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Review article

Molecular endotypes of pediatric asthma: a novel language directs asthma treatment!

Malak Shaheen
Professor of Pediatrics, Ain Shams University, Cairo, Egypt

Introduction

Pediatric bronchial asthma has been clinically recognized as a heterogeneous disease for decades, including descriptions of patients with allergy-associated disease, viral induced asthma and exercise-induced asthma[1].

Despite this recognition, treatment approaches were similar, involving the use of nonspecific agents, such as inhaled corticosteroids (CS) and beta 2- agonists. These drugs, which were generally effective in the majority of asthmatic children, also failed in many. In each case, the molecular under-pinnings of the heterogeneity or the varied responses to treatment were unknown[2].

In the middle of the 20th century, a significant increase in the prevalence of bronchial asthma (BA) was reported worldwide. This rise particularly referred to BA of pediatric onset; therefore asthma became one of the most prevalent chronic inflammatory diseases in pediatrics[3].

BA is described as a chronic inflammatory disorder of the airways with inflammatory symptoms causing a variable degree of airflow limitation and is accompanied by an increased sensitivity to multiple pharmacological and non-pharmacological stimuli. The bronchial obstruction seen is often reversible, either spontaneously or with treatment[4].

BA is considered a complex disorder based on a multifactorial interaction of genetic predisposition and diverse environmental influences[5].

The Transition to "Asthma Molecular Immunological Endotypes"

Recently, great progress has been made with regard to new insights into the heterogeneity of asthma on a molecular-biological level using unsupervised hierarchical clustering.

Through molecular-immunological endotyping, a change of gene expression was identified, with the gene products representing potential biomarkers[6].

Clustering of asthmatic patients based on differential gene expression profiling from bronchial brushings and induced sputum samples revealed at least three distinct asthma endotypes[5]; Classic and non-Classic TH 2 endotypes and non TH2 endotype[7].

Gene expression analysis was used to perform endotyping at a transcriptional level. mRNA was extracted from induced sputum of BA patients and gene expression was examined using whole-genome microarray analysis genes were found to be differentially expressed in patients as compared with healthy controls[8]. In particular, gene expression profile was analyzed in the different subgroups through genome-wide profiling after isolation of mRNA and microarray evaluation. The analysis led to the identification of a panel of differentially expressed genes, which were strongly over-expressed in subjects with asthma compared with healthy controls, smokers and ICS-treated asthmatics. The genes that were most strongly induced in asthma were:

- Chloride channel, calcium activated, family member 1 (CLCA1), (6.2 fold)
- Serine peptidase inhibitor, clade B (ovalbumin), member 2 (SerpinB2) also known as plasminogen activator inhibitor-2 (PAI 2), (3.5 fold)
- Periostin (POSTN), (4.4 fold)

Validation of gene-expression was performed by PCR, and validation of protein expression was performed by immunostaining and enzyme-linked immunosorbent assay (ELISA).

In order to obtain further information regarding the regulation of these genes, additional in vitro experiments were carried out on human respiratory epithelium, which showed that IL-13 induced the identified genes while dexamethasone prevented this effect or inhibited the gene expression[8].

TH2-high and TH2-low gene signatures and endotypes

According to the preliminary molecular clustering studies of bronchial asthma, asthmatic children were further evaluated for the presence or absence of a TH 2 cytokine molecular signature. This TH 2 molecular signature was first identified in vitro, in epithelial cell cultures, where three genes, periostin, CLCA1, and SERPINEB2, were found to be up-regulated by IL-13[9].

E-mail: *drmalak_shaheen@med.asu.edu.eg*

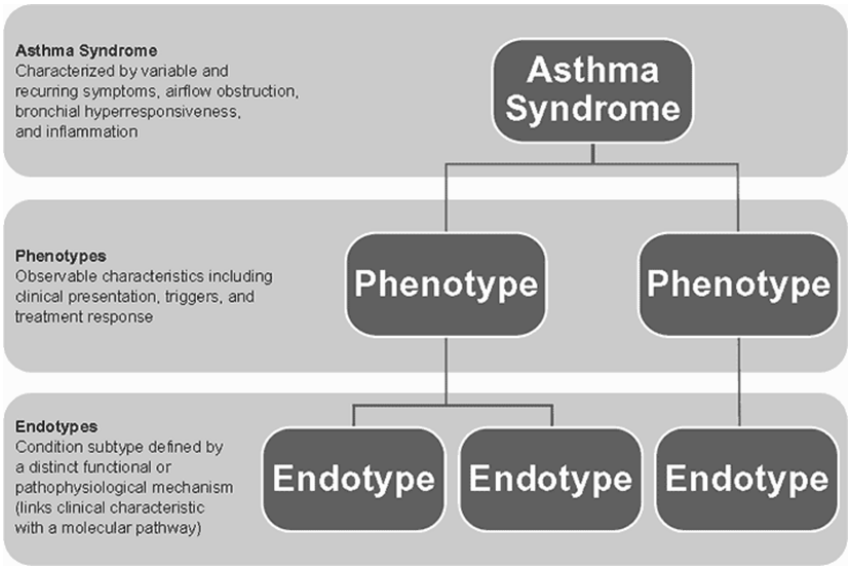


Figure 1. Molecular Asthma Classification[7]

The expression of this Type 2 molecular signature was then evaluated in freshly brushed epithelial cells obtained from mild, CS naïve asthmatics, where clear differences were observed in the presence or absence of this Type 2 signature. The Type 2 signature was present in only about 50% of the mild asthmatics and was not seen in the non-atopic healthy controls. Whereas the absence of this Type 2 signature in many mild asthmatics was perhaps surprising, the presence of the signature was associated with relevant clinical and biological phenotypic characteristics, including more lung eosinophils and mast cells, higher IgE levels, more bronchial hyper-responsiveness, and, importantly, higher tissue expression of IL-5 and -13[10]. Similar to the association with tissue eosinophils reported in atopic asthma, the Type 2 signature was associated with a thicker subepithelial basement membrane, supporting considerable overlap between a Type 2 cytokine molecular phenotype and the eosinophilic phenotypes. Importantly, the presence of this Type 2 molecular signature also predicted a robust response to moderate-dose inhaled CS, whereas no response was seen in those without the Type 2 cytokine signature[11].

