aspirate. Notification of cases was obtained by consultation of the electronic registry of all patients of the COPD cohort; inquiries were repeated every 4

weeks until November 14th, 2020. Clinical and functional data refer to the last follow-up visit for each patient before the pandemic outbreak.

In patients who developed symptomatic COVID-19, the following clinical outcomes were considered: disease severity, defined as the need for intensive care (ICU), length of hospitalization, in-hospital mortality and mortality at 6-months. Mann-Whitney, $\chi 2$ and Fisher's exact-test were performed for comparisons between groups, as appropriate; bivariate correlations were estimated using Spearman's rank-correlation coefficient. Statistics were performed using SPSS (v26, IBM Armonk, NY, USA) (level of significance $p < 0.05$).

RESULTS

Twenty-two patients out of 370 (5.9%) developed COVID-19 (COPD/COVID-19+), while 348 did not (COPD w/o COVID-19). Table 1 shows that age, gender, and smoking history were similar in the two groups and that COPD/COVID-19+ had a higher prevalence of cardio-metabolic comorbidities (obesity, hypertension, diabetes, dyslipidemia, metabolic syndrome) compared to COPD w/o COVID-19. Conversely, lung function, presence of emphysema (Figure 40A), history of exacerbations, COPD treatment and blood counts were similar in the two groups (Table 22).

Out of the 22 COPD/COVID-19+ subjects, 20 (90%) were hospitalized and 2 (10%) home treated. Among hospitalized patients, 10 (50%) needed ICU. Table 2 shows the characteristics of subjects needing (IC) or not needing ICU (No-IC). Dyslipidemia and metabolic syndrome were significantly more prevalent in IC patients than in No-IC. FEV1 was not related to the need for ICU, however IC subjects had lower DLCO at baseline and higher prevalence of emphysema (Figure 30B). When first seen at the Emergency Room, PaO2/FiO2 Ratio was the only parameter associated to IC and it was inversely related with hospitalization length ($r = -0.63$; $p = 0.013$) (Table 23). In-hospital mortality rate was 18.1% (4/22), three patients died in IC and 1 in No-IC. Among in-hospital deceased patients, 3 of 4 had emphysema and a very low DLCO (median 35%pred.) even if with mild obstruction (median FEV1 76%pred). At 6-months, follow-up mortality raised to 36.3% (8/22), 4 patients in IC group (40%), 4 in no-IC (33.3%).

Table 22. Demographic, functional and clinical characteristics of COPD subjects who developed COVID-19 (COPD/COVID-19+) and COPD subjects who did not (COPD w/o COVID-19).

	COPD/COVID-19+	COPD w/o COVID-19	p-value
Subjects, n (%)	22	348	-
Gender male, n (%)	16 (72)	247 (71)	n.s.
Age, years	79 [75-85]	77 [72-83]	n.s.
Smoking: current/ex/never, n (%)	4 (18) / 18 (82) / 0(0)	94(27)/ 243(70)/ 11(3)	n.s.
Smoking: pack-years	40 [19-51]	40 [22-51]	n.s.
Comorbidities, n (%) ● Obesity ● Hypertension ● Type 2 diabetes ● Dyslipidemia ● Metabolic Syndrome	 8 (36) 22 (100) 9 (41) 14 (63) 9 (41)	 47 (14) 207 (59) 62 (18) 76 (22) 45 (13)	 0.004 <0.001 0.002 <0.001 <0.001
FEV_1, %pred.	69 [54-90]	67 [51-86]	n.s.
FEF_{25-75}, %pred.	36 [27-54]	28 [18-44]	n.s.
DLCO, %pred.	45 [32-85]	71 [50-84]	n.s.
Blood eosinophils, cells/μL	120 [37-262]	180 [100-267]	n.s.
Blood lymphocytes, cells/μL	1820 [1445-2302]	1860 [1430-2427]	n.s.
AE in the previous year, n	0 [0-1]	0 [0-1]	n.s.
Emphysema on CT-scan*, n (%)	12 (60) *n=20*	133 (58) *n=226*	n.s.
Inhaler therapy, n (%) ● None ● LAMA or LAMA/LABA ● LAMA/LABA/ICS or LABA/ICS or LAMA+LABA/ICS	 4 (18) 9 (41) 9 (41)	 16 (5) 164 (47) 168 (48)	 n.s. n.s. n.s.

