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Biomarkers in the Management of Difficult Asthma

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Abstract: Difficult asthma is a heterogeneous disease of the airways including various types of bronchial inflammation and various degrees of airway remodeling. Therapeutic response of severe asthmatics can be predicted by the use of biomarkers of Type2-high or Type2-low inflammation. Based on sputum cell analysis, four inflammatory phenotypes have been described. As induced sputum is time-consuming and expensive technique, surrogate biomarkers are useful in clinical practice.

Eosinophilic phenotype is likely to reflect ongoing adaptive immunity in response to allergen. Several biomarkers of eosinophilic asthma are easily available in clinical practice (blood eosinophils, serum IgE, exhaled nitric oxyde, serum periostin). Neutrophilic asthma is thought to reflect innate immune system activation in response to pollutants or infectious agents while paucigranulocytic asthma is thought to be not inflammatory and characterized by smooth muscle dysfunction. We currently lack of user-friendly biomarkers of neutrophilic asthma and airway remodeling.

In this review, we summarize the biomarkers available for the management of difficult asthma.

Keywords: Airway remodeling, Biomarker, Difficult asthma, Inflammation, Phenotype, Severe.

INTEREST OF PHENOTYPING DIFFICULT TO TREAT ASTHMA

Difficult to treat asthma is defined by inadequate asthma control or frequent severe exacerbations despite high-dose inhaled corticosteroids or the need for oral corticosteroids often associated with other controller medication such as long-acting β2 agonists, leukotriene receptor antagonists or theophylline [1, 2]. Refractory asthmatics are patients in whom alternative diagnoses have been excluded, co-morbidities have been treated, trigger factors have been removed and adherence with treatment has been checked. This subgroup of asthmatics represents around 10% of the general asthmatic population.

Severe asthma is heterogeneous. Phenotyping allows to predict who best will respond to therapies and optimise quality of life by reducing the risk of exacerbations. Based on sputum cell analysis, four inflammatory phenotypes have been described in the literature: eosinophilic, neutrophilic, paucigranulocytic and mixed granulocytic phenotype [3]. According to values found in our healthy population, we defined eosinophilic asthma as having more than 3% eosinophils in the sputum, neutrophilic asthma as more than 76% neutrophils in the sputum, mixed granulocytic asthma when both inflammatory cells are increased and paucigranulocytic asthma if both inflammatory cells are below the thresholds. The importance of these inflammatory phenotypes is that the underlying molecular mechanisms are different. While the eosinophilic phenotype is likely to reflect ongoing adaptive immunity in response to allergen with Th2-cytokines Interleukin-4 (IL-4), IL-5, IL-9 and IL-13 playing a key role [4], the neutrophilic is thought to reflect innate immune system activation in response to pollutants or infectious agents [5, 6]. Paucigranulocytic asthma has been poorly studied. It is thought to be not inflammatory and is characterized by smooth muscle dysfunction with bronchial hyperresponsiveness. The main trait is their bronchial hyperresponsiveness to methacholine. Therefore it is conceivable that phenotypes actually require different therapeutic molecular approaches. Numerous studies showed that regular treatments with inhaled corticosteroids (ICS) sharply and quickly reduce the percentage of eosinophils contained in the sputum from asthmatics [7, 8], repress the release of Th2 cytokines from lymphocytes [9], and eotaxin from epithelial cells [10]. ICS are particularly effective in combating Th2-driven inflammation featuring mast cell and eosinophilic airway infiltration. Their effect on innate immunity-driven neutrophilic inflammation is rather poor. Moreover, targeting airway eosinophilic inflammation was shown to reduce exacerbations [11, 12].

Induced sputum is an expensive, technically demanding, time consuming and not widely available technique. In this review, we classified patients according to sputum inflammatory cell counts and wanted to highlight the biomarkers available in clinical practice to predict the presence of airway inflammation or airway remodeling in difficult to treat asthma. This inflammatory phenotyping allows the prediction of response to targeted biotherapies and the exacerbation risk.

EOSINOPHILIC ASTHMA

The raised airway granulocytic inflammation is a common finding in severe asthma [13]. Duncan [14] has demon-

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strated that both reduced eosinophil apoptosis and increased sputum eosinophilia significantly correlate with asthma severity. In a cohort of asthmatics encompassing the entire disease severity spectrum, the functional index which best correlates with sputum eosinophils is the FEV_1/FVC ratio. We have previously shown that eosinophilic asthma (sputum eosinophils ≥3%) represents the predominant phenotype (55%) in severe asthma [15]. Mixed granulocytic asthma was found in 6% of Belgian severe asthmatics, thus raising the proportion of severe asthmatics exhibiting more than 3% eosinophilis in their sputum to 61%. A trait suggesting type2-high, either increased induced sputum eosinophils, blood eosinophil count, Immunoglobulin E (IgE) or exhaled nitric oxide (FENO) levels, was identified in the majority of severe asthma despite treatment with high doses of ICS and oral corticoids in a subgroup [15]. Moreover, patients with eosinophilic inflammation are at high risk of severe exacerbations [16, 17].

BIOMARKERS FOR EOSINOPHILIC DIFFICULT ASTHMA

Sputum Eosinophil Count

Eosinophilic airway inflammation can be assessed directly by broncholaveolar lavage [18] and bronchial biospies [19, 20] or induced sputum [21-23]. Induced sputum is generally considered as the gold standard non-invasive method [3] for assessing airway inflammation in asthma to identify inflammatory phenotype.