This was linked to the traditional allergic clinical phenotype to an underlying Th2 signature (Fig. 3A). As noted, in addition to tissue eosinophils, mast cells have also been reported in airway tissue in relation to Type 2 inflammation. Importantly, there is an increase in epithelial mast cells expressing the enzyme hematopoietic prostaglandin (PG) D synthase and its product PGD2. PGD2 activates several receptors, one of which, chemo-attractant receptor homologous molecule expressed on Th2 cells, is expressed on eosinophils, Th2 lymphocytes, and, more recently, ILC2 cells, suggesting that mast cells/their products could integrate several cell types/pathways, including those related to innate immunity[8]. ILC2 cells are of particular interest for their role in Type 2 asthma, as they could both initiate and perpetuate Type 2 cytokine responses, with some evidence for their existence in asthmatic airways; their importance in Type 2 asthma, however, remains unknown[12]. Importantly, however, the IL-33 pathway has been strongly linked to ILC2 cells. Polymorphisms in both IL-33 and its receptor, IL1RL1, have been consistently linked to early-onset allergic asthma in genome- wide association studies, consistent with a role in some Type 2 phenotypes[13].

Based on the above findings; it is suggested that products of the POSTN, CLCA1 and SERPINB2 genes could serve as a surrogate marker for a TH2-driven immune-response. The experiments and studies revealed that these three genes were over-expressed only in a subset of asthmatics. To define this subset precisely, unsupervised hierarchical clustering was applied[14] to the findings of the gene array analysis based on the expression level of the genes POSTN, CLCA1 and SERPINB2.

Almost half of the subjects showed high expression level TH2-induced genes, while the other half exhibited a normal expression level. A comparison of all BA subjects and healthy controls revealed a general over-expression of the three genes. However after hierarchical clustering about half of the asthma-patients remained

undistinguished from the healthy controls using these surrogate markers[14].

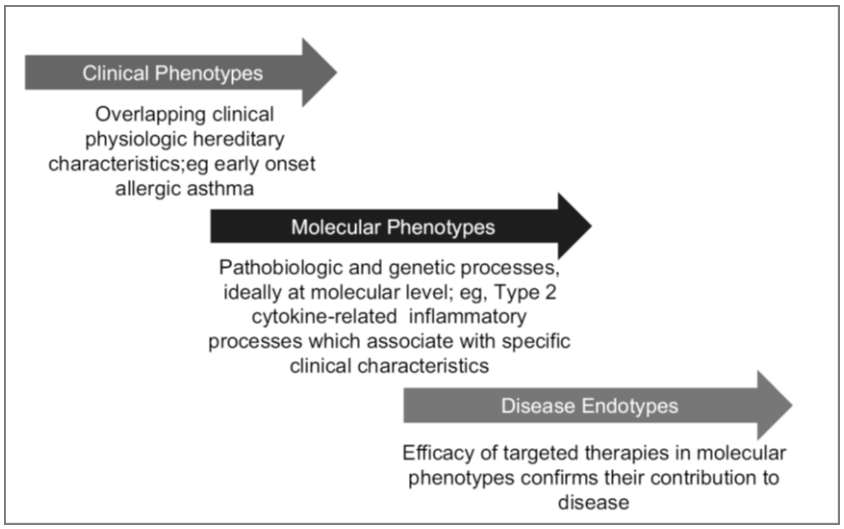


Figure 2. Definitions for the transition from clinical phenotypes to molecular phenotypes to endotypes

As a result the following two clusters could be separated; in cluster 1 the markers POSTN, CLCA1 and SERPINB2 were highly up-regulated, which was not the case in cluster 2, representing the other half of the participants and the healthy controls. These studies together with additional analysis of the characteristics of airway inflammation helped to define the TH2-high and the TH2-low endotypes[8].

At one end of the spectrum it is possible to define an endotype with an up-regulated TH2 response mediated by the typical TH2 cytokines IL-5, IL-13 and IL-4 measured by the surrogate-markers POSTN, CLCA1 and SERPINB2; and at the other end resides the endotype which is not associated with a TH2 response. This provides further evidence that the population of asthmatics is heterogeneous on a molecular level[5]. It has been suggested that the TH2 endotype shows a higher degree of bronchial hyper-reactiveness, higher serum IgE concentrations and a higher eosinophilia in blood and bronchoalveolar lavage (BAL). There is also a higher number of mast cells in the TH2 endotype[15]. Patients with the TH2- endotype benefit from ICS treatment as measured by improvement of the FEV1, while the TH2-low subset is more resistant to steroid treatment[16].

Molecular endotyping obviously has important therapeutic implications. While the TH2 endotype, in particular the eosinophilic subtype, largely contains responders to steroid-treatment, TH2-low endotype asthma, which probably is consistent with the neutrophilic phenotype, is less understood with regard to the molecular pathogenesis[17]. Based on this concept, a randomized placebo-controlled study was held using a monoclonal antibody against IL-13 (lebrikizumab), a prototypic TH2 cytokine, which is pathogenetically important in the TH2 endotype. A total of 219 asthmatics were stratified according to TH2 endotype (high versus low) using serum *periostin* level as a surrogate marker of IL-13 (and hence TH2 status). The primary endpoint was FEV1. Treatment with anti-IL-13 monoclonal antibody was given once a month for 6 months, with the result that when compared with placebo, there was a significant improvement in FEV1 only in the TH2-high endotype (p=0.03) which was not seen in the TH2-low endotype (p=0.61) [18].

Periostin as a potential novel biomarker of a TH2 inflammation /as biomarker of a TH2 endotype.

Biological/physiological basics of periostin, inducibility and effects Periostin is a POSTN-gene encoded, disulfide bridge-bearing, 90 kDa, matricellular protein, which was originally isolated from osteoblasts and described as osteoblast-specific factor 2. Periostin is secreted into the extracellular matrix of the bronchial tissue, and not via the apical membrane into the lumen of the airway, which allows systemic measurement. Periostin is inducible in the airway epithelium by IL-4 and IL-13[15] and is secreted by lung fibroblasts into the extracellular matrix[11].

