

BIOMARKERS IN THE DIAGNOSIS AND THERAPY OF ALLERGIC DISEASES AND ASTHMA

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Abstract

Modern healthcare requires a proactive and individualized response to diseases, combining precision diagnosis and personalized treatment. Accordingly, the approach to patients with allergic diseases encompasses novel developments in the area of personalized medicine, disease phenotyping and endotyping and the development and application of reliable biomarkers. A detailed clinical history and physical examination followed by the detection of IgE immunoreactivity against specific allergens still represents the state of the art. However, nowadays, further emphasis focuses on the optimization of diagnostic and therapeutic standards and a large number of studies have been investigating the biomarkers of allergic diseases, including asthma, atopic dermatitis, allergic rhinitis, food allergy, urticaria and anaphylaxis. Various biomarkers have been developed by omics technologies, some of which lead to a better classification of the distinct phenotypes or endotypes. The introduction of biologicals to clinical practice increases the need for biomarkers for patient selection, prediction of outcomes and monitoring, to allow for an adequate choice of the duration of these costly and long-lasting therapies. Escalating healthcare costs together with questions on the efficacy of the current management of allergic diseases requires further development of a biomarker-driven approach. Here, we review biomarkers in diagnosis and treatment of asthma, atopic dermatitis, allergic rhinitis, viral infections, chronic rhinosinusitis, food allergy, drug hypersensitivity and allergen-immunotherapy with a special emphasis on specific IgE, microbiome and epithelial barrier. In addition, EAACI guidelines on biologicals are discussed within the perspective of biomarkers.

Keywords

Biomarkers, asthma phenotypes and endotypes, allergic rhinitis, food allergy, allergen immunotherapy, biologicals.

Introduction

Allergic diseases represent a group of conditions caused by hypersensitivity of the immune system to allergens present in the environment.¹ These diseases include food allergies, asthma, atopic dermatitis (AD), allergic rhinitis (AR), conjunctivitis, and other co-morbidities and complications, such as chronic rhinosinusitis with or without nasal polyposis (CRSwNP).²⁻⁴ The 100 year old personalized allergen-specific management of allergic diseases has been a particular advantage in our specialty contributing to the early awareness of personalized approaches and precision medicine. The use of multiple omics, big data, and systems biology have demonstrated a profound complexity and dynamic variability and enabled the discovery of novel biomarkers.⁵

Biomarkers represent measurable indicators linking an underlying pathway to a phenotype or endotype of a disease.⁶⁻⁸ Regrettably, current biomarkers are not precise in selecting the specific endotype that will respond to a targeted treatment. A good example is the observation that blood eosinophilia predicts therapeutic response to all currently available or future-targeted interventions in severe asthma (i.e. anti-IL-5, IL-4 /IL-13, anti-IgE-targeted treatment, CRTH2 antagonists).^{8,9} Precision medicine in allergic diseases demands accurate diagnoses¹⁰, which mostly rely on the combination of the clinical history and respective gold standards, which are all subject to the operator, observer and interpretation variability^{11,12}. Some of the approaches are time-consuming, and *in vivo* challenges may result in severe side effects and, in rare cases, even death. Therefore, the discovery, validation and clinical applicability of molecular biomarkers become increasingly important.¹³

The cellular, biochemical, or molecular changes in allergic patients which are measurable in blood, sputum or nasal secretions can be considered as biomarkers.¹⁴ These biomarkers are used for disease diagnosis, selection of targeted therapy, disease monitoring and prediction of prognosis.¹⁵ Except for the well-known biomarkers (e.g. IgE, blood or sputum eosinophilia, fractional exhaled nitric oxide [FeNO]),¹⁶⁻¹⁸ research focusing on pro-inflammatory mediators, genes, epithelial barrier and microbiomes are now emerging, which highlight more potential biomarkers for allergic diseases.^{19,20} Some of the biomarkers showing a strong ability in identifying disease endotypes or phenotypes may also act as therapeutic targets.^{21,22} This article reviews the biomarkers identified to date and potential targeted therapy in allergy. In addition, it briefly reviews the biomarkers taking place in EAACI guidelines.