The recent ERS/ATS guidelines suggest that induced sputum can be used in the management of severe asthma in specialised centres with a dedicated laboratory [2]. The success rate of the collection of airway cells and lining fluid by sputum induction in severe asthma was 77% according to our experience [15].

Surrogates for Sputum Eosinophil Count

A biomarker is a surrogate measurement designed to characterize and quantify an underlying disease process. As induced sputum is a complex technique, several biomarkers have been suggested to assess sputum eosinophilia in asthma (Table **1**).

Exhaled Nitric Oxide

Nitric oxide (NO) has been found to be correlated with sputum eosinophil count [24].

NO is mainly produced by epithelial cells in asthma but can also be released by mast cells, macrophages, endothelial cells and vascular smooth muscle cells. The synthesis of NO is mediated by constitutive (endothelial NOS or neuronal NOS) and inducible NO synthase (iNOS). Its production is due to oxidation of L-arginin to L-citrullin. iNOS is the only isoform correlated with exhaled nitric oxide [25]. IL-13 can induce iNOS [26]. NO is measured by chemoluminescence using a nitric oxide monitor set at an exhalation flow rate of 50ml/s according to the ERS/ATS recommendations.

Both FENO and sputum eosinophil count were found to be strongly reduced by treatment with ICS [8, 27]. In a general population of asthmatics, we found that FENO threshold that best identified a sputum eosinophil count ≥3% in patients receiving high dose of ICS was 27ppb [24]. In this study, FENO was found to be more sensitive than the sputum eosinophil count to ICS as patients receiving higher doses of ICS had lower FENO values than the other groups, a phenomenon not observed with the sputum eosinophil count. Consequently, the FENO threshold associated with sputum eosinophilia ≥3% was lower in patients receiving high doses of ICS. In severe asthmatics [15], we confirmed that 27ppb was the threshold exhibiting the best sensitivity and specificity for identification of eosinophilic severe asthma.

The median FENO value was found to be in the lower part of the grey zone (25-50ppb) [28]) in the Belgian severe asthma cohort [15]. This is probably due to higher doses of inhaled corticosteroids in this sub-population. In the Belgian severe asthma registry, we found a modest but significant correlation between FENO and sputum eosinophil count ($r=0.37$, $p<0.001$). We found FENO levels >27ppb in 49% of severe asthmatics FENO levels >27ppb suggesting persistent eosinophilic inflammation.

It is highly likely that FENO and induced sputum are different facets of bronchial inflammation. Jatakanon has indeed shown that the change in sputum eosinophil count was better than the change in FENO in predicting loss of asthma control [22]. The administration of mepolizumab in patients with refractory eosinophilic asthma led to a decrease in sputum eosinophilia without any change in FENO levels [29].

In a recent paper, it was shown that, in severe asthma, FENO had a lower accuracy than blood eosinophils to identify eosinophilic asthma [30] but increased FENO levels have been associated with a good response to ICS [31], oral corticosteroids, anti-IgE [32, 33] and anti-IL-4 and anti-13 [34]. FENO and blood eosinophil counts measurement gives complementary information that suggests a value in combining these two biomarkers. Hanania et al [32] indeed evaluated omalizumab (anti-IgE) effects in relation to FENO, blood eosinophils, and serum periostin levels at baseline. Patients were divided into low- and high-biomarker subgroups. The difference in exacerbation frequency between omalizumab and placebo was greatest in the three high-biomarker subgroups, probably associated with the greater risk for exacerbations in high subgroups. In the same way, patients with serum periostin, FENO or blood eosinophil counts above the median had significant improvements in lung function as assessed by forced expiratory volume in one second (FEV_1) in the lebrikizumab arm as compared with the placebo arm [34]. Treatment with lebrikizumab also normalized FENO levels.

As compared to other biomarkers of sputum eosinophil count, FENO has the advantage to sample the airways. Studies on anti-IL-5 enrolling only patients who had various combinations of elevated sputum eosinophils, blood eosinophils, or FENO showed that mepolizumab could significantly reduce the rate of severe asthma exacerbations in those patients [29, 35, 36]. IL-5 inhibition did not decrease FENO levels. This suggests that FENO reflects IL-13 activity more than sputum eosinophil count.

Blood Eosinophils

Blood eosinophil count measurement is biologically plausible surrogate marker for sputum eosinophilia since the infiltrating granulocytes in the airway are bone marrow-derived cells that access the airway through diapedesis from the circulation. A previous study showed that patients with high blood eosinophilia (>250 cells per mm^3) had lower FEV_1 values and worse asthma control than those with normal blood eosinophil counts [37]. Recently, Volbeda *et al.* [20] have shown that patients with uncontrolled asthma exhibit higher eosinophil numbers in peripheral blood.

Like Hastie et al [38], in the Belgian severe asthma registry, we demonstrated a significant correlation between blood eosinophil count (/mm^3) and sputum eosinophil count (%). In this population of severe asthmatics, the blood eosinophil count threshold that best predicts the presence of uncontrolled airway eosinophilia was found to be 188/mm^3 with 72.3% sensitivity and 72.7% specificity for identifying a sputum eosinophil count ≥3%. According to this threshold, 58% of severe asthmatics in Belgium exhibited eosinophilic asthma.