Periostin is a matricellular protein, which contains a cysteine-rich domain at the N-terminal end and four repetitive fascillin- 1-domains and, therefore, belongs to the fascillin family[18]. Periostin functions as an adhesion molecule. It interacts with different integrin molecules on the cell surface such

as alpha-V/beta1, alpha-V/beta3 and alpha-V/beta5, and facilitates signals for tissue differentiation, growth and remodeling[16]. As a ligand for the mentioned integrins it supports cell adhesion and migration[15].

Furthermore it is a highly inducible product in lung fibroblasts following increased IL-4 and IL-13 and plays a role in the development of fibrosis in asthma. Periostin directly regulates the accumulation of eosinophils in TH2-associated mucosal inflammation, also in the airways. Periostin enhances the emergence of fibrosis through binding to proteins of the extracellular matrix. Another effect of periostin is the counter-regulation of mucus production. It was found that a POSTN-deficiency leads to an enhancement of the expression of Muc5ac and Gob5, and, consequently to an enhanced mucus production[19].

These novel observations suggest that periostin not only promotes inflammation. This is further supported by the observation that periostin can increase the production of IFN-gamma[20]. How these pro- and anti-inflammatory effects of periostin are regulated and controlled requires further study and whether periostin is important in the pathogenesis of other allergic conditions, or even in other chronic inflammatory diseases, steel needs to be investigated.

Periostin as biomarker of TH2 inflammation

Key question:

Is it possible to use markers of TH2 inflammation as biomarkers for stratification prior to therapeutic interventions?

A distinct eosinophilia is a reliable predictor for response to ICS. Particularly with regard to molecular-based therapies, differentiation in TH2-high and TH2-low profiles is required and studies of mepulizumab and reslizumab (two monoclonal antibodies against the TH2 cytokine IL-5) demonstrate the importance and the benefit of selection of patients to obtain a clinically significant effect of treatment with targeted therapeutics[21].

There is recent evidence[1] that periostin levels in serum are significantly increased in patients with asthma. This was found particularly for the eosinophilic type of airway inflammation compared with patients with only minimal eosinophilic airway inflammation. Periostin levels seem to correlate well with the TH2-high gene signature, and with the TH2 endotype.

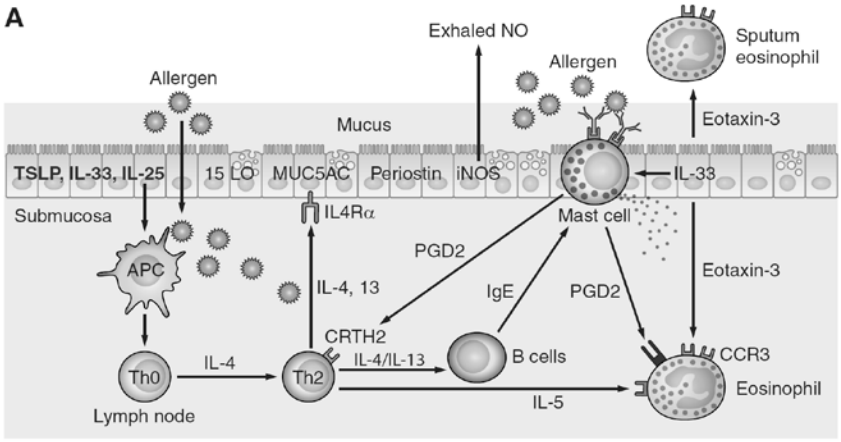
This leads to the question of whether serum periostin levels may serve as a good predictive parameter for stratifying patients prior to asthma treatment.

The next important step is the development of reliable assay systems for such new biomarkers. For this purpose an *ELISA assay for serum periostin* is just developed[1]. The assay was tested in a variety of asthmatic patients, and significantly elevated serum periostin levels were found in a subset of asthmatics together with a correlation with eosinophilic airway inflammation.

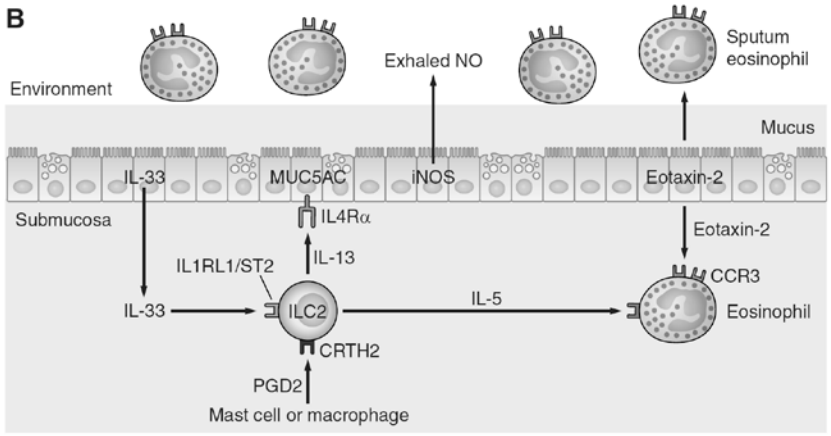
Although treatment with ICS has been found to reduce periostin levels as well as the airway eosinophilia in patients with mild-to moderate asthma, a subset of asthmatics showed persistent eosinophilic airway inflammation and symptoms despite high doses of ICS[4]. This subset could be divided into a subpopulation with sputum- or tissue-eosinophilia (ensured by biopsy) and a subpopulation without these criteria (sputum eosinophils <3% and tissue eosinophils <22/mm^2). The median periostin levels were significantly higher in individuals with high sputum- or tissue-eosinophilia as compared with individuals with lower eosinophil counts. Individuals with a periostin level above a cut-off of 25 ng/mL had a greater than 85% risk of having an increased amount of eosinophils either in sputum or in bronchial tissue[7]. These eosinophil-low and eosinophil-high subpopulations could be distinguished by means of a periostin cut-off level of 25 ng/mL with a positive predictive value of 93%[21].

These analyses may serve as a starting point for further investigation in order to establish periostin as a systemic biomarker of eosinophilic airway inflammation.