Biomarkers in asthma

In the past decades, it has been increasingly recognized that asthma is a highly heterogeneous disorder with different underlying mechanisms and pathways translating into variable responses to standard treatment across the different subsets or clinical phenotypes.^{23,24} Unbiased approaches and cluster analyses identified four major clinical phenotypes, *i.e.* : (1) early-onset allergic asthma, (2) early-onset allergic moderate-to-severe asthma, (3) late-onset non-allergic eosinophilic asthma, and (4) late-onset non-allergic non-eosinophilic asthma.²⁵ The late-onset subsets tend to present as more severe or more difficult-to-treat than early onset asthma. To promote an adequate treatment strategy, asthma can be subdivided into Type 2 (high) and non-Type 2 (or Type 2 low) endotypes based on their underlying inflammatory pathways.²⁶ As part of a more general syndrome often including nasal polyps with or without NSAID-Exacerbated Respiratory Disease (NERD),^{27,28} Type2 asthma currently comprises the best defined asthma subset(s) in terms of underlying immunopathology, corresponding biomarkers⁸ and targeted treatment options with biologicals and small molecules.^{13,29,30}

In parallel with the available (targeted) treatment options, biomarkers have been validated along the corresponding inflammatory pathways aimed for pheno/endotyping and to guide treatment for Type 2 asthma.⁸ Clinically applicable point-of-care biomarkers include blood eosinophils, or whenever feasible, sputum eosinophil counts, serum specific IgE and FeNO.^{8,31} Although overlapping in type 2 biomarkers may

occur within patients, all biomarkers represent different aspects of the type2 inflammatory pathways with IgE associating to allergy, while FeNO is linked to the IL-13 pathway and epithelium-derived inflammation.⁸ Based on these point-of-care biomarkers in combination with clinical characteristics (age of onset, comorbidities, exacerbations, need for maintenance systemic corticosteroids) and physiological parameters (lung function, airway hyperresponsiveness, etc.), current guidelines have now adapted algorithms which can help to predict a response to (targeted) treatments and/or can be used to monitor the subsequent treatment response.^{23,32,33} In this context, some confounders have been recognized for the existing point-of-care biomarkers, *i.e.* , for FeNO mainly related to ICS use, smoking, dietary nitrate intake, virus infections and bronchoconstriction, while for blood eosinophils circadian variation, parasites and systemic corticosteroids were found to be the most common perturbing factors.^{23,34} In this context, a clinically relevant issue has been raised, *i.e.* , whether “true” non-Type 2 (non-eosinophilic) asthma really exists among patients with severe asthma, given the fact that high-dose inhaled and oral corticosteroids may potentially mask pre-existing Type 2 inflammation interfering with its biomarkers, especially blood eosinophils and FeNO.^{35,36} Currently ongoing corticosteroid-tapering studies (RASP-UK) in patients with non-Type 2 severe asthma should answer this question. Alternatively, airway neutrophilia (“neutrophilic asthma”) may often reflect (subclinical) airway infection.³⁶⁻³⁸

In contrast, for non-Type 2 asthma which is by default defined as asthma without type2 biomarkers, underlying pathways and, hence, clinically applicable biomarkers and targeted treatment options are still largely under exploration.^{36,39} Apart from most patients with mild clinically stable asthma,^{26,40} clinical phenotypes frequently associated with non-Type 2 asthma include very late-onset asthma (women), obesity-associated asthma, smoking-associated neutrophilic asthma and pauci-granulocytic asthma. Although generally based on increased sputum neutrophils or absence of normal levels of (sputum) eosinophils and neutrophils (paucigranulocytic) with normal levels of other Type 2 markers, the diagnosis of non-type2 asthma is difficult to establish as often based on cross-sectional data potentially affected by confounders including respiratory infections or anti-inflammatory therapies.³⁶ In the absence of targeted biologicals, in non-Type 2 asthma treatable traits should be targeted,⁴¹⁻⁴³ *e.g.*: obesity, smoking habits, psychological aspects, neutrophilia as a potential indicator of respiratory infection and airway narrowing or airway hyperresponsiveness as an indicator of ASM dysfunction, while corticosteroids may not be effective and should be tapered off (Figure 1).²³