The blood eosinophil counts predict the response to anti-IL-5 therapy [36] and anti-IgE [39]. In a recent study [30], Wagener et al found that blood eosinophils had the highest accuracy (best Receiver operating characteristic curve (ROC) – Area under the curve (AUC)) in the identification of sputum eosinophilia even in more severe asthma with a cut-off value of 270/mm^3.

Measurement of Sputum and Blood Eosinophils

In a previous study [17], we found that patients exhibiting both local and systemic eosinophilic inflammation had more severe asthma reflected by lower baseline lung function, higher bronchial responsiveness to methacholine, poorer asthma control and quality of life, and a greater number of exacerbations in previous year. This suggests that the global magnitude of eosinophilic inflammation is a significant factor in disease severity and that measurement of eosinophils in both compartment give additional information to the clinician.

Serum IgE

IgE is one of the most important biomarker of atopy. In a general population of asthmatics, IgE was found to be an independent factor associated with the presence of sputum eosinophilic inflammation. Woodruff et al conducted a study in which a 3-gene signature composed of periostin, chloride-channel regulator-1 and serpin peptidase inhibitor clade B member 2 in airway epithelial cells was used as a surrogate marker to discriminate between TH2-high and TH2-low inflammation. The TH2-high cluster phenotype was characterized by increased serum IgE levels and eosinophilic inflammation [40]. In severe asthma, it is necessary to measure total blood IgE levels when a treatment with anti-IgE is started but there is no clear association between IgE levels and omalizumab efficacy.

Serum Periostin

Periostin is an extracellular matrix protein induced by IL-4 and IL-13 from airway epithelial cells and lung fibroblasts. Serum periostin was proposed as a systemic biomarker of eosinophilic inflammation due to the correlation found with sputum eosinophils in uncontrolled severe asthma and the prediction of steroid responsiveness [41, 42]. This measurement requires ELISA kits and is not yet widely available. In Wagener's paper, serum periostin was not able to distinguish eosinophilic from non-eosinophilic airway inflammation [30]. The authors concluded that periostin was not associated with sputum eosinophilia. This does not exclude complementary information by periostin to sputum eosinophil count as this type-2 high biomarker was found to be associated with a better response to anti-IL-13 therapy [34].

Sputum and Sputum Cell Culture Supernatants

Primary information of induced sputum is inflammatory cell count but sputum supernatant analysis might offer information relevant to molecular biomarkers of inflammation. Various soluble mediators are eosinophil-derived proteins. Eosinophil cationic proteins (ECP) [43, 44], eosinophil-derived neurotoxin (EDN) [45] and eosinophil peroxidase (EPO) [46] were found in increased levels in sputum supernatant of asthmatics with eosinophilic phenotype. Our group previously showed that eosinophilic asthma phenotype was associated with raised sputum IgE, IL-5 and IL-13 overproduction [47].

Tseliou et al [48] found a weak association between angiopoietins-1 and percentage sputum eosinophils in severe refractory asthma. Increased levels of osteopontin were also found in sputum supernatant of severe refractory asthma with significant association between log osteopontin and sputum eosinophils [49]. Increased levels of eotaxin-2 were associated with eosinophilic phenotype [38] and eosinophilic patients showed higher levels of sputum IL-5 and granulocyte macrophage colony stimulating factor (GM-CSF) [50]. IL-13 has been detected in sputum supernatant and inversely correlated with provocative concentration of methacholine suggesting a relationship between IL-13 and airway hyperresponsiveness. Moreover, sputum cells from eosinophilic asthmatics released more IL-4 and less TNF-α than healthy subjects [51]. Jang et al found a positive correlation between NO metabolites and sputum eosinophils and a decrease in NO metabolites, ECP and IL-5 levels following anti-asthmatic treatment [52].

Volatile Organic Compounds (VOCs)

VOCs might be useful in the assessment of asthma severity. Paredi et al indeed found elevated levels of exhaled ethane in steroid-naïve compared to steroid-treated asthmatics and ethane was also found in higher levels in severe as compared to mild asthmatics [53]. Moreover, Ibrahim showed that VOCs profiles were also markers of sputum eosinophilia in a small asthmatic series [54]. The usefulness of VOCs profile in assessing asthma inflammatory phenotypes still needs to be confirmed.

MANAGEMENT OF EOSINOPHILIC ASTHMA.

Most of the new biological treatments available for severe asthma are monoclonal antibodies directed against specific immunologic processes and in particular against cyto-

kines. Several cytokines are involved in the pathophysiology of asthma including Th2 cytokines. When the diagnosis of severe asthma is confirmed, it is critical to assess inflammation to predict the response to targeted therapies and reduce the risk of exacerbations.

ANTI-IgE

In those refractory asthmatics with moderately elevated total serum IgE and sensitisation to a perennial allergen, omalizumab, a humanised monoclonal antibody against IgE has proved to be effective in reducing exacerbation rate and improving quality of life [55, 56] and asthma control scores although part of the effect in quality of life improvement seen in clinical practice is likely to be due to a placebo effect and a careful follow-up of the patient inherent in the mode of drug administration [57]. It has also recently been shown that the presence of eosinophilic airway inflammation but not IgE is predictive of treatment efficacy [32, 33] (Table **2**).

