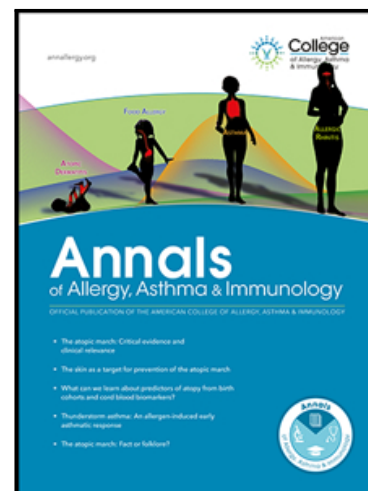


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Use of Biomarkers to Identify Phenotypes and Endotypes of Severe Asthma

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Asthma

Bromotyrosine

Dipeptidyl peptidase-4

Endotype

Eosinophil

Eosinophil peroxidase

Fraction of exhaled nitric oxide

Immunoglobulin E

Neutrophil

Periostin

Phenotype

Tryptase

Abbreviations:

AERD Aspirin exacerbated respiratory disease

ABPA Allergic bronchopulmonary aspergillosis

ABPM Allergic bronchopulmonary mycosis

DPP4 Dipeptidyl peptidase-4

EPX Eosinophil peroxidase

FeNO Fraction of exhaled nitric oxide

Ig Immunoglobulin

IL Interleukin

KIT KIT proto-oncogene receptor tyrosine kinase

SCF Stem cell factor

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Figures: 1

Tables: 2

Key Messages

- A number of different biomarkers can be applied to identify severe asthma phenotypes and endotypes.
- Biomarkers are not yet pathognomonic for severe asthma phenotypes or endotypes, and therefore need to be utilized thoughtfully and in combinations.
- Biomarkers, while useful to identify severe asthma phenotypes and endotypes, may have even greater impact when used to predict response to targeted biologic therapies.
- Novel biomarkers in development, which assess specific inflammatory pathways or identify activated inflammatory cells, may prove to more accurately identify severe asthma phenotypes or endotypes.

Introduction:

Severe asthma is a complex, heterogeneous set of diseases. The European Respiratory Society and American Thoracic Society Task Force define severe asthma as asthma that requires or remains uncontrolled despite treatment with systemic corticosteroids or high dose inhaled corticosteroids plus another controller such as a long acting beta agonist¹. Lack of control can be determined by symptoms, lung function, or exacerbations, accounting for inter-individual variability in disease state and outcomes. With this recognition of heterogeneity among patients with severe asthma, and with the burden of identifying next-generation therapies to treat these patients, clinicians and researchers increasingly acknowledge the importance of better and more precise classifications of severe asthma into its subtypes. In the field of severe asthma, these subtypes are classified into phenotypes, referring to the observable characteristics of the disease in an individual, and endotypes, referring to the specific biological mechanisms that cause those observed properties of any given phenotype^{2,3}. Determining a patient's disease phenotype or endotype becomes crucial, particularly when selecting targeted biologic or other immunomodulatory therapeutics, thus the need for biomarkers that will help to distinguish among these groups.

Biomarkers

In 1998, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic

responses to a therapeutic intervention.⁴” Biomarkers can be generally categorized into groups that describe their utility and function; common terms used include diagnostic, predictive, dynamic, and stable. Diagnostic biomarkers can determine if a disease exists, such as the presence of low hemoglobin to diagnose anemia. Predictive biomarkers are used to determine a differential response to an intervention, such as the likelihood of a specific patient to benefit from a specific medication. Dynamic biomarkers are expected to change with disease variables, such as change in severity or response to treatment. One possible way to implement use of a dynamic biomarker is to monitor changes with a therapy, to determine or define response to an intervention. Stable biomarkers, such as gene polymorphisms, while of limited diagnostic utility in complex diseases like severe asthma, may be applied, for example, to pharmacogenomics, to assist in predicting response or harm from different therapeutics⁵.