In conclusion, despite substantial progress in our understanding, applicable biomarkers and targeted treatment options for Type 2 asthma, further characterization of molecular pathways by omics technologies,⁴⁴⁻⁴⁶ sophisticated imaging⁴⁷ and innovative anatomical approaches⁴⁸ should help to further unravel the complexity of asthma and to define reliable (composite) biomarkers and therapeutic strategies for patients non-responsive to currently available (targeted) treatment options including non-Type 2 asthma.

Biomarkers linked to Microbiome and Asthma

An enormous variety of microbes colonize mucosal body surfaces and these microbes are organized within complex community structures, utilizing nutrients from other microbes, host secretions and the diet. Modern lifestyles, medications and social interactions have fundamentally altered and disrupted the human microbiome metacommunity and, as a consequence, risk of immune-mediated diseases such as allergy and asthma.^{49,50} The mechanisms that mediate host-microbe communication are highly sophisticated and are being intensely investigated by many research groups across the world. However, there is accumulating evidence that microbiome composition and metabolic activities within the gut and the airways can influence asthma pathogenesis.⁵¹⁻⁵³ Here, we summarize some of the key recent findings that identify specific microbes or associated metabolites that may be useful as biomarkers to predict asthma risk, asthma severity or guide existing or novel therapies.

Alterations in the gut microbiota within the first year of life have been associated with asthma risk in multiple birth cohort studies.¹⁹ The lower relative abundance of genera including *Lachnospira* , *Veillonella* , *Faecalibacterium* , *Rothia* , *Bifidobacterium* and *Akkermansia* in the gut during early life have been associated with the development of asthma.^{54,55} While fewer studies have examined pre-school children (2-4 years of age), a

recent study in this age group demonstrated that certain bacterial genera within the gut were still associated with wheezing (*Collinsella* and *Dorea*) or subsequent development of asthma (*Gemmiger* and *Escherichia*).⁵⁶ In addition to the gut microbiota, studies are also showing changes in the microbial populations of the airways. Microbial diversity and the relative abundances of *Veillonella* and *Prevotella* in the airways at age one month were associated with asthma by age 6 years.⁵⁷ Interestingly, higher relative abundance of these bacteria was associated with reduced TNF- α and IL-1 β and increased CCL2 and CCL17 within the airways. A switch from a *Corynebacterium* and *Dolosigranulum* cluster in the upper-airways to a *Moraxella* cluster was associated with a higher risk of severe asthma exacerbation in children with asthma.⁵⁸ In adults, increased relative abundance of the phylum *Proteobacteria* (including *Haemophilus*, *Comamonadaceae*, *Sphingomonadaceae*, *Nitrosomonadaceae*, *Oxalobacteraceae* and *Pseudomonadaceae*) is often associated with asthma or with worse asthma control.⁵⁹ Microbial changes within the gut, upper and lower airways of adult asthma patients are magnified in obese asthma patients and in those with severe disease.⁶⁰ Bronchoalveolar lavage levels of IL-5 and eosinophils correlated with a variety of microbes within the airways. Of note, severe asthma negatively correlated with fecal *Akkermansia* levels and oral administration of *Akkermansia* to murine models significantly reduced airway hyper-reactivity and airway inflammation (Figure 2).