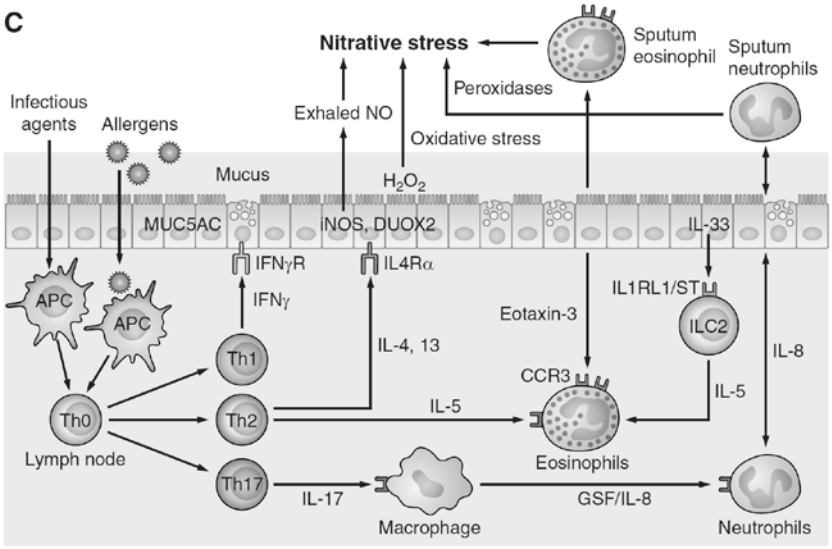
Of all previously examined non-invasive biomarkers such as blood eosinophils, FeNO or IgE, periostin shows the highest probability as a predicator of a TH2-driven inflammation[5]. The ability of periostin to serve as a suitable biomarker for the identification of theTH2-endotype was illustrated in the previous study[18] by Corren et al, in which 219 asthmatic patients were stratified into a TH2-high and a TH2-low-endotype as measured by their peripheral periostin levels. After treatment with lebrikizumab, an anti-IL-13 monoclonal antibody, subjects with a high peripheral periostin level showed a significant improvement in FEV1 compared with placebo that was not observed in subjects with low periostin levels.



A: Traditional (classical) TH 2 Endotype: view of Type 2/Th2 inflammation in early-onset, allergic asthma. iNOS, inducible nitric oxide synthase; PGD, prostaglandin D; TSLP, thymic stromal lymphopoietin; CCR, CC chemokine receptor; MUC5AC, mucin 5AC.



B: Non-traditional (non-classical) TH 2 Endotype: possible underlying pathobiology in patients with nontraditional (non-classical) Type 2 inflammation associated with late onset, eosinophilic disease. ILC2; innate lymphoid cells.



C: Mixed TH2 Endotype: possible underlying pathobiology in complicated/mixed Type 2 severe asthma (Th2/Th17 pathway). APC, antigen-presenting cell; GCSF, granulocyte colony-stimulating factor; DUOX2, dual oxidase 2.

Figure 3. Illustrations of asthma molecular endotypes[1]

The initial identification of Type 2 cytokine-associated asthma relied on an invasive bronchoscopic approach. For this molecular phenotype to become more widely applicable, identification of more easily measured biomarkers was needed.

Numerous less invasive biomarkers have now been reported in relation to a Type 2 asthma phenotype. Serum levels of periostin, the protein associated with one of the three epithelial Type 2 signature genes, have been reported to better predict sputum eosinophilia than other biomarkers. Interestingly, the relation of serum periostin to epithelial expression of the Type 2 molecular signature has not yet been reported. The predictive value was maintained even in a multivariate approach, which included fractional exhaled nitric oxide (FeNO) and was seen in both CS-treated and -untreated patients.

It also was reported to predict response to treatment with anti-IL-13 (lebrikizumab) molecularly targeted therapy[17].

Sputum assessment has also been used to less invasively identify this Type 2 Hi phenotype. The mean expression of the Type 2 cytokines IL-4, -5, and -13 in sputum was reported to strongly correlate with the Type 2 epithelial signature genes, CLCA1 and periostin (but not SERPINEB2), which are thought to be arising from epithelial cells in sputum and to predict both blood and sputum eosinophilia, at least in patients with milder asthma. The utility of this sputum Type 2 signature in more severe CS-treated patients or as a predictor of response to Type 2-targeted therapies remains to be determined [1, 22].

Elevated levels of FeNO have been associated with asthma and allergy for many years. Inducible NO synthase (iNOS), the enzyme primarily responsible for generation of NO in the airway epithelium, is strongly induced by IL-13 in vitro, and FeNO is consistently decreased following treatment with anti- IL-4/-13 therapies[17]. It is also known to be upregulated by the Type 1 cytokine interferon-^{3}, such that elevated FeNO is not specific to Type 2 inflammation[12]. This may explain the observation that FeNO can be elevated in atopic healthy controls as well as severe CS-treated patients[23]. Although it has been associated with eosinophilic inflammation, selective reduction in blood and sputum eosinophils following anti-IL-5-targeted therapy does not decrease FeNO, supporting important differences between these two Type 2 cytokine-associated biomarkers[20]. Whether differences in identification of Type 2 sub-phenotypes or prediction of responses to different Type 2-targeted biological therapies will emerge remains to be investigated.

The presence of type 2 molecular phenotypes is supported by responses to type 2-targeted therapies.

Determining the clinical significance of any molecular phenotype requires evidence that the identified molecular pathway responds to targeted molecular therapies and that clinically meaningful responses occur. Trials of Type 2-targeted therapies for asthma were generally not successful in non-phenotyped populations[24]. However, studies of Type 2 cytokine-directed therapies have confirmed the importance of these molecular pathways in predefined patients with Type 2 biomarker-associated asthma and suggest that clinically identifiable phenotypes may be driven by common underlying molecular pathways.

When a Type 2 biomarker is utilized to identify a Type 2 asthma phenotype, all recent studies of Type 2 cytokine targeted therapies have been clinically successful. Thus confirmation of biological importance of a molecular pathway to a clinically and molecularly identifiable disease is the first step toward identifying disease endotypes[25].

TH 2 asthma: confirming the role of IL-5

In this regard, IL-5, a canonical Type 2 cytokine, is one of the most pro-eosinophilic cytokines yet identified. When mepolizumab, a monoclonal antibody to IL-5 that had failed in previous nonselective asthma studies, was targeted to patients with moderate to severe asthma with historical sputum eosinophilia, a significant reduction in asthma exacerbations occurred over a period of a year[23].