Applying biomarkers to identify phenotypes and endotypes of severe asthma may have a variety of purposes. For example, the currently recognized and published phenotypes and endotypes of severe asthma are largely defined by observable and measurable characteristics of the disease or patient and not yet by specific diagnostic biomarkers^{2,3}(Table 1). For example, early onset, allergic asthma is characterized by the presence of allergen-specific immunoglobulin (Ig) E, and late-onset, hypereosinophilic asthma is characterized by elevated peripheral blood and sputum eosinophils. Eosinophil and IgE levels can be used as diagnostic biomarkers for these phenotypes. However, blood eosinophils are commonly present in early onset allergic asthmatics, and allergen-specific IgE may be present in those late-onset, hyper-eosinophilic asthmatics. Therefore, while these commonly used biomarkers may give clues as to the phenotype or endotype

of a severe asthmatic patient, they are not pathognomonic or mutually exclusive and do not address the molecular mechanisms underlying endotype. Notably, asthma endotypes are not yet well described nor mechanisms well defined, perhaps contributing to these limitations.

Combinations of biomarkers may be more helpful to fully characterize patients into one of these categories, and novel biomarkers in development may provide improved specificity for one or multiple phenotypes. Further, diagnostic, predictive and dynamic biomarkers are more recently valued and utilized to drive therapy with targeted biologic agents. Clinicians already have access to a variety of clinical biomarkers that may be useful in phenotyping severe asthma, including age of onset, duration of disease, symptom triggers, and obesity. In the following sections, we will discuss the use of commonly implemented, commercially available, objectively measured biomarkers to identify phenotypes and endotypes of severe asthma and highlight novel biomarkers that may improve our ability to identify accurately phenotypes and endotypes of severe asthma.

Eosinophils and Eosinophil-related Biomarkers

Eosinophils may be detected in one or multiple system compartments, including the peripheral blood, the airway submucosa, and the airway lumen. Interestingly, the presence of eosinophilia is not always consistent among these compartments^{6,7}. Eosinophils are primarily tissue-dwelling leukocytes, and therefore blood counts do not necessarily indicate the entire extent of eosinophil involvement in the affected tissues^{6,8}. A systematic review and meta-analysis of blood biomarkers found that peripheral blood

eosinophil counts had a sensitivity of 71% and specificity of 77% for detecting sputum eosinophils of $>3\%$ ⁹. The extremes of blood eosinophil levels may be more sensitive and specific for identifying sputum eosinophils¹⁰⁻¹². For example, patients with blood eosinophil counts less than 90 eosinophils per microliter are highly unlikely to have airway eosinophilia; alternately, almost all patients with more than 400 eosinophils per microliter of blood can be expected to have significant sputum eosinophilia¹³. Reference values for sputum eosinophil percentages have been determined through epidemiologic studies of healthy patients^{14,15}. In general, sputum eosinophil levels of >2 to 3% , and blood eosinophil counts of > 300 per microliter (uL) may be used to define eosinophilic disease^{12 16}.

The phenomenon of airway eosinophilia occurs in approximately 50% of asthma patients¹⁷. Eosinophilia can be detected across the entire spectrum of asthma severity and in multiple phenotypes. For example, eosinophilic inflammation is a characteristic of and a diagnostic biomarker for both childhood-onset allergic asthma and later-onset asthma¹⁸ and is especially prominent in severe asthmatics with the hypereosinophilic, late onset disease phenotype¹⁹. While eosinophil count does not distinguish between these phenotypes, when used in combination with clinical characteristics such as allergic comorbidities and age of onset of asthma, the phenotype becomes better defined. Eosinophilia has been linked to a variety of specific clinical characteristics and outcomes of asthma including worsening lung function and incidence of fatal asthma²⁰⁻²³. Eosinophil accumulation in the airways in severe asthma correlates with markers of local tissue and extracellular matrix remodeling; airway and blood eosinophilia identify those at risk for more frequent exacerbations²⁴⁻²⁷.