In addition to microbiota composition, microbial metabolites may also be useful biomarkers in asthma. The fecal metabolome of children at increased risk of asthma contained increased levels of pro-inflammatory metabolites, among which 12, 13 DiHOME was able to induce IL-4 production in CD4⁺T cells and decreased the abundance of Tregs.⁵⁵ High levels of short chain fatty acids (SCFAs), such as butyrate and propionate, at one year of age were associated with reduced risk of atopic sensitization and asthma by school age.⁶¹ Multiple immune modulatory effects have been described in murine models for SCFAs, which include the promotion of Treg development and the inhibition of pulmonary ILC2 functions and subsequent development of airway hyper-reactivity.⁶² In adults, an increased abundance of histamine secreting bacteria were observed within the gut of patients with asthma, while disease severity correlated with high levels of the histamine secreting microbe *Morganella morganii*.⁶³ Murine models have demonstrated that bacterial-derived histamine within the gut can influence inflammatory responses within the lungs.⁶⁴

In the future, the application of recent advances in metagenomic sequencing technologies and bioinformatics will likely lead to the identification of novel functional traits and metabolites within the gut and airway microbiome of asthma patients.⁶⁵ In addition, future asthma studies should include the microbiome as potential biomarkers that predict or associate with responses to biologics, as already observed for *Faecalibacterium*, *Bifidobacterium* and *Akkermansia* that associate with immunotherapy responses in certain groups of cancer patients.⁶⁶

Skin bacterial microbiome as clinical biomarker in atopic eczema

Diagnosis of atopic eczema (AE) severity is still today a semi-quantitative clinical score based on subjective information from the patients together with doctor's subjective estimation on severity of the skin lesions and patient's history of itching and sleep loss.^{67,68} In the era of targeted therapy, and thus more complex therapy management requirements, more objective criteria are urgently needed. A diagnostic biomarker would also have the potential to differentiate between the different subgroups of AE. AE, likewise, lacks a prognostic biomarker: AE⁶⁹ affects 30% of children but only 5% of adults – thus the question remains who keeps the disease, who emerges from it, and who embarks on the full career of an atopic individual. Skin microbiome dysbiosis, measured either as microbiome diversity or more reliably as abundance of *S. aureus*, was shown to correlate with both the AE clinical score and the expression of skin barrier molecules.⁷⁰ It is still a matter of scientific debate whether the relative frequency of various bacteria (e.g., *S. aureus* frequency as obtained from 16S based NGS) is an adequate biomarker or rather the absolute microbial load (e.g., as obtained from qPCR) is better. Furthermore, is it enough to quantify the DNA abundance from non-standardized amounts of skin samples, or rather is the absolute microbial load of standardized skin samples needed?

S. aureus is important for AE pathogenesis even though it is still a matter of debate whether overgrowth of *S. aureus* is a cause or a result of barrier disruption.⁷¹ Thus, microbiome analysis, at least on the species level, but ideally on the strain level, would enable us to identify personalized biomarkers. This highlights

a methodological drawback, as currently tools for annotation on species level are not reliable. Furthermore, the current methods for skin microbiome measurement are not standardized; testing the same material in different laboratories is prone to give different results. For skin microbiome to be used clinically as a biomarker, standardized methodology needs to be developed and validated so it can be reliably used across different laboratories.⁷² Combinatory biomarkers between skin microbiome and biomarkers of type 2 immunity would also be of great potential.⁷³ Recently, biofilm propensity of *S. aureus* skin isolates – as a cause and possible target has become more and more of a central issue.⁷⁴ Thus, resolving the enigma of skin-microbe interaction as a function of skin homeostasis has to take more players into the.⁷⁵

In conclusion, skin bacterial microbiome shows great potential to be used as a clinically important biomarker for atopic eczema. To reach this aim, we need to perform prospective clinical trials and large longitudinal registries that include skin microbiome testing. Furthermore, it is critical to advance standardized and foremost quantitative methodologies for skin bacterial microbiome analysis. New technologies, such as single molecule real time , need to be further developed and tested in order to improve skin microbiome analysis with higher accuracy and/or longer sequencing length. Collaboration between large academic consortia and pharmaceutical companies is essential for such endeavors.