ANTI-IL-5

An example of the interest of phenotyping severe asthmatics was given by the studies on treatment targeting IL-5, a very specific pathway in asthma. This treatment did not significantly improve an unselected population of severe asthmatics while it improved asthma control and reduced exacerbation in selected patients exhibiting eosinophilic phenotype [36, 58]. The importance of IL-5 in driving the persistent systemic and airway eosinophilic inflammation has been demonstrated by the efficacy of mepolizumab, an anti-IL-5 monoclonal antibody, to further decrease eosinophilic inflammation in those patients with refractory asthma despite high dose of corticosteroids [29, 35] (Table **2**). The clinical relevance of the persistent eosinophilic inflammation is demonstrated by the reduction in exacerbation rate and the improvement in quality of life observed in those patients receiving mepolizumab, even if no effect is observed on airway calibre and bronchial hyperresponsiveness [29]. Studies with reslizumab, another anti-IL-5, have however shown positive effects on asthma control and FEV_1 [58] reinforcing the role of eosinophils in several asthma outcomes.

ANTI-IL-4 AND ANTI-IL-13

Many investigations focusing on the potential actions of IL-4 and IL-13 targeted therapies showed discordant results.

IL-13 promotes the production of IgE by plasma cells, the release of eosinophils chemoattractant and airway smooth muscle contraction. It is increased in severe asthma and causes corticosteroid resistance so is a logical target. This cytokine exerts similar function as IL-4 by binding and activating IL-4 receptor.

Some studies on anti-IL-13 (lebrikizumab, tralokinumab) have been disappointing with little physiological effect and no effect on symptoms or exacerbations [59, 60]. Anti-IL-13 monoclonal antibody lebrikizumab was found to slightly improve FEV_1 and decrease asthma exacerbations in patients exhibiting high levels of FENO and periostin [34, 61].

Blocking the specific epitope for IL-4 receptor (IL-4-R) showed reduced fall in FEV_1 after allergen inhalation challenge [62]. Pitrakinra (anti-IL-4R) decreased severe adverse events during allergen challenge in asthmatics [63] and it was found that variation in the human IL-4 receptor α gene was associated with therapeutic response to inhaled pitrakinra [64].

The recently published studies on anti-IL-13 have highlighted relevant limitations with partial effects that could be due to overlapping biological actions of IL-4 and IL-13.

The combination approach dupilumab inhibiting both IL-4 and IL-13 was likely to be more effective. In patients with difficult-to-control asthma, dupilumab can markedly decrease asthma exacerbations (to an overall 87% reduction) and improve respiratory symptoms and lung function (exceeding 200ml FEV_1) with significant reductions in T-helper 2-associated inflammatory biomarkers such as FENO, serum IgE levels and eotaxin-3 but not in blood eosinophil count [65] (Table **2**).

NON-EOSINOPHILIC ASTHMA

Among severe asthmatics, a subgroup characterized by non-eosinophilic inflammation was described [66]. Patients with a non-eosinophilic phenotype can be further split in two inflammatory phenotypes depending on the level of their airway neutrophilic inflammation: paucigranulocytic and neutrophilic [67]. In the Belgian severe asthma registry, paucigranulocytic asthma accounted for 17% and neutrophilic asthma for 22% of patients [15].

The association between neutrophilic inflammation and severe asthma has been demonstrated in various studies [13, 68-71]. Sputum neutrophil count is a potentially important biomarker in asthma, as it is negatively associated with FEV_1/FVC ratio [3], pre- and post-bronchodilator FEV_1 [72]. In a general population of asthmatics of more than 500 patients including severe asthmatics, it was shown that only FRC was an independent predictor of increased sputum neutrophilic inflammation [73].

No consensus exists to define an abnormally high percentage of sputum neutrophils, and cut-off values vary in studies from 49% to 93%, most authors using a threshold between 61% and 76% [74].

Numerous clinical studies have linked *Chlamydia pneumoniae* [75] and *Haemophilus influenzae* [76] infections to neutrophilic asthma due to the strong neutrophilic inflammation and potent Th1 and Th17 responses induced by those bacteria [77]. One study in a small number of patients showed that in neutrophilic asthmatics, 43% were colonized by bacteria with a higher frequence of Haemophilus influenzae [78]. It was previously shown that asthmatics with higher load of bacteria displayed increased sputum neutrophil [79]. Haemophilus influenzae was detected in 60% of these patients. Together, these studies provide strong associations between infections that induce Th1/Th17 responses and neutrophilic inflammation with steroid-insensitivity in severe asthma.

BIOMARKERS OF NEUTROPHILIC ASTHMA

Specific biomarkers to discriminate neutrophilic asthma from other inflammatory phenotypes do not exist yet. Sev-

eral biomarkers have however been identified in the airways of neutrophilic asthmatics (Table **1**).

IL-8

IL-8 is a chemoattractant and activator of neutrophils [80], and was found in higher level in sputum supernatants of severe asthmatics [69]. Other studies evidenced a higher sputum mRNA expression of IL-8 [78] and a higher IL-8 level in sputum supernatants of neutrophilic asthmatics [79], which correlated with the sputum neutrophil count [81]. IL-8 chemokine (C-X-C motif) ligand (CXCL8) and other neutrophil chemoattractants like growth-regulated protein alpha (GRO-α, CXCL1) and Epithelial-derived neutrophil-activating peptide 78 (ENA78, CXCL5) act through the CXC chemokine receptors (CXCR1 and CXCR2) [82, 83]. Expression of both those receptors were increased in the sputum of neutrophilic asthmatics [79].