Eosinophil levels can be used as a predictive biomarker for response to multiple classes of drug therapy for severe asthma. Increased blood eosinophil counts are a good predictor of treatment response to corticosteroids in asthma^{28,29}. For example, in children who require controller therapy for asthma, phenotyping with aeroallergen sensitization and blood eosinophils >300/uL was useful for identifying those with a high exacerbation probability for whom treatment with daily inhaled corticosteroids is beneficial³⁰. Furthermore, blood eosinophil levels can decrease significantly in asthmatics treated with inhaled and systemic corticosteroids, supporting the importance of eosinophil reduction for asthma control^{31,32}. Similarly, sputum eosinophilia can identify patients with clinical response to systemic³³ and inhaled corticosteroids^{28,34}. Gene expression patterns relevant to eosinophil markers can also identify individuals who are most likely to have clinical benefit from corticosteroids. Sputum and airway gene expression of the type 2 inflammatory cytokines, interleukin(IL)-4, IL-5, and IL-13, correlate with sputum and blood eosinophils³⁵ and can identify some patients who benefit from inhaled corticosteroids^{36,37}. Increased expression of the gene encoding for the eosinophil's Charcot-Leyden crystal protein can also identify the eosinophilic inflammatory phenotype, and was observed to predict clinical response to inhaled corticosteroids³⁸. However, it is important to note that patients without eosinophils may also benefit from corticosteroid treatment.

Evidence of eosinophilic inflammation related to asthma can help guide selection of targeted treatments for severe asthma, particularly those related to the main stimulant of eosinophil recruitment and differentiation, IL-5. Currently, in the United States, three anti-IL-5 monoclonal antibodies are FDA approved for the treatment of severe

eosinophilic asthma. Mepolizumab (GlaxoSmithKline) is administered subcutaneously as a once monthly standard dose; reslizumab (Teva) is administered once monthly as a weight-based intravenous infusion. Phase three studies supported the use of these medications for both reduction of exacerbation frequency and corticosteroid sparing effects in patients with peripheral blood eosinophil counts of $>300\text{--}400/\mu\text{L}$ ³⁹⁻⁴¹. Eosinophils may be also a dynamic biomarker to determine response to these therapies, as changes in sputum and blood eosinophil counts related in one study to clinical benefit from these biologics⁴². Benralizumab (AstraZeneca), an IL-5-receptor antagonist that blocks IL-5 signaling and induces rapid eosinophil depletion through antibody-dependent cell-mediated toxicity, was more recently FDA approved for the treatment of severe eosinophilic asthma^{43,44}. Benralizumab has shown efficacy toward decreasing exacerbations, improving symptoms and improving lung function in patients with severe eosinophilic asthma. However, not all patients with eosinophilic disease will show clinical improvement with these three IL-5-pathway biologics, underscoring the pathobiologic complexity of eosinophilic endotypes of asthma.

Another limitation of the eosinophil as a predictive biomarker for diagnosis or treatment may include failure to identify the presence of eosinophil functional stimulation and activity related to the severe asthma. Markers of eosinophil activation may therefore be more pathophysiologically relevant as diagnostic and predictive biomarkers for response to therapy or other disease characteristics. For example, eosinophil peroxidase (EPX) is one of the major granule contents released by eosinophils upon activation⁴⁵. In sputum, EPX is highly correlated with sputum eosinophil levels, increases with allergen challenge, and is specific for those with eosinophilic disease⁴⁶. Interestingly, EPX is also

readily detected in nasal and pharyngeal swabs, and both values are highly correlated with sputum eosinophils in poorly controlled eosinophilic asthmatics⁴⁷. As a potential dynamic biomarker, sputum EPX also decreases with anti-eosinophil biologics^{42,46}. Airway EPX measurement therefore could be utilized as a diagnostic, predictive and dynamic biomarker of eosinophilic asthma.