There were no effects on other phenotypic characteristics, such as symptoms or lung function. There was a small effect on airway wall thickening measured by CT scan observed, in line with the previously observed effect of reductions in extracellular matrix components, eosinophils, and TGF-^{2} expression in bronchoscopic biopsy specimens collected from patients with milder asthma[23]. Clinical characteristics of this eosinophilic IL-5-responsive phenotype included the presence of late-onset disease, nasal polyps, and sinus disease, clinical characteristics known to be related to eosinophilic inflammation[25].

A similar antibody to IL-5 (resilizumab) was evaluated in moderate to severe asthma with persistent high levels of sputum eosinophilia (e 3% on 2 occasions). There was a tendency for improvement in symptoms and lung function, but

the study was not of long enough duration to address exacerbations[26]. Most recently, anti-IL-5 was targeted to eosinophilic systemic CS-dependent severe asthmatics[18]. Forty percent of these primarily adult-onset patients, eosinophilic despite systemic CS, were able to reduce their CS dose by >75% while maintaining asthma control and decreasing exacerbations compared with placebo. Interestingly, the remaining subjects had little overall difference compared with placebo, suggesting that IL-5 plays a critical role in some, but perhaps not all, eosinophilic asthmatic patients. Overall, these studies confirm the molecular relationship of the Type 2 cytokine IL-5 to an eosinophilic inflammatory process, with some suggestions that responsive patients (later onset, highly eosinophilic) may differ from the traditional (classic) early-onset Th2 molecular allergic phenotype (Fig. 3 B).

A type 2 molecular phenotype: confirmation of a role for IL-4/-13:

Like IL-5, IL-4 and -13 are reported to be increased in asthma[24]. However, associations with clinical characteristics other than disease severity or atopy have not been not reported. Therefore, it was perhaps not surprising that studies targeting IL-4, alone or in combination with IL-13, although efficacious in early small trials, were not successful in larger studies[11] (Fig. 4).

Despite these negative studies, and unlike anti-IL-5, which had failed in an allergen challenge model in humans, a mutant IL-4 (pitrakinra) that blocked the IL-4 receptor complex and a monoclonal antibody to IL-13 showed efficacy in inhaled allergen challenge studies. These challenge studies suggested that targeting IL-4/-13 could be efficacious in the presence of a prototypical allergic/Type 2 inflammatory process[12].

These allergen challenge studies were followed by studies of patients with chronic asthma, in which Type 2 status was not initially addressed. An antibody to the IL-4 receptor was not effective in unphenotyped moderate asthma[27, 28].

Anti-IL-13 (lebrikizumab) was also only marginally effective in all enrolled patients with moderate to severe asthma[23]. However, lebrikizumab was more effective in improving lung function/forced expiratory volume in 1 s (FEV1) in patients with high serum periostin levels, a biomarker of a Type 2 phenotype, compared with those with low levels. Greater improvements in lung function were also seen in those patients with high compared with low FeNO levels, with the greatest improvements seen in those with both high periostin and FeNO[28]. Although there was a suggestion of marginal effects on asthma exacerbations, there were no effects on symptoms or asthma control, supporting a selective role of IL-13 on the phenotypic characteristic of airflow limitation. Similar results were seen with the anti-IL-13 antibody tralokizumab, which was min had greater efficacy in improving FEV1 in those patients with detectable sputum IL-13 levels[25].

Blood and sputum eosinophils (as Type 2 biomarkers) were used to prospectively identify an eosinophilic moderate to severe asthma population for a trial of a monoclonal antibody to the IL-4 receptor-± (dupilumab)[26]. Most patients were included on the basis of blood eosinophils e300/mm^3 despite use of moderate- to high-dose inhaled CS and long-acting ² 2-agonists (LABA). In population of generally severe asthmatics, treatment with dupilumab resulted in an 87% reduction in protocol-defined exacerbations while improving asthma control, FEV1, asthma and rhinitic symptoms compared with placebo both when added to background therapy and when that therapy was withdrawn.

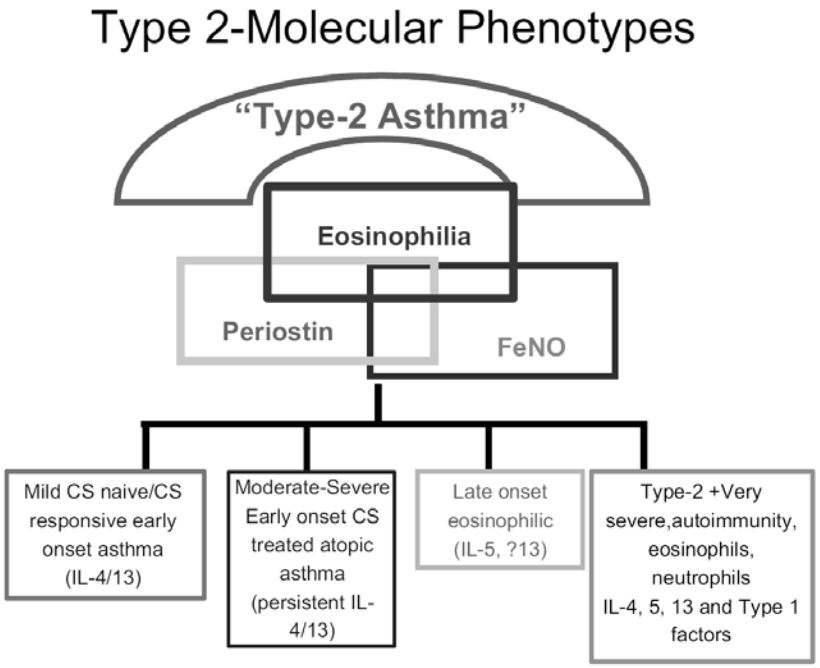
Type 2 cytokine-associated molecular endotypes (Figure 3).