TH-17 Cytokines

TH-17 associated cytokines (IL-17A and IL-17F) expression has been observed in airway tissues of severe asthmatics [84]. It was shown *in vitro* that human bronchial epithelial cells stimulated with IL-17 produced IL-6 and IL-8, and *in vivo* that IL-17 recruits and activates neutrophils in the respiratory tract [85]. In sputum from asthmatic patients, IL-17 (or IL-17A) mRNA levels correlated with IL-8 mRNA levels and both these mRNA levels were correlated with the percentage of sputum neutrophils [86].

TNF-α

Sputum mRNA expression of TNF-α was also increased in neutrophilic asthmatics, compared with paucigranulocytic asthmatics [78]. In severe refractory asthmatics, TNF-α is likely to be an important cytokine [87, 88]. TNF-α is a cytokine of the innate immune response and acts through an increased transcription of several genes, such as IL-1β, IL-6, IL-8 and TNF-α [88]. In mild asthmatics, inhalation of recombinant human TNF-α was associated with an increased airway hyperresponsiveness and a rise in sputum neutrophils and eosinophils [89].

MPO and Neutrophil Elastase

MPO (myeloperoxidase) and sputum NE (neutrophil elastase) are markers of neutrophil activation. Levels of sputum MPO were increased in severe asthma patients [69] and levels/activity of sputum NE were associated with neutrophilic asthma [79, 90].

Other Biomarkers

Sputum mRNA expression of Toll-like receptors 2 and 4 (TLR-2 and TLR-4) as well as CD14 was higher in neutrophilic asthmatics than in other inflammatory phenotypes of asthma [78]. It was also shown that severe asthmatics whose airways were colonized with potentially pathogenic microorganisms (PPMs) were characterized by sputum neutrophilic inflammation [91]. Finally, the neutrophilic phenotype of asthma was characterized by higher systemic inflammation (through plasma C-reactive protein (CRP) and IL-6 levels) than non-neutrophilic asthma, in the study of Wood et al [79].

BIOMARKERS OF PAUCIGRANULOCYTIC ASTHMA

To the best of our knowledge, there has not been many studies focusing on biomarkers of paucigranulocytic asthmatics since there are rather considered as non-inflammatory. However, it is interesting to notice that paucigranulocytic asthmatics may show, like eosinophilic asthmatics, disturbance in oxidative stress pathways such as a raised glutaredoxin 1 protein level in sputum supernatants [92]. Clearly, other aspects than granulocytic infiltration should be investigated in those patients called paucigranulocytic asthmatics.

Table 1. Surrogate biomarkers for inflammatory phenotypes defined by induced sputum cell counts in severe asthma.

	Eosinophilic Asthma	Neutrophilic Asthma	Airway Remodeling
Biomarkers validated in clinical practice	Exhaled nitric oxide		
	Blood eosinophil count		
	Serum total IgE		
	Serum periostin		
Potentially useful biomarkers	Supernatant ECP, EDN, EPO, IgE, eotaxin-2 and IL-5	Supernatant IL-8, MPO and NE Serum CRP and IL-6	MMPs
			Sputum FGF2 and galectin-3
			Plasma IL-8/TIMP1
Promising biomarkers	Supernatant angiopoietins-1, osteopontin, GM-CSF and IL-13	Supernatant CXCR1 and CXCR2	Sputum CC16/IL-8
	VOCs	Supernatant IL-17 and TNF-α, TLR 2 and 4 mRNA	KL-6, SP-D, SP-A, YKL-40, CCL18

MANAGEMENT OF NON-EOSINOPHILIC ASTHMA

Low Sensitivity to ICS

It has been shown that patients with non-eosinophilic phenotype were poorly sensitive to corticosteroids [93].

In a population of refractory asthmatics from secondary care, a cluster analysis identified a specific subgroup characterized by a minimal eosinophilic inflammation associated with lots of symptoms [16]. In these patients, a treatment strategy based on sputum analysis allowed to substantially reduce the ICS dose without increasing the rate of severe exacerbations, compared with usual care [16].

Specific Treatment Strategies

Some treatment strategies focusing on neutrophilic inflammation or mediators of the innate immunity have been described. With all molecules aiming at reducing neutrophilic inflammation, adverse events should be carefully monitored because it is known that patients with a reduced neutrophil count are at risk of severe infections [94].

Macrolides

Macrolides have antibacterial, anti-inflammatory and immunomodulatory properties [95]. The use of macrolides has been validated in several respiratory diseases presenting neutrophilic inflammation, such as chronic obstructive pulmonary disease (COPD), cystic fibrosis, non-cystic fibrosis bronchiectasis, and diffuse panbronchiolotis [95].