Bromotyrosine is an oxidation product of EPX. Elevated levels of bromotyrosine can be identified in the airway of clinically stable asthmatic patients⁴⁸, and can be induced by airway allergen challenge⁴⁹. Bromotyrosine is a stable compound that is excreted in the urine⁵⁰ where it can be non-invasively measured. Elevated urinary levels of bromotyrosine strongly correlate with uncontrolled asthma and risk of asthma exacerbations in children⁵¹. As might be expected, urinary bromotyrosine can also be used as a predictor of clinical response to inhaled corticosteroids in asthma²⁹. Further underscoring the distinction between the pathobiologic contribution of eosinophils and activated eosinophils, despite prominent airway eosinophilia in the aspirin-exacerbated respiratory disease endotype, urinary bromotyrosine levels are not induced by aspirin challenge, supporting the relative importance of the leukotriene/prostaglandin pathways over eosinophilic activation in this disease⁵².

IgE and related biomarkers

IgE is an important marker of airway inflammation, in particular playing a key role in allergic asthma. The presence of allergen-specific IgE is required to define the phenotype of early-onset allergic asthma, with panels of clinically relevant aeroallergen skin testing or specific IgE-serology functioning as essential biomarkers of allergic

asthma, and may be present and relevant in adult onset asthma. Total and specific serum IgE are considered core outcomes for interventional and observational studies of asthma related to their relevance in asthma phenotyping⁸ and, independent of specific aeroallergen sensitization, total IgE is a marker of asthma risk⁵³. Binding of IgE to high affinity receptors, followed by subsequent exposure to allergens, induces mast cell activation and initiates the inflammatory cascade, including release of cytokines, chemokines, and lipid metabolites, all of which contribute to acute and chronic symptoms of allergic asthma⁵⁴. Both specific aeroallergen sensitization and elicitation of symptoms upon exposure to that aeroallergen should be present in the clinical syndrome of allergic asthma. This requires clinician assessment of exposure-related symptoms, in addition to specific IgE testing through percutaneous skin testing or specific IgE serum assays.

Allergic bronchopulmonary aspergillosis, or the more general term of allergic bronchopulmonary mycosis (ABPM), is an endotype of severe asthma. Pathognomonic diagnostic and dynamic biomarkers of ABPM include markedly elevated serum total IgE, specific IgE to molds (detected by percutaneous skin testing or serum analysis, most commonly to *Aspergillus fumigatus*), in the setting of severe asthma, mucus plugging, and often central bronchiectasis. For patients with ABPM, the biomarkers are necessary for diagnosis. Precipitating anti-mold antibodies of the IgG or IgE subtype, as well as airway and blood eosinophilia, are commonly present. However, the presence of total or allergen specific IgE can fluctuate according to the disease stage. Elevated levels of IgE during acute or exacerbation phases may normalize or markedly decline during periods of remission or fibrosis, or during adequate medical treatment⁵⁵. Repeated measurements of

these biomarkers is therefore often required for diagnosis of disease, for response to therapy, and to predict exacerbations⁵⁵.

Omalizumab is a monoclonal anti-IgE antibody developed for the treatment of moderate to severe allergic asthma. Omalizumab may blunt the allergen-induced early asthmatic responses, producing inhibition of allergen-induced bronchoconstriction, and suppressing the effect of allergen-induced, immediate hypersensitivity responses in the airway⁵⁶. Package guidelines approved by the FDA suggest that treatment with omalizumab, which is dosed based on weight and serum total IgE level, be restricted to those with IgE between 30-700 kU/mL. However, omalizumab can benefit patients with IgE outside that range, particularly for those with ABPM⁵⁵. While omalizumab functionally addresses the early onset allergic severe asthma phenotype and depletes IgE from airway tissue, treatment with omalizumab can also decrease sputum and blood eosinophils, and therefore may also have a role in preventing exacerbations in those with eosinophilic phenotypes^{57,58}.