Elements of a Type 2 signature can be identified over a range of asthma severity levels and treatments[1,18]. However, the characteristics associated with a Type 2 signature may differ by severity level and clinical phenotype, such that a Type 2 signature alone may not predict disease severity or associated clinical phenotype. Whereas it identifies a traditional early-onset allergic asthma phenotype in patients with milder/CS-naïve asthma, it may also associate with a late-onset, severe-CS-dependent clinical phenotype[29]. Previous studies suggested that lung (tissue and sputum) eosinophils, as a Type 2 (IL-5) biomarker, were more prominent in adult-onset, less allergic, and more severe asthma, a group where anti-IL-5 appears to be quite effective[3] (Fig. 3*B*). Interestingly, this adult-onset Type 2 molecular phenotype was also reported to be specifically associated with higher epithelial levels of eotaxin-2/CCL24, one of the potent pro-eosinophilic chemokines compared with early onset allergic asthma, potentially suggesting a mechanism for the increased eosinophilia[21]. In contrast, an anti-IL-5 was ineffective in an allergen challenge model, whereas inhibitors of IL-4/-13 significantly impacted these responses[2]. Thus, although the contributions of different Type 2 cytokines to disease may vary by clinical phenotype, an improved understanding of which phenotype is more strongly linked to which anti-cytokine will

clearly advance our understanding of molecular phenotypes.

The presence of Type 2 molecular subphenotypes is also supported by in vitro lymphocytic expression studies, which report differences in expression of Type 2 cytokines by cellular phenotype. IL-4, -5, and -13 may arise from different cell types, with IL-5 expression being associated with both a more differentiated ("super") Th2 lymphocyte[9]. Alternatively, it (as well as IL-13) can be expressed by ILC2 cells. Thus a subphenotype identified by high levels of IL-5 with lesser association with IL-4 (and perhaps IL-13) potentially exists, which could selectively respond to IL-5-targeted therapy (Fig. 3*C*). However, this molecular pathobiology has not yet been confirmed in vivo[29].

In contrast, BAL eosinophilia was less in two clusters of early-onset asthmatics but more allergic mild to severe asthmatics than in the late-onset cluster. The efficacy of biologics targeted to IL-4/-13 in allergen challenge studies suggests that IL-4/-13-targeted therapies may be more effective in this Type 2 subphenotype, where eosinophilia is less central to disease outcomes (Fig. 3*A*).



The increasingly granular umbrella for Type 2 cytokine-associated asthma molecular phenotypes, all of which encompass some Type 2 inflammatory biomarkers. [CS, corticosteroid; FeNO, fractional exhaled NO]

Figure 4. Asthma endotypes in clinical context[29]

Importantly, however, a fourth patient cluster identified the most severe patients with the most systemic CS use. In these patients, BAL eosinophils and neutrophils were persistently elevated, similar to previously reported associations of some patients with severe asthma with a mixed granulocytic inflammation[27]. FeNO (as an additional Type 2 biomarker) was also highest in this severest patient cluster, despite more modest elevations in BAL eosinophils and low levels of blood eosinophils, supporting inconsistent associations of these Type 2 biomarkers. These inconsistent relationships between Type 2 cytokine biomarkers were also seen in a multivariate regression analysis of severe asthmatic patients in the SARP cohort. This analysis identified elevated FeNO as the most significant independent risk factor for chronic systemic CS use, despite very low blood eosinophil levels[13]. The presence of this apparently more complex Type 2 severe asthma subphenotype (neutrophils, lung eosinophilia, and high FeNO despite systemic CS) strongly supports a contribution from additional molecular processes, including those related to autoimmunity and Type 1 and perhaps Type 17 cytokines (Fig. 3*C*).

The Th2/Th17 predominant endotype of severe asthma is associated with increased IL1², C3 and their downstream signaling molecules in bronchoalveolar lavage as manifested by increased expression of MEK (mitogen-activated protein

kinase kinase). The expression of two MEK-inducible and steroid resistance-inducing transcription factors—c-Fos and JunB was elevated in Th2/Th17 cells. The MEK inhibitor trametinib (anticancer treatment) inhibited Th2/Th17 pathway. Also; BAL with Th2/Th17 cells had increased Interferon regulatory factor 4 (IRF4) and Batf - JUN family protein complexes, two transcription factors that are associated with chromatin reorganization and transdifferentiation of Th2 and Th17 cells[5, 29].

This group may include the recently defined subset of patients with asthmatic granulomatosis, consisting of patients with severe, systemic CS-dependent asthma with elevations in FeNO, blood and lung eosinophils, as well as interstitial granulomas[14]. Intriguingly, a family history of autoimmune disease is common in these patients, and they generally respond to non-steroidal immunomodulating therapies, including azathioprine (Immuran) and mycophenolate (CellCept or Myfortic).

Persistent elevations of FeNO despite systemic CS in patients with severe asthma have also been seen in association with elevations of concomitant Type 1 cytokines (interferon-³ in particular). IL-4 and/or IL-13 in combination with interferon-³ synergistically enhance iNOS expression[19]. This combination contributes to a high degree of nitro-oxidative stress in the airway epithelium and, potentially, to severe disease[5].

The specific contribution of Type 1 inflammation to certain Type 2 sub-phenotypes remains to be determined but may make this very severe Type 2 sub-phenotype more refractory to both CS and Type 2-directed therapy than other phenotypes. This complex immunity may explain the inability of another monoclonal antibody to IL-13 to improve outcomes in patients with more severe asthma than those previously studied[24].

Non-Type 2 cytokine-associated asthma

Although substantial studies have been made in identifying molecular markers and even nascent molecular sub-phenotypes of Type 2 cytokine associated asthma, the same cannot be said for non-Type 2 asthma. In fact, the very definition of non-Type 2 asthma is the absence of any evidence for activated Type 2 pathways (low levels of FeNO, eosinophils, or Type 2 signature genes), without links to other molecular pathways/phenotypes[1].

Thus identification of these non-Type 2 phenotypes is limited by an overall lack of association between clinical characteristics, responses to therapy, and underlying pathology, including the presence of lung neutrophils. Except for the poorly studied smoking-associated asthma, the underlying disease present in most non-Type 2 asthmatics may be less severe on the whole than those with persistent Type 2-related inflammation[30]. Rather, co-morbidities, including obesity, sinus disease, gastroesophageal reflux, and vocal cord dysfunction may make it appear more severe[11, 20]. Interestingly, one of the unifying characteristic of these non-Type 2 phenotypes, even in milder disease, is their relative CS unresponsiveness, perhaps attributable to lack of CS-responsive inflammatory elements[10].