*Data refer to the last visit available before COVID-19. Data are expressed as median (interquartile range) or absolute numbers (percent). The p-value is referred to the Mann-Whitney-U or Fisher Exact-tests comparisons, as appropriate. *Data available on 246 out of 370 patients. FEV1, Forced Expiratory Volume in the 1st s; FEF25−75, forced mid-expiratory flow; DLCO, diffusing capacity of the lung for carbon monoxide; AE, acute exacerbations; CT, computed tomography; LAMA, long acting muscarinic antagonist; LABA, long-acting beta2-agonist; ICS, inhaled corticosteroid.*

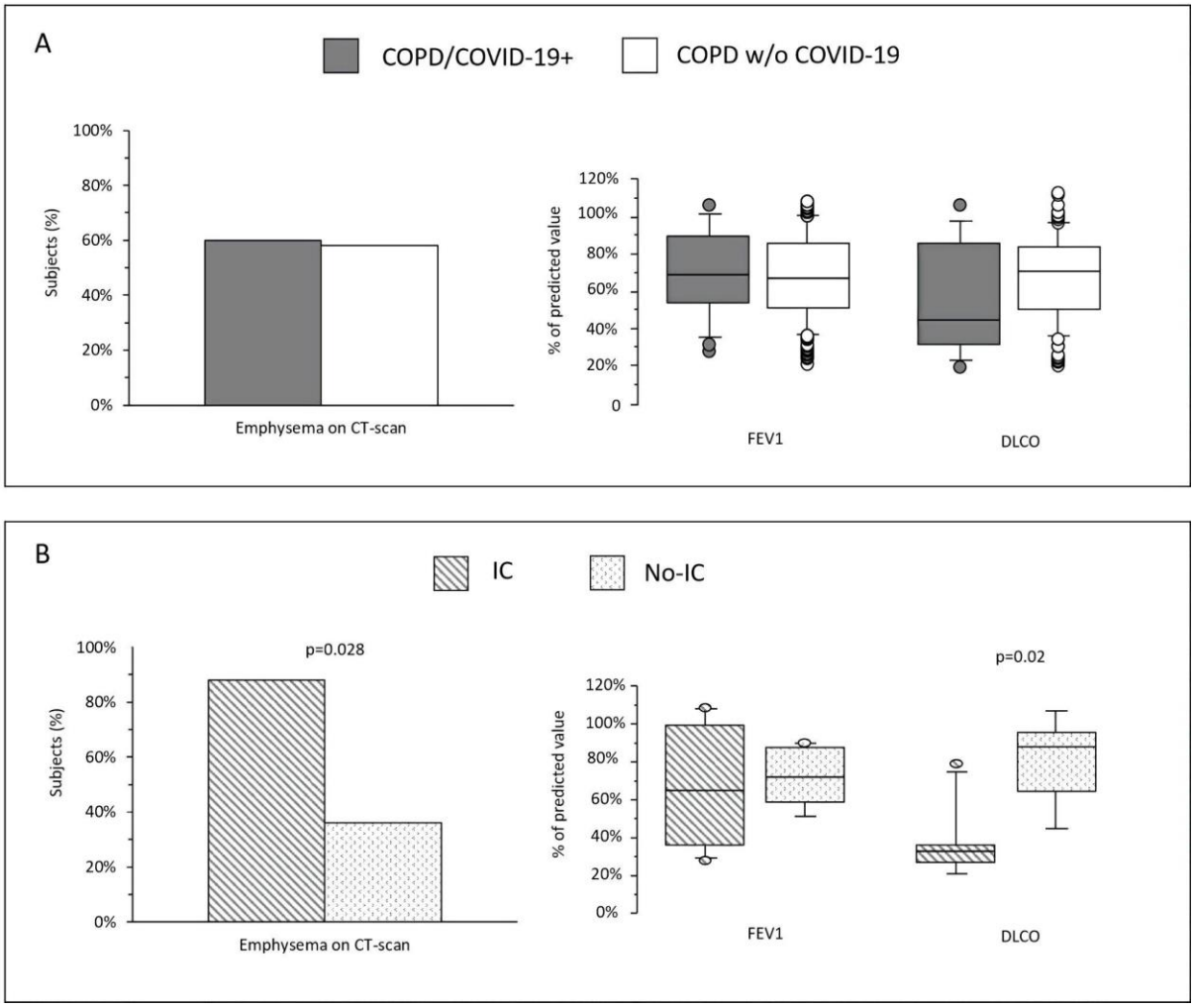

Figure 40. (A) Prevalence of emphysema and values of FEV1 and DLCO in COPD who developed COVID-19 (COPD/COVID-19+) and COPD without COVID-19 (COPD w/o COVID-19). (B) Prevalence of emphysema and values of FEV1 and DLCO in COPD/COVID-19+ subjects who needed intensive care (IC) and in those who did not (No-IC).

Table 23. Demographic, functional and clinical characteristics of COPD subjects who developed COVID-19 categorized in subjects who needed intensive care (IC) and in those who did not (No-IC).

	COPD/COVID-19+		p-value
	IC	No-IC	
Subjects, n (%)	10	12	
Gender male, n (%)	7 (70)	9 (75)	n.s.
BASELINE			
Age, years	78.2 [75.9-80]	79 [72-86]	n.s.
Smoking: current/ex, n (%)	2 (20)	2 (16)	n.s.
Smoking: pack-years	45 [32-57.5]	32 [15-45]	n.s.
Comorbidities, n (%) ● Obesity ● Hypertension ● Type 2 diabetes ● Dyslipidemia ● Metabolic Syndrome	 6 (60) 10 (100) 6 (60) 9 (90) 7 (70)	 2 (16.6) 12 (100) 3 (25) 5 (41.6) 2 (16.6)	 n.s. (0.074) n.s. n.s. (0.1) 0.032 0.027
FEV_1, %pred.	65 [36.5-99.5]	72 [58.7-87.7]	n.s.
FEF_{25-75}, %pred.	39 [28-77]	36 [26-52.7]	n.s.
DLCO, %pred.	32 [28-35]	88 [64.5-95.7]	0.02
Blood eosinophils, cells/μL	110 [10-270]	120 [90-205]	n.s.
Blood lymphocytes, cells/μL	1755 [1450-2290]	1820 [1440-2325]	n.s.