The production of antigen-specific IgE requires class switching within activated B cells, which is induced by IL-4 and to a lesser extent IL-13. Therefore, for patients with allergic asthma phenotypes and endotypes for whom allergen-specific IgE contributes pathophysiologically, identification and targeting of IL-4 and IL-13 signaling could prove therapeutically useful. Dupilumab (Sanofi-Regeneron) is a monoclonal antibody targeting the IL-4 receptor alpha chain, which is also a subunit of the IL-13 receptor and therefore blocks signaling of both molecules. Clinical and inflammatory characteristics supporting the activity of this type-2 inflammatory pathway, such as comorbid atopic disease, elevated IgE, or elevated FeNO, may identify patients who preferentially benefit from

this drug⁵⁹. While type-2 pathways in asthma can also cause elevated peripheral blood or airway eosinophils, the presence of eosinophils is not absolutely required to demonstrate efficacy of this IL-4/IL-13 inhibitor⁶⁰. Alternative biomarkers for type 2 inflammation have been identified through gene expression studies. Both periostin and dipeptidyl peptidase-4 are induced by IL-13 expression, therefore elevated levels of each of these markers may identify patients with asthma who benefit from blockade of the IL-13 and/or IL-4 signaling pathway⁶¹.

Exhaled Nitric Oxide

Nitric oxide (NO) is produced by NO synthases, some of which can be induced by cytokines, including interferon-gamma, IL-1beta, tumor necrosis factor-alpha, IL-4, and IL-13⁵⁰. While these latter Th2 cytokines are not required for NO production, elevated levels of NO can be detected in diseases with prominent IL-4 and IL-13 expression, such as in type-2 asthma²⁹. The measurement of the fraction of exhaled NO (FeNO) is used clinically as a biomarker of airway inflammation related to IL-4 and IL-13-mediated pathways. This relationship is supported by the observation that FeNO levels decline with IL-4 and IL-13 inhibition^{59,62}. Higher levels of FeNO are related to severe, early onset, allergic and eosinophilic asthma and can therefore help to identify patients with these phenotypes⁶³. However, FeNO has only moderate diagnostic accuracy for sputum eosinophilia, with an estimated sensitivity of 66%⁹. Further, FeNO is a predictive and dynamic biomarker to determine likelihood of therapeutic benefit from corticosteroids²⁹, and potentially for biologics targeting the IL-4/IL-13 pathway. FeNO is also biomarker for monitoring treatment responses⁶⁴, or adherence⁶⁵, to corticosteroids. FeNO is

therefore often measured as a point of care test in the clinical setting through commercially available device systems, to phenotype patients with airway inflammation or to adjust inhaled corticosteroid therapy⁶⁶.

Mast cell-related biomarkers

Aspirin exacerbated respiratory disease (AERD) is a well-defined endotype of severe asthma characterized by the triad of severe asthma, recurrent and severe nasal polyposis, and the development of acute respiratory (and often systemic) symptoms upon exposure to nonsteroidal anti-inflammatory drugs⁶⁷. These patients generally have marked tissue eosinophilia and have corticosteroid responsive or corticosteroid dependent disease. The pathognomonic chemical pathways involved in this disease relate to arachidonic acid metabolism, with overproduction of cysteinyl leukotrienes and prostaglandin D₂, and relative lack of the bronchoprotective prostaglandin E₂. Upon exposure to cyclooxygenase-1 inhibitors, imbalance of these chemicals become exaggerated, with loss of prostaglandin E₂ and upregulation of the leukotrienes, causing the clinical syndrome of worsening upper and lower airway inflammation. Urinary measurement of leukotriene E₄, which is the stable metabolite of the cysteinyl leukotrienes, can accurately identify patients with AERD⁶⁸. Further, these and urinary prostaglandin D₂ levels increase with nonsteroidal anti-inflammatory drug exposure and correlate with severity of bronchoconstriction subsequent to exposure⁶⁷. While the primary source of these arachidonic acid metabolites is the activated mast cell, eosinophils and platelet-adherent neutrophils may also produce cysteinyl leukotrienes and therefore actively

contribute to the disease state. Therefore, these mast cell-related metabolites, measured in the urine, can be both descriptive and dynamic.