In the randomized control trial (RCT) of Simpson et al [96], clarithromycin (500mg twice daily for 8 weeks) significantly reduced the sputum IL-8 concentration (primary endpoint) and the neutrophil count in severe refractory asthmatics. Treatment also improved quality of life scores, but not symptom scores and FEV_1 (Table **2**). A subanalysis showed a highest anti-inflammatory effect in patients with non-eosinophilic asthma (sputum eosinophil proportion <1.01%). In a population of mild-to-moderate asthmatics whose control was not achieved with a low dose of ICS, an add-on treatment with clarithomycin (500mg twice daily for 16 weeks) was not superior to placebo in terms of asthma control, asthma quality of life and FEV_1 [97]. In the AZISAST study [98], severe asthmatics at risk of exacerbations received azithromycin (250 mg once daily for 5 days and then 3 times a week) versus placebo for 6 months. No significant difference was observed between both groups for the primary endpoint, which was the rate of severe exacerbations and lower respiratory tract infections. Interestingly, in the subgroup of non-eosinophilic asthma (FENO<upper limit of normal according to Travers et al [99] and blood eosinophils $\leq 200/\mu L$), the rate of severe exacerbations and lower respiratory tract infections was significantly reduced with azithromycin whereas it was increased in the subgroup of eosinophilic asthmatics.

These results highlight the importance to phenotype severe asthmatics when treatments target specific inflammatory pathways [100].

Bacterial resistance is a concern of treatment with macrolides [95, 98] and requires more research [100]. Precautions should also be taken with macrolides: treatment is contraindicated in case of allergy, suspicion of mycobacteria-associated pulmonary infection [95] and patients should be selected and monitored to reduce the risk of cardiac toxicity [98, 100].

More research is needed to confirm the effect of macrolides in non-eosinophilic severe patients with frequent exacerbations, and to investigate the accuracy of sputum neutrophils as biomarker of macrolide responsiveness [95].

Anti-IL-17

A recent RCT in severe asthmatics assessed the safety and efficacy of a 12 week treatment with Brodalumab, a human anti-IL-17 receptor A monoclonal antibody, blocking the activity of IL-17A, IL-17F, IL-17A/F and IL-17E (IL-25) [101]. Results were however disappointing, with no improvement in Asthma Control Questionnaire (ACQ) score (primary endpoint), FEV_1 and symptom score in the active group, compared with placebo. This study did however not stratified patients according to the airway inflammatory phenotypes and we would certainly be interested to know about the effect of anti-IL-17 in neutrophilic asthmatics.

CXCR2 Antagonists

Early studies showed that CXCR2 antagonists were able to reduce the sputum neutrophil count after inhalation of lipopolysaccharide (LPS) in healthy subjects, compared with placebo [82]. In the proof-of-concept study of Nair *et al.* [83], a CXCR2 antagonist was tested for 4 weeks (versus placebo) in severe asthmatics with a sputum neutrophil count > 40% and a total cell count < 10 million cells/g. Treatment with the CXCR2 antagonist was safe and associated with a reduction in sputum neutrophil count (primary oucomes). A reduction of mild exacerbations and trend towards improvement in ACQ score were also observed. This study was not designed to evidence a clinical efficacy in asthma, but these results support further investigating the association between a reduction in sputum neutrophils and clinical improvement in asthma.

Anti-TNF-α

In the RCT of Berry *et al.* [87], a treatment of 10 weeks with etanercept in refractory asthmatics was associated with a reduction in airway hyperresponsiveness, an increase in asthma quality of life (primary endpoints) and an increase in post-bronchodilator FEV_1 (secondary endpoint). Interestingly, these improvements in primary endpoints were positively correlated with the baseline expression of membrane-bound TNF-α by peripheral-blood monocytes on one hand, and with the reduction of this expression by etanercept on the other hand. This suggests that expression of TNF-α by peripheral-blood monocytes could be a predictive biomarker of the efficacy of etanercept [88]. In the RCT of Morjaria *et al.* [102], a treatment with etanercept for 12 weeks in severe refractory asthmatic showed only a small improvement in asthma control, but no significant effect on asthma quality of life and airway hyperresponsiveness. In the multicentric study of Wenzel *et al.* [103], golimumab, another anti-TNF-α, was studied in severe persistent asthma for a scheduled

Table 2. Biomarkers predicting the response to treatment.

	Value of baseline biomarker	Population studied	Value of change in biomarker	Outcomes
Inhaled corticosteroids	Sputum eosinophils >3%	Moderate to severe asthma	Sputum eosinophil count <3%	Exacerbations↓[11]
	Sputum eosinophils >3%	Moderate asthma		ACQ↓, BHR↓, FEV1↑[127]
	Sputum eosinophils ≥2%	Mild asthma		ACQ↓, BHR↓, AQLQ↑, FENO↓[128]
	FENO>33ppb	Mild asthma		BHR↓[128]
	FENO>35ppb	Unselected population	FENO↓<40%, FENO↑>30%	ACQ↓[129] ACQ↑
Oral corticosteroids	Sputum eosinophils ≥4%	Moderate to severe asthma		FEV1↑≥15%[130]
	FENO	Moderate to severe asthma	FENO↑10ppb	FEV1↑≥15%[130]
	Sputum eosinophils↑, Blood eosinophils ↑	Severe asthma		FEV1↓, symptoms↑[131]
	Blood and sputum eosinophil count	Severe asthma		FEV1↑, BHR↓, AQLQ↑ [132]
Anti-IgE	FENO≥19ppb	Severe asthma		Exacerbations↓[32]
	Blood eosinophils count≥260/mm³	Severe asthma		Exacerbations↓[32]
	Serum periostin seuil ≥50ng/ml	Severe asthma		Exacerbations↓[32]
Anti-IL-5	Blood eosinophil count>300/mm³	Severe asthma		Exacerbations↓[36,133], ACQ-6[133] and FEV1[133]
Anti-IL-13	Serum periostin ≥median (50ng/ml) FENO ≥ median	Moderate to severe asthma		FEV1↑[34]
Anti-IL-4 and 13	FENO Blood eosinophil count≥300/µl	Moderate to severe asthma		Exaverbations↓[65] FEV1↑[65] ACQ↓[65]
Clarithromycin	Sputum neutrophil >61%	Severe asthma	Sputum neutrophil	AQLQ ↑[96]
Azithromycin	FENO<upper limit of normal and blood eosinophils ≤200/µl	Moderate to severe asthma		Exacerbations↓[98]
Thermoplasty	Smooth muscle	Moderate to severe asthma	Smooth muscle	Exacerbations ↓[134;135] AQLQ↑[134]