AE in the previous year, n	0.5 [0-2]	0 [0-0.5]	n.s.
Emphysema on CT-scan, n (%)*	8 (88.8) *n=9*	4 (36.3) *n=11*	0.028
Inhaled therapy, n (%) ● None ● LAMA or LAMA/LABA ● LAMA/LABA/ICS or LABA/ICS or LAMA+LABA/ICS	 1 (10) 5 (50) 4 (40)	 3 (25) 4 (33) 5 (42)	 n.s. n.s. n.s.
ON ADMISSION			
P/F ratio	180 [124-296]	310 [262-364]	0.02
C-Reactive protein, mg/dl	39 [18-117]	43 [17-130]	n.s.
D-dimer, ng/L	238 [167-669]	258 [181-596]	n.s.
BNP, ng/L	224 [94-319]	174 [100-201]	n.s.
Blood lymphocytes, cells/μL	815 [570-1590]	695 [610-890]	n.s.
OUTCOME			

Hospitalization length, days (all patients)	21 [8-46]	8.5 [6-11.12]	0.05
Hospitalization length, days (discharged alive only)	36 [13.7-53.5]	8 [5.25-10.5]	0.01
In-hospital mortality, n (%)	3 (30)	1 (8)	n.s.
6-month mortality, n (%)	4 (40)	4 (33)	n.s.

*Baseline data refer to the last visit available before COVID-19. Data are expressed as median (interquartile range) or absolute numbers (percent). The p-value is referred to the Mann-Whitney-U or Fisher Exact tests comparisons as appropriate. *Data available on 20 out of 22 patients. IC: subjects who needed intensive care. No-IC: subjects who did not need intensive care. FEV1, Forced Expiratory Volume in the 1st s; FEF25−75, forced mid-expiratory flow; DLCO, diffusing capacity of the lung for carbon monoxide; AE, acute exacerbations; CT, computed tomography; LAMA, long acting muscarinic antagonist; LABA, long-acting beta2-agonist; ICS, inhaled corticosteroid; P/F, PaO2/FiO2 ratio; BNP, brain natriuretic peptide.*

DISCUSSION

The first evidence that emerged from the research activity during my PhD program is that both asthma and COPD are heterogeneous diseases and that dissecting the molecular bases of such heterogeneity may significantly improve the clinical outcomes of the disease and optimize the therapeutic approach we can offer.