Mast cells are fully differentiated, long-lived hematopoietic cells that reside in tissues and express tryptase, a serine protease, which is constitutively leaked in immature forms and released in mature form upon mast cell activation and degranulation. Mast cell development, proliferation and survival are dependent on stem cell factor and its receptor, the KIT proto-oncogene receptor tyrosine kinase (KIT). The presence of airway mast cells, as well stem cell factor and KIT levels presumably indicating mast cell presence, correlate with hyperresponsiveness and asthma disease severity^{69,70}. To target this pathway, imatinib, a KIT-inhibitor, has been proposed as a potential therapeutic strategy for patients with poorly controlled severe asthma and persistent airway hyperresponsiveness⁷¹, for which evidence of mast cell burden or activity could serve as predictive biomarkers.

Neutrophils

A significant proportion of patients with severe asthma will have airway granulocytes, including neutrophils and eosinophils⁷². Patients with few inflammatory are considered paucigranulocytic. Those with significant proportions of neutrophils (literature ranging from 40-76% of total sputum cells) but lacking significant airway eosinophils are considered to have the neutrophilic phenotype of severe asthma. Therefore, sputum granulocyte counts can be used as a diagnostic biomarker for this severe asthma phenotype. However, the use of systemic or inhaled corticosteroids at the time of sputum collection, as these treatments induce neutrophilia, could make interpretation of this finding more challenging. This phenotype is not well understood but may be driven by

chronic infection, such as with atypical bacteria⁷³ or occupational exposures. Other potential biomarkers related to neutrophilic inflammation in severe asthma are being explored. For example, elevated airway IL-6 levels can be detected in some patients with neutrophilic asthma, which may contribute to disease through airway remodeling in this phenotype⁷⁴. IL-8 levels in sputum of severe asthmatic children may identify those with neutrophilic disease and atypical infection⁷⁵. Smoking asthmatics also tend to have higher neutrophil counts in the sputum, associated with less corticosteroid-responsiveness⁷⁶. The presence of sputum neutrophils is not accurately predicted by other biomarker surrogates⁷, including peripheral blood neutrophil levels¹¹. As neutrophilic asthma does not currently have a targeted therapy, the use of sputum neutrophils or related biomarkers currently lack a clinical application.

Conclusion

Putting it all together: Biomarkers and Severe Asthma

The current application of biomarkers to identify phenotypes and endotypes of severe asthma is limited, as none of the currently available biomarkers are specific to diagnosis or management of one phenotype or endotype. Indeed, many of the discussed biomarkers play multiple roles, as described in Table 2. Further, true endotyping of asthma through biomarkers has not yet been achieved, except perhaps through those biomarkers specific for AERD. However, these biomarkers, when used in conjunction with the clinical characteristics of each patient, may help identify a phenotype in some cases. An algorithm using the commercially and clinically available biomarkers to determine severe asthma phenotype is proposed in Figure 1. Biomarkers which support, but may be less

accessible for measurement, are noted in dashed-lined boxes. Using algorithms such as this, and adding novel biomarkers that are still in development, will better inform clinicians about the inflammatory processes driving each patient's asthma and guide clinicians toward improving therapeutic strategies and ultimately, improving patient outcomes.

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Figure 1. Biomarker-based approach to identifying phenotypes/endotypes of severe asthma