period of 52 weeks. No significant changes in FEV_1 and severe asthma exacerbations were shown, and the study was prematurely stopped due to an unfavorable risk-benefit balance. Another RCT assessing the effect of 12 weeks etanercept in moderate to severe asthmatics could neither evidence an effect on prebronchodilator FEV_1 and other secondary endpoints [104].

Responses to anti-TNF-α treatments therefore seem to be variable in unselected severe asthmatics, and the development and validation of biomarkers potentially identifying a small subgroup of sensitive asthmatics is necessary [88].

Finally, an important concern with these anti-TNF-α treatments is the risk of adverse event such as solid organ malignancy and infections, and the risk-benefits balance should be assessed [88].

PDE4 Inhibitors and Kinase Inhibitors

Other therapies targeting neutrophilic inflammation include phosphodiesterase 4 (PDE4) inhibitors and kinase inhibitors[a]. Roflumilast, a PDE4 inhibitor, is already registered in the treatment of severe COPD patients with a history of exacerbations, and this drug could also be interesting in se-

vere neutrophilic asthma with frequent exacerbations [105], A concern with roflumilast and with other drugs of this class is the presence of side effects like diarrhea, nausea and headache [105, 106]. Future research is needed to extent the indications of roflumilast to severe asthma, and to develop molecules of this class with a better tolerability [106]. Regarding kinase inhibitors, such as p38 mitogen-activated protein (MAP) kinase inhibitors, side effects after systemic administration is of concern, and further development is needed [105].

AIRWAY REMODELING IN SEVERE ASTHMA

In contrast to what has been found for eosinophilic asthma there have not been many studies focusing on user-friendly biomarker of airway remodeling in asthma, a component likely to be found in most of severe asthmatics (Table **1**). The phenotype with fixed airway obstruction has been highlighted as being one key feature of many severe asthmatics [107]. Recent data from the Belgian national severe asthma registry found 60% of patients with post bronchodilator ratio FEV_1/FVC lower than 70% [15]. Fixed airway obstruction is thought to be a consequence of airway remodeling.

BIOMARKER OF CURRENT AIRWAY REMODELING

Bronchial Biopsy

The real demonstration of airway remodeling requires biopsy analysis, hence the need for an invasive procedure to perform bronchial biopsies. Airway remodeling in severe asthma with fixed airway obstruction mainly features smooth muscle hypertrophy and mucosal glands hypertrophy together with increased number of fibroblasts and collagen-3 deposition within bronchial wall [108].

Imaging and Functional Parameters

There have been several attempts to link imaging obtained by Chest CT to physiological marker of airway obstruction. Overall bronchial thickness failed to significantly correlate with airway caliber measured by expiratory flow [109] and the predictive value of the FEV_1/FVC ratio (threshold of 75%) to show bronchial alteration (bronchiectasis or wall thickness) is rather weak with sensitivity and specificity of 67% and 65% respectively [110].

Matrix Metalloproteases

There have been several studies investigating the performance of a biomarker that reflects the airway remodeling underlying the fixed airway obstruction. Most of these markers belong to the matrix metalloprotease (MMPs) and to the growth factor family. The sputum ratio MMP-9/TIMP-1 was first proposed since being inversely correlated with the FEV_1 [111] and with a remodeling score on the high resolution CT scan in asthmatics [112]. A recent study has investigated the correlation between several sputum cytokines, growth factors and matrix metalloproteases measured by ELISA in sputum supernatant and signs of airway remodeling on bronchial biopsies.

Fibroblast Growth Factor-2 and Galectin-3

Among a list of potential candidates, fibroblast growth factor-2 (FGF2) has proved to be the biomarker that most convincingly correlated with physiological and pathological findings of airway remodeling in asthmatics [113]. It has also recently been shown, through a proteomic analysis, that galectin -3, an IgE-binding protein, was a reliable, stable and predictive biomarker of airway remodeling modulation identified on bronchial biopsies of severe asthmatics treated with omalizumab [114].

Ratio IL-8/TIMP1

Whilst studies in asthma have already provided some results, the discovery of interesting biomarkers may also come from studies conducted in COPD and lung fibrosis, diseases associated with severe airway and parenchymal remodeling. Patients with COPD had significantly higher plasma levels of IL-8 and significantly lower levels of TIMP-1 than smokers without COPD and non-smokers. Therefore the ratio IL-8/TIMP1 would strongly correlate with the fixed airway obstruction [115].