Large cohort studies around the world such as the SARP study (US, 105), the U-BIOPRED study (EU, 198) and the Leicheste's cohort study (UK, 199) converge in defining age of symptom onset as one of the main variable for discriminating asthma phenotypes. For example, in the SARP study, five different asthma phenotypes were identified of which three, less severe and often associated with atopy, had an early onset and two, more severe, often associated with obesity and female sex, had an adult onset. In our study I, the comparison between adult patients with early and late onset asthma routinely seen at our outpatient asthma clinic confirmed the existence of two different phenotypes with distinct clinical and functional characteristics. Of note, we observed a clear discriminating impact of comorbidities in early and late onset phenotypes, which differed both in the pattern of distribution of comorbidities and as for their impact on disease control. In early onset asthmatics, we observed a crucial role of allergic rhinitis and atopic status on disease control and pulmonary function. The prevalence of rhinitis was much higher in this phenotype who clearly showed systemic hallmarks of type 2 inflammatory pattern activation (increased serum IgE and blood eosinophils). Our findings also fit well with observations from cohorts of children and adolescents (104, 200). On the contrary, in late onset asthmatics, obesity rather than atopy had an impact on clinical and functional disease control. Moreover systemic traits of neutrophilic inflammation have been observed in this late onset phenotype. This results, consistent with other studies, suggesting an added inflammatory contribution of obesity to asthma, possibly with involvement of leptin produced by adipose cells, with consequent activation of systemic inflammation linked to IL-17 (201).

Although both our experience and data in the literature associate early-onset asthma (i.e. pediatric asthma) with atopy, our studies on the pediatric cohort of children with wheezing have highlighted that the high heterogeneity that we observed in adult asthma is already present from the beginning of the disease natural history. Studies n. II, III, and even more study V, indicate that in wheezing children, atopy is not always present but, even at this young age, multiple phenotypes and endotypes exist. Our work then focussed on clarifying the significance of these different endotypes, and the role of the major risk factors in the context of the evolutivity of wheezing into asthma. In Study II, we sought to assess which clinical and pathological factors might predict the future development of asthma in wheezing children. We observed that among clinical factors, reduced birthweight and reduced duration of breastfeeding and among pathological factors basement membrane thickness and airway eosinophilia were most closely associated with asthma at

follow-up. Of note, atopy was only a weak predisposing factor for asthma development in this population: in fact, we observed only a nonsignificant trend for IgE values, while the presence of other atopic conditions (allergic rhinitis and atopic dermatitis) was not associated with the subsequent development of asthma at follow-up in our cohort. These results contrast with the literature since the Tucson study (115), but the comparison with epidemiological studies is always indirect since they have a different design and different inclusion criteria. Recent data confirm that pediatric asthma can be non-atopic, and usually displays more severe characteristics when non-atopic, and that the influence of atopy is age dependent (202, 203). On this line, in study III, that was focussed on the determinants and biomarkers of eosinophilic airway inflammation in children, we observed that atopy is not a prerequisite for airway eosinophilia. Indeed, when wheezing children were stratified by the presence of eosinophilic airway inflammation, we did not observe increased prevalence of atopic conditions (allergic rhinitis or dermatitis) nor higher IgE levels in children with airway eosinophilia compared to those without eosinophilia. Conversely, we observed that the atopic status was significantly related to circulating levels of eosinophils, suggesting that distinct immunopathogenic mechanisms are responsible for peripheral and central eosinophilia. Indeed, recent studies have shown that different subpopulations of eosinophils exist, with different surface receptor patterns and, then, different cytokine susceptibility (204, 205). For instance very recent results from Medina et. al. suggest that airway eosinophils differentiate into a CD66b-HIGH CD15-HIGH subpopulation in atopic asthmatics (206). Of note, the results of those studies in asthma should be also discussed in the context of COPD, as was done in Study IX, where in a well characterized cohort of COPD patients with no history of asthma or atopy, blood eosinophils counts did not differ from the general population nor were they associated with the disease severity. Given all these results, we directly investigated the heterogeneity of pediatric wheezing from phenotypic/endotypical perspective in Study V. In particular, our goal was to investigate the role of innate lymphoid cells type 2 in asthma immunopathogenesis and its evolution in the natural history of the disease. Thus, in collaboration with Ghent University, we developed an immunohistochemical methodology for the identification of ILC2 in biopsies (Study IV and V). The results of the main analysis demonstrated that T2 High atopic, T2 High non-atopic and T2 Low endotypes exist in wheezing children, as in adult asthmatics. The 3 different groups showed peculiar phenotypic traits: older age was characteristic of the T2 High atopic group; female gender was more prevalent in the T2 High non-atopic, while male gender was more prevalent in T2 Low. Again, we confirmed that blood eosinophils were strictly related to atopy since they were increased in the T2 High atopic endotype, but not in other endotypes. All endotypes of wheezing children show the typical structural hallmarks of asthma (thickened basement membrane and destroyed epithelium) indicating that airway remodeling is an early event that is present in wheezing independently from the inflammatory and atopic background. When we quantified ILC2, we observed that they were selectively associated to the non-atopic endotype (and almost absent in T2 high atopic and T2 low endotypes)