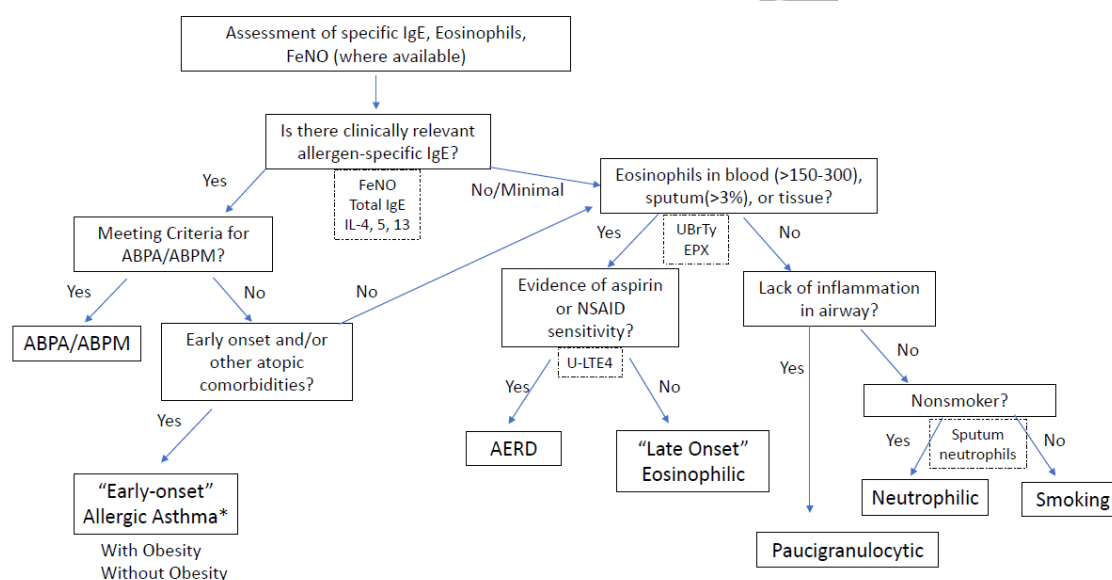


Figure Legend:

Dashed lines indicate accessory biomarkers

ABPA: Allergic bronchopulmonary aspergillosis

ABPM: Allergic bronchopulmonary mycosis

AERD: aspirin exacerbated respiratory disease

UBrTy: urinary bromotyrosine

EPX: eosinophil peroxidase

FeNO: Fraction of exhaled nitric oxide

U-LTE4: Urinary leukotriene E4

* May be childhood or adult in onset

Table 1. Clinically determined and measured biomarkers for the Phenotypes and Endotypes of Severe Asthma (adapted from reference²)

Phenotype	Clinically determined biomarkers and characteristics	Measured biomarkers
Early-Onset, Allergic Asthma	Childhood onset Allergen triggers Allergic rhinitis	Aeroallergen-specific IgE Elevated FeNO Eosinophilia
Early-Onset, Obesity-exacerbated	Childhood onset Allergen triggers Obesity	Aeroallergen-specific IgE Eosinophilia
Aspirin-Exacerbated Respiratory Disease	Adult onset Nasal polyposis Reactivity to NSAIDs	Eosinophilia Leukotrienes
Allergic Bronchopulmonary Mycosis	Adult onset Allergy to mold Pronounced mucus production	Markedly elevated total IgE Mold-specific IgE Eosinophilia
Severe Late-Onset Hyper-eosinophilic	Adult onset Severe exacerbations Less atopic Sinusitis Nasal polyps	Eosinophilia
Exacerbation-prone	Frequent exacerbations Sinusitis GERD	Eosinophilia
Neutrophilic	Adult onset Variable severity	Elevated neutrophils in blood and sputum
Obesity-Induced, non-eosinophilic	Adult onset Obesity Predominantly female Very symptomatic	Lack of Th2 biomarkers Elevated IL-6 Elevated Leptin
Paucigranulocytic	Mild and severe	Lack of airway inflammation
Asthma with smoking	Current or former tobacco smoke exposure Worse quality of life and more symptoms Corticosteroid insensitivity	Less eosinophilic, more neutrophilic

Table 2. Biomarkers can perform multiple functions towards diagnosis and treatment of severe asthma.

Biomarker	Diagnostic	Predictive	Dynamic	Stable
IgE		x	x	
Specific IgE	x			
Eosinophils	x	x	x	
Neutrophils	x			
Fraction of exhaled nitric oxide		x	x	
Eosinophil peroxidase	x	x	x	
Bromotyrosine	x	x	x	
Stem cell factor/KIT		x		
Urinary Leukotriene E4	x	x	x	
Periostin		x		
Single Nucleotide Polymorphisms		x		x