Epithelium-Derived Proteins

Although IL-8 is a well-accepted marker for injured airway epithelium, Clara cells and their major secretory product CC16 protein have only recently emerged as potential small airways repair markers. The CC16/IL-8 ratio measured in sputum is a potentially valuable biomarker for non-invasive assessment of small airway remodeling in smokers as there was a striking negative correlation between CC16/IL-8 levels and whole airway thickness at morphometry [116]. Promising biomarkers in lung fibrosis are lung epithelium-derived proteins such as KL-6 (Krebs von den Lungen-6), SP-D (surfactant protein-D), SP-A (surfactant protein-A), YKL-40 (chitinase-3-like protein 1 [CHI3L1] or cytokines such as CCL18 [chemokine (C-C) motif ligand 18]) [117]. All these markers deserve to be considered and validated in remodeled asthma.

BIOMARKER PREDICTIVE OF FUTURE AIRWAY REMODELING

Ideally one of the purposes of asthma management and treatment would be to prevent evolution towards fixed airway obstruction that may happen in some patients. Several studies have identified some features that associate with accelerated lung function decline in asthma. Among those there are chronic hypersecretion, tobacco smoking [118], high exhaled NO [119] and sputum eosinophils [120]. Genetic studies have shown that polymorphism in the ADAM33 gene [121], oestrogen receptor [122], plasminogen activator [123] were associated with greater loss in expiratory flow over time. Recent emphasis has been placed on periostin, a matricellular protein secreted from airway epithelial cells in response to IL-4 and IL-13 signaling, as a marker of rapid lung function decline. It has been shown that high serum periostin level correlated with annual change in FEV_1, which might be linked to a polymorphism in the periostin gene [124]. As established in COPD, it has been shown that occurrence of severe exacerbation is predictive of greater loss in % predicted FEV_1 in asthmatics. In these

cases inhaled corticoids seem to be able to slow down loss of lung function, which is not the case in a general population of asthmatics [125]. Whether some biomarkers might be taken into account to adjust the treatment in order to prevent fixed airway obstruction has not been often investigated yet although high total serum IgE was also found to predict a significant effect of ICS in attenuating lung function decline [126].

Management of Remodeled Asthma

There is currently no drug able to convincingly reverse airway remodeling. Thermoplasty has been proposed as a non pharmacological treatment in asthmatics who remained uncontrolled despite inhaled corticoids. Thermoplasty is the first treatment which specifically targets some facet of airway remodeling and the supposed mechanisms is a reduction in the amount of airway smooth muscle thereby reducing the airway twitchiness (Table **2**). This has resulted in a reduction of exacerbation and sometimes hospitalization. It must however be noticed that thermoplasty failed to reverse baseline airway obstruction as it did not improve FEV_1.

CONCLUSION

The heterogeneity of severe asthma includes various types of airway inflammation and different degrees of airway remodeling. Several biomarkers have been described in the literature to assess severe asthma characteristics. The use of such biomarkers can be helpful in difficult to treat asthma to predict the response to targeted therapies. Sputum eosinophil is a good biomarker to adjust treatment with ICS and can be satisfactorily approached by FENO and blood eosinophil counts in clinical practice. FENO and serum periostin are indeed markers of the potential response to omalizumab, anti-IL-13 and anti-IL-4 while blood eosinophils are best predictor for a response to anti-IL-5 therapy. Neutrophilic asthma is characterized by a low response to ICS and higher loads of bacteria and seems to be better targeted by macrolides. We currently lack of airway neutrophilic inflammation biomarkers easily available in clinical practice. There is not yet user-friendly biomarker of airway remodeling. It is probably more relevant to use biomarkers predictive of future airway remodeling in order to prevent this evolution.

LIST OF ABBREVIATIONS

ACQ	=	asthma control questionnaire
AUC	=	area under the curve
CHI3L1	=	Chitinase-3-like protein 1
COPD	=	chronic obstructive pulmonary disease
CRP	=	C-reactive protein
CXCL	=	chemokine (C-X-C motif) ligand
CXCR	=	CXC chemokine receptor
ECP	=	eosinophil cationic protein
EDN	=	eosinophil-derived neurotoxin
ENA78	=	epithelial-derived neutrophil-activating peptide 78
EPO	=	eosinophil peroxydase
FENO	=	fraction exhaled nitric oxide
FEV_1	=	Forced expiratory volume in one seconde.
FGF	=	fibroblast growth factor.
FVC	=	forced vital capacity.
GM-CSF	=	granulocyte macrophage colony stimulating factor
GROα	=	growth-regulated protein alpha
ICS	=	Inhaled corticosteroids
IgE	=	Immunoglobulin E
IL	=	Interleukin
iNOS	=	inducible nitric oxide synthase
KL	=	kit ligand.
LPS	=	lipopolysaccharide
MAP	=	mitogen-activated protein
MMP	=	matrix metalloproteinase
MPO	=	myeloperoxidase
NE	=	neutrophil elastase
NO	=	nitric oxide
PDE4	=	phosphodiesterase 4
PPMs	=	pathogenic microorganisms
RCT	=	randomized control trial
ROC	=	receiver operating curve
SP	=	surfactant protein
TIMP	=	tissue inhibitor of metalloproteinase
TLR	=	Toll-like receptor.
TNF-α	=	Tumor necrosis factor α
VOC	=	volatile organic compound

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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