Potential molecular phenotypes related to obesity

Obesity in Western societies has become epidemic, with several epidemiological studies suggesting that obesity predisposes to asthma[7]. Additionally mouse studies suggest that obese mice (obese because of leptin or leptin receptor deficiency) develop more AHR to ozone or allergen challenge, but the relationship to inflammatory mediators and cellular inflammation is less clear[4, 8]. The effect on AHR does not appear to be leptin dependent, and, similar to obese humans with asthma, the reasons for the increase in AHR are not clear[1, 15].

Whether obesity causes a distinct molecular asthma phenotype or whether it worsens existing disease in humans is controversial. One study reported that obesity in childhood onset asthma is directly proportional to duration of disease, suggesting that it is a co-morbidity but not a driver of disease[6]. In contrast, the degree of obesity in asthma that develops later in life is not related to disease duration. Rather, it associates strongly with symptoms, health care utilization, and molecularly with low FeNO, eosinophils, and IgE levels[9], supporting a lack of Type 2 immune background. Supporting this, weight loss through bariatric surgery had a greater therapeutic effect on late-onset/non-Type 2 obese asthma compared with early-onset/Type 2-associated severe asthma[27].

Although highly preliminary, these studies suggest that obesity, with its associated metabolic and oxidative stress, may contribute to the development of a late-onset obese asthma molecular phenotype. Although there are few specifics regarding the oxidative stress pathway in obese asthma, obesity in general induces a higher oxidative stress level[15]. If there is an underlying, perhaps genetic increase in AHR, the addition of oxidative stress could make the condition worse. Preliminary studies have suggested that a compound increased in obesity and metabolic

syndrome, *asymmetric dimethylarginine* (ADMA), is increased in late-onset obese asthma[7]. ADMA is a known inhibitor of iNOS, which can switch iNOS from metabolizing arginine to NO to generating superoxide, which could then contribute to oxidative stress observed in late-onset obese asthma[1, 8]. Confirming the importance of this or other oxidative pathways to obese, non-Type 2 molecular (perhaps metabolic) phenotype of asthma will require specific antioxidative/ metabolic pathway approaches[29].

Do IL-17 or TNF-± contribute to molecular phenotypes?

The lack of CS response in association with neutrophilia has suggested that the IL-17 pathway may be important in some poorly controlled non-Type 2 molecular phenotypes[2, 26]. IL-17 has been related to neutrophilia and steroid resistance, as well as enhanced allergic/complement-associated responses in mouse models. Although one study suggested a relation to smoking-associated asthma, with its generally neutrophilic phenotype, other recent data suggest that an active Type 17 (and neutrophilic) immune process may actually contribute to less severe disease[1]. In fact, a monoclonal antibody to the IL-17 receptor, which inhibits all isoforms of IL-17, including the Type 2-associated cytokine IL-25 (brodalumab), was modestly efficacious only in patients with asthma with a robust bronchodilator response[14].

There was no relation to neutrophilic or eosinophilic inflammation. Similarly, TNF-± and related Toll-like receptor pathways have been suggested to contribute to neutrophilic asthma by gene expression array profiling[6]. However, the confirmation of the relevance of these pathways to a clinically identifiable phenotype that responds to selective molecularly targeted therapies does not yet exit. The few studies that have evaluated anti-TNF-± approaches in larger studies of moderate to severe asthma were unable to demonstrate efficacy in the total population and unable to identify neutrophilia (or eosinophilia) as a specific responder phenotype. Interestingly, subgroup analyses of the efficacy of anti-TNF-± identified robust bronchodilator responsiveness, later-onset disease, and sinusitis as elements of a responder phenotype in very severe asthma[10]. However, despite the association of obese asthma with increases in TNF-±, the efficacy of the anti-TNF-±, golimumab, was not modified by obesity[28]. Thus the relevance of specific immunological factors to some phenotypes of non-Type 2 asthma remains unknown.

The molecular underpinnings of smoking-related asthma are also not clear. It is likely that, in some cases, a Type 2 immune process was and still is present, perhaps exacerbated by effects of oxidative stress, neutrophilic inflammation, epithelial damage, and infection, particularly in those with early-onset disease[18]. Interestingly, active smoking lowers FeNO levels although whether the mechanisms include inhibition of Type 2 cytokine pathways, increased levels of nitrative stress = reactive nitrogen species (RNS) (as opposed to FeNO), or simply activation of non- Type 2 cytokine pathways, including neutrophilic processes, is not clear[27,30].

Conclusion

The view that bronchial asthma (BA) is a single disease is rapidly disappearing. Recognition of the heterogeneity of BA together with the definition of clinical phenotypes, pathogenetic endotypes and the development of novel biomarkers has opened a new chapter in pediatric asthma clinical care.

Although the vast majority of asthmatic children are well controlled with guideline based anti-inflammatory therapies, there is a strong medical need for improved therapies in the remaining portion of patients.

As better understanding of asthma phenotypes and endotypes emerges, it is hoped that, in the future, the findings will enhance the development of personalized and asthma subtype-specific therapies.

REFERENCES

1. **Ray A, Oriss TB, Wenzel SE.** Emerging molecular phenotypes of asthma. Am J Physiol Lung Cell Mol Physiol 2015; 308:130–40.
2. **Al-Alwan A, Bates JH, Chapman DG, Kaminsky DA, Desarno MJ, Irvin CG, et al.** The nonallergic asthma of obesity. A matter of distal lung compliance. Am J Respir Crit Care Med 2014; 189: 1494–1502.
3. **Spears MMC, Chaudhuri R, Weir CJ, Dewet C, Thomson NC.** Smoking in asthma is associated with elevated levels of corticosteroid resistant sputum cytokines-an exploratory study. PLoS One 2013; 8: e71460.
4. **Wu W, Bleecker E, Moore W, Busse WW, Castro M, Chung KF, et al.** Unsupervised phenotyping of Severe Asthma Research Program participants using expanded lung data. J Allergy Clin Immunol 2014; 133: 1280–1288.