and that they really represent innate drivers of type 2 inflammation as they were directly related to T2 effectors such as airway eosinophils and IL-5 expression. These results are in agreement with those of Study IV (performed as part of our international collaboration with Gent University) where we demonstrated a predominant involvement of ILC2s and the regulating miRNA miR-155 in a model of asthma non IgE mediated (TDI asthma). Both the mouse model and human bronchial biopsies of subjects with diisocyanate-induced asthma, remarked the putative pivotal role of ILC2s in T2High non-atopic endotype as main drivers of type 2 inflammation.

Of importance, when we performed a longitudinal follow-up of our cohort after 5 years, we observed that the T2 High non-atopic endotype had a significant risk of evolution to atopic asthma and that ILC2 at baseline were predictive of atopic asthma development. The observation that ILC2 are predominant in T2 High non atopic wheezing children and are likely immature drivers of type 2 inflammation which may lead to adaptive immunity activation during growth is consistent with recent literature. Indeed, Chen et al. (207) demonstrated in adult asthmatics the role of ILC2s as first initiators of eosinophilic inflammation in allergic asthma, while Smith et al. (208), assessed that ILC2s can promote the persistence of airway eosinophilia in patients with severe asthma in comparison to mild-atopic asthma.

An immunopathogenetical role of ILCs (and in particular of ILC1) has been demonstrated also in COPD (study XI), although differently from literature (209) we did not find differences according to disease severity, probably for limited sample size. The heterogeneity of COPD, although less remarkable than asthma, is also evident from our study IX where we showed that phenotypes such as emphysematous, bronchiectasis and chronic bronchitis have different profiles of systemic inflammation: the first is characterized by lower levels of blood lymphocytes, the second and the third by higher levels of blood neutrophils suggesting possibly different immunopathogenic mechanisms, as suggested by previous observations (175).

Given the previous results on the relation between atopy and innate immune inflammation we then focused on detailing the role of other important risk factors for asthma, assessing the link between viral infections and air pollution and pathological traits examined in our unique cohort of patients at the onset of their disease. Epidemiological studies have shown that asthma is a complex multifactorial disorder where the environmental influence acts on a genetic background in the genesis of the disease (210). However, the precise effect of environmental factors in immunopathogenesis is still unknown.

Data from epidemiological studies show a close relationship between viral infection susceptibility and asthma development (211). Starting from the observation by ourselves and other groups (study II,212, 213) that type 2 eosinophilic inflammation

is present in wheezing children since preschool age and is predictive of definite asthma development at school age, we wanted to explore the relation between airway inflammation and epithelial responses to viral infections. In study VI we demonstrated at the beginning of the natural history of asthma that asthmatic children had an impairment of interferon antiviral innate response, in terms of decreased production of IFNs which results in increased viral replication. Because we were interested in dissecting the role of asthma and atopy in the abnormal epithelial responses to viral infections, we extended our investigation to children with either atopic and non- atopic asthma and even to atopic children without asthma. We demonstrated that the deficient production of interferon by epithelial cells in response to rhinovirus, which was previously documented in adults with atopic asthma, can be seen not only in atopic and nonatopic asthmatic children but also in atopic children without asthma symptoms. The common link to the deficient immune response to rhinovirus in these conditions seems to be the airway type 2 inflammatory profile that correlates with the decreased interferon production. These findings are in line with the known inhibitory effects of type 2 cytokines on innate immune responses (214, 215). Furthermore, we were able to demonstrate in a longitudinal follow-up that the impaired rhinovirus-induced IFN production seen in the original population could be a risk factor for the persistence of asthma later in life, at school age. On this line, it has been suggested that an impaired immune response of the airway epithelium may be the primary disturbance in asthma, which may even anticipate atopy (216).

In addition, in a research line conducted in collaboration with the Unit of Biostatistics and Epidemiology of our Department, we wanted to explore the relationship between previous exposure to air pollution, i.e. nitrogen dioxide (NO2) and particulate matter (PM10), and the pathological traits typical of asthma in our cohort of children living in North-East of Italy, one of the most polluted regions in Europe.

In study VII, we found that exposure to high levels of particulate matter in wheezing children is associated with increased eosinophilic inflammation. Our study illustrates for the first time, in vivo, a significant association between chronic air pollution exposure and histopathological changes in children. This result is in line with the findings of many in vitro or animal model studies demonstrating that air pollution enhances a type 2 cytokine environment through the release of IL-33 or IL-13 or through modulation of the antigen presentation process (132, 217). Of interest, to underline the importance of susceptibility status associated with wheezing, we observed that chronic exposure to air pollution was related to a different inflammatory pattern in children without wheezing in our cohort. Indeed, children without wheezing who lived in the more polluted areas show reduced numbers of eosinophils and neutrophils in bronchial biopsy specimens. Recent evidence *in vitro* showing that air pollutants may impair innate immune responses to influenza virus infection, particularly by downregulating type 1 interferons, downregulating IL-6, and preventing NLRP3 inflammasome formation (218, 219). This concept was also

underlined by our study VIII, where we observed *in vivo* an impairment of interferon production in association with short-term exposure to PM10 and even more to NO2.

Moreover, in study VII we did find that children with wheezing who live in areas with poor air quality (i.e., those exposed to high levels of PM10) have not only increased eosinophilic inflammation, but also a significant thickening of the reticular BM. By this study, although retrospectively, we demonstrated for the first time *in vivo* an association between PM10 exposure and BM thickness increase, that suggests that air pollution may have an important putative role on the activation of the epithelial-mesenchymal cross-talk in asthma.

An important observation raised by our studies is that, despite the heterogeneity of asthma, basement membrane thickening as an element of homogeneity throughout different endotypes of the disease. Indeed, we observed that the thickening of the reticular basement membrane is an early pathological sign of asthma at the beginning of its natural history that transcends the inflammatory endotype. Thickening of the reticular BM represents a key structural change in bronchial asthma whose pathogenetic role has been increasingly appreciated in recent decades. It has been identified as a marker of epithelial–mesenchymal cross-talk, which may even anticipate inflammatory changes (220). In study II, we observed that BM thickening was the factor most strictly associated to the development of asthma at follow-up. Importantly, when our cohort of wheezing children was stratified by age, BM thickness was the only factor that was associated with asthma at follow-up in children who were ≤3 years of age at baseline. Our results suggest that structural alterations can even precede inflammation in the natural history of the disease and are consistent with two other controlled studies (221, 222) that demonstrated that asthmatic children had a thickened RBM in the absence of a prominent eosinophilia.

In summary, our studies show that the thickening of the basement membrane is probably a pivotal element in the development of the disease since: (a) it can be found in children with wheezing, therefore at the beginning of the natural history of the disease, (b) it is always present regardless of the inflammatory endotype. We know from the literature that the activation of the epithelial-mesenchymal trophic unit is the result of the balancing between the antagonist pathways of epidermal growth factor (EGF) and transforming growth factor-beta (TGF-b) and that in asthmatics both pathways are overexpressed which results in collagen deposition (223).

Another point that has emerged from our studies is the presence of a derangement of the innate antiviral response in the pathogenesis of COPD. Recent evidence suggests that common respiratory viruses (in particular rhinovirus) are responsible for a high proportion of exacerbations both in asthma and in COPD, and may even trigger subsequent bacterial infections (224). Accumulating evidence from experimental work with epithelial cells in vitro but also some preliminary data coming from in vivo models of COPD exacerbation support also the presence of impaired

immune response (225, 226) and in particular of IFNs signaling, may occur in vivo in peripheral lungs of COPD. We have already argued about our observations that children with wheezing have a deficient antiviral response that is more pronounced in relation to the presence of a type 2 inflammatory response, is predictive of the subsequent development of asthma, and is retrospectively associated with increased exposure to air pollutants (study VI and VIII). In COPD, our results in Study X demonstrated that increased expression of the IFN responsive transcription factor IRF-3 is present in all smokers in our cohort, both COPD and smokers with normal lung function, indicating that cigarette smoke per se activates lung innate immunity. However, among subjects with COPD, lower levels of the innate immune mediator were present in subjects with chronic bronchitis and greater airflow obstruction, suggesting that those patients with more severe clinical manifestation of the disease are unable to mount a proper innate response. Our results complement that of previous studies that reported a decreased expression of IFN and IRF-7 in the lungs of smokers with COPD, particularly in those with very severe COPD (227). Moreover, we observed an increased infiltration of cells expressing the activating natural cytotoxicity receptor NKp46 in COPD subjects. This result is in line with other authors that reported that NK cells in patients with COPD were activated, marked by increased expression of granzyme B and perforin, and are involved in epithelial and parenchymal destruction (228, 229). Even in the case of NKcells, subjects with the most severe airflow limitation and chronic bronchitis had the lowest numbers of NK cells. These results can be integrated with results of Study XI, a study of Ghent University we collaborated on, where we observed an increase of innate lymphoid cells type 1 (ILC1), a major source of IFN, in subjects with COPD. Furthermore, we observed in the murine model that the expansion of the ILC1 population is related to a contraction of NK. This would support the conclusions that while smokers with normal lung function and mild COPD are able to mount a proper innate immune response, this capacity is lost in most severe forms.

On this line of reasoning of the interaction between COPD and viral infections some interesting observations are remarked by the results of Study XII where we investigated the risk factors for the development and severity of COVID-19 in patients with COPD. We observed that in COPD, cardio-metabolic conditions (hypertension, dyslipidemia, obesity, diabetes) were the main risk factors for development of COVID-19 in COPD patients. Besides metabolic comorbidities, emphysema and low DLCO, were related to a more severe disease and need for intensive care, remarking a possible relationship between COPD severity and impaired antiviral responses.

Take home messages

Key evidences that emerged from the research projects pursued during my PhD are:

1) The paradigm that defines asthma and COPD solely on the basis of clinical symptoms and functional profile without considering phenotypic and endotypic heterogeneity is limited. Indeed, we highlight the presence of multiple phenotypes and endotypes not only on adult asthmatics, but also on wheezing children and in COPD patients.
2) The idea of atopy as a crucial factor in the pathways regulating the transition between early wheezing and confirmed asthma should be rediscussed. While atopy was usually considered a prerequisite to pose a diagnosis of asthma in wheezing children and considered indispensable for type 2 inflammation, our studies highlighted the role of innate responses, and ILC2 in particular, in driving type 2 inflammation at the beginning of the natural history of the disease.
3) The possible existence of a pathogenetic intersection between atopy, air pollution, viral infections and type 2 inflammation in asthma. We have dissected the potential impact of air pollution on airway pathological changes, as well the altered airway milieu that occurs in asthmatic airways during viral infections from different, complementary, perspectives.
4) The putative importance of epithelial-mesenchymal cross-talk in the pathogenesis of asthma. Our results remark thickening of the basement membrane as the earliest pathological trait of asthma, which is transversal to different endotypes and has prognostic value for asthma persistence with growth. These observations first refute the classical view of remodeling as a consequence of chronic inflammation, then suggest a pivotal role of the epithelial-mesenchymal unit in asthma pathogenesis (Figure 41).
5) A deranged antiviral innate response has a pathogenic role both in asthma and in COPD. Indeed, an impairment of interferon-mediated antiviral innate response is present in asthma at the beginning of its natural history and it is related to symptoms persistence through growth. Furthermore, in COPD an impaired antiviral response mediated by NK is associated with a more severe disease. These findings provide a mechanistic explanation for the broad evidence coming from epidemiological studies that viral infections are a major risk factor for the development of these diseases.

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