Biomarkers in diagnosis of allergic rhinitis

With deeper insights into mechanisms of AR, novel biomarkers have recently been identified in its diagnosis. Furthermore, several immune cells and mediators, genes and metabolites have been studied to explore their potential utilization in diagnosis of AR.

Immune cells and mediators

Several potential immune cells (granulocytes, lymphocytes, etc.) and mediators might serve as diagnostic biomarkers of AR.^{14,76} Izuhara and colleagues have reported that induction and increased expression of periostin reflect type 2 inflammation and remodelling, and could be regarded as an emerging biomarker for allergic diseases.⁷⁷ One study has demonstrated that allergen-induced surface CD203c expression on basophils exhibits a time-of-day-dependent variation, and allergen-specific basophil reactivity shows daily variations depending on circadian clock activity in basophils, which could partly be responsible for temporal symptomatic variations in AR.⁷⁸ One recent study has suggested that circulating group 2 innate lymphoid cells (ILC2s) may play an important role in the pathology of AR, particularly as increased levels of ILC2s correlated with symptom scores and IL-13 levels in house dust mite (HDM)-sensitized AR patients,⁷⁹ and these cells produce large amounts of proinflammatory mediators in response to Th2 cytokines.^{80,81} Indeed, a more recent study by Tojima and colleagues found that prostaglandin D2 (PGD2) and cysteinyl leukotriene (cysLTs) might induce ILC2s to produce Th2 cytokines such as IL-5 and IL-13.⁸² Similarly, ST2-expressing pathogenic memory T helper (Th) 2 cells, producing substantial amounts of IL-33-induced IL-5 and IL-13, have been shown to be linked to sensitization and the onset and progression of AR (Figure 3).⁸³

Genes

Epigenetic modifications, particularly DNA methylation and microRNAs (miRNAs), might have the potential to identify AR patients. One recent study has demonstrated changes in DNA methylation in tryptase gamma 1 (*TPSG1*), schlafen (*SLFN12*), and mucin 4 (*MUC4*), following controlled allergen challenge, and suggested that baseline epigenetic status may act as a potential biomarker for AR symptom severity.⁸⁴ Another recent study has indicated that nasal epigenome associated with asthma, FeNO and IgE may serve as a sensitive biomarker of asthma, allergy and airway inflammation in children.⁸⁵ Other studies have reported that subsets of circulating miRNAs are solely expressed in the blood of patients with AR and asthmatics and may therefore be used as non-invasive biomarkers for diagnosis and characterization of these diseases.^{86,87}

Metabolites

Metabolites have also been proposed as biomarkers for AR. A very recent study of serum metabolomics has demonstrated that at least nine metabolites (13(S)-HPODE, bilirubin, leukotriene D4, hypoxanthine, L-stercobilinogen, N-succinyl-L-diaminopimelic acid, chlorophyll b, 15-hydroxyeicosatetraenoic acid, and urate)

were significantly altered in the serum of AR patients, and therefore may provide a better understanding of the metabolic pathways involved in the aetiology of AR.⁸⁸ Additionally, decreased serum lactoferrin level has been reported to be associated with the phenotype of *Dermatophagoides pteronyssinus* (*Der p1*)-sensitive AR, and in combination with serum *Der p1*-specific IgE level, may serve as a serologic biomarker for early detection of AR.⁸⁹

Biomarkers in therapy of allergic rhinitis

Currently, optional therapeutic measures for AR involve patient education, environmental control, pharmacotherapy, allergen immunotherapy (AIT), and surgery.^{90,91} Traditional medications include nasal corticosteroids, antihistamines, mast cell stabilizers, decongestants, etc. MP29-02, a combination of nasal corticosteroid and antihistamine, is a novel topical medication which has proved to be effective in reducing nasal hyperreactivity and nasal mediators such as substance P, in patients with AR.⁹² As ILC2s have been shown to produce significant amounts of proinflammatory mediators in response to