5. **Amelink M, De Nijs SB, De Groot JC, Van Tilburg PM, Van Spiegel PI, Krouwels FH, et al.** Three phenotypes of adult-onset asthma. Allergy 2013; 68: 674–680.
6. **Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, et al.** Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med 1992; 326: 298–304.
7. **Barnig C, Cernadas M, Dutile S, Liu X, Perrella MA, Kazani S, et al.** Lipoxin A4 regulates natural killer cell and type 2 innate lymphoid cell activation in asthma. Sci Transl Med 2013; 5: 174.
8. **Bel EH, Wenzel SE, Thompson PJ, Prazma CM, Keene ON, Yancey SW, et al.** Oral glucocorticoid-sparing effect of mepolizumab in eosinophilic asthma. N Engl J Med 2014; 371: 1189–1197.
9. **Lotvall J, Akdis CA, Bacharier LB, Bjermer L, Casale TB, Custovic A, et al.** Asthma endotypes: A new approach to classification of disease entities within the asthma syndrome. J Allergy Clin Immunol , 2011; 127: 355–360.
10. **Castro M, Mathur S, Hargreave F, Boulet LP, Xie F, Young J, et al.** Reslizumab for poorly controlled, eosinophilic asthma: a randomized, placebo-controlled study. Am J Respir Crit Care Med 2011; 184: 1125–1132.
11. **Amelink M, De Groot JC, De Nijs SB, Lutter R, Zwinderman AH, Sterk PJ, et al.** Severe adult-onset asthma: A distinct phenotype. J Allergy Clin Immunol 2013; 132: 336–341.
12. **Chibana K, Trudeau JB, Mustovich AT, Hu H, Zhao J, Balzar S, et al.** IL-13 induced increases in nitrite levels are primarily driven by increases in inducible nitric oxide synthase compared with effects on arginases in human primary bronchial epithelial cells. Clin Exp Allergy 2008; 38: 936–946.
13. **Guo FH, Uetani K, Haque SJ, Williams BR, Dweik RA, Thunnissen FB, et al.** Interferon gamma and interleukin 4 stimulate prolonged expression of inducible nitric oxide synthase in human airway epithelium through synthesis of soluble mediators. J Clin Invest 1997;100: 829–838.
14. **Dougherty RH, Sidhu SS, Raman K, Solon M, Solberg OD, Caughey GH, et al.** Accumulation of intraepithelial mast cells with a unique protease phenotype in T(H)2-high asthma. J Allergy Clin Immunol 2010; 125: 1046–1053.
15. **Fajt ML, Gelhaus SL, Freeman B, Uvalle CE, Trudeau JB, Holguin F, Wenzel SE.** Prostaglandin D (2) pathway upregulation: Relation to asthma severity, control, and TH2 inflammation. J Allergy Clin Immunol 2013; 131: 1504–1512.
16. **Moore WC, Hastie AT, Li X, Li H, Busse WW, Jarjour NN, et al.** Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis. J Allergy Clin Immunol , 2014; 133: 1557–1563.
17. **Chapman DG, Tully JE, Nolin JD, Janssen-Heininger YM, Irvin CG.** Animal models of allergic airways disease: Where are we and where to next? J Cell Biochem 2014; 115: 2055–2064.
18. **Corren J, Busse W, Meltzer EO, Mansfield L, Bensch G, Fahrenholz J, et al.** A randomized, controlled, phase 2 study of AMG 317, an IL-4 Ralpha antagonist, in patients with asthma. Am J Respir Crit Care Med 2010;181: 788–796.
19. **Busse WW, Holgate S, Kerwin E, Chon Y, Feng J, Lin J, Lin SL.** Randomized, double-blind, placebo-controlled study of brodalumab, a human anti-IL-17 receptor monoclonal antibody, in moderate to severe asthma. Am J Respir Crit Care Med 2013; 188: 1294–1302.
20. **Corren J, Lemanske RF, Hanania NA, Korenblat PE, Parsey MV, Arron JR, et al.** Lebrikizumab treatment in adults with asthma. N Engl J Med 2011; 365: 2433–2334.
21. **De Boever EH, Ashman C, Cahn AP, Locantore NW, Overend P, Pouliquen IJ, et al.** Efficacy and safety of an anti-IL-13 mAb in patients with severe asthma: A randomized trial. J Allergy Clin Immunol , 2014; 133: 989–996.
22. **Deckers J, Branco Madeira F, Hammad H.** Innate immune cells in asthma. Trends Immunol 2013; 34: 540–547.
23. **Hazenberg MD, Spits H.** Human innate lymphoid cells. Blood 2014; 124: 700–709.
24. **Dharajiya N, Vaidya SV, Murai H, Cardenas V, Kurosky A, Boldogh I, Sur SA.** FcgammaRIIb inhibits allergic lung inflammation in a murine model of allergic asthma. PloS One 2010; 5: 9337.
25. **Dixon AE, Pratley RE, Forgione PM, Kaminsky DA, Whittaker- Leclair LA, Griffes LA, et al.** Effects of obesity and bariatric surgery on airway hyperresponsiveness, asthma control, and inflammation. J Allergy Clin Immunol 2011;128: 508–515.
26. **Wenzel S, Ford L, Pearlman D, Spector S, Sher L, Skobieranda F, et al.** Dupilumab in persistent asthma with elevated eosinophil levels. N Engl J Med 2013; 368: 2455–2466.

27. **Voraphani N, Gladwin MT, Contreras AU, Kaminski N, Tedrow JR, Milosevic J, Bleecker ER, et al.** An airway epithelial iNOS-DUOX2-thyroid peroxidase metabolome drives Th1/Th2 nitrative stress in human severe asthma. Mucosal Immunol 2014; 7: 1175–1185.

28. **Baines KJ, Simpson JL, Wood LG, Scott RJ, Gibson PG.** Transcriptional phenotypes of asthma defined by gene expression profiling of induced sputum samples. J Allergy Clin Immunol 2011; 127: 153–160.

29. **Peters MC, Mekonnen ZK, Yuan S, Bhakta NR, Woodruff PG, Fahy JV.** Measures of gene expression in sputum cells can identify T2-high and T2-low subtypes of asthma. J Allergy Clin Immunol 2014; 133: 388–394.

30. **Martin RA, Ather JL, Daggett R, Hoyt L, Alcorn JF, Suratt BT, et al.** The endogenous Th17 response in NO2-promoted allergic airway disease is dispensable for airway hyperresponsiveness and distinct from Th17 adoptive transfer. PLoS One 2013; 8: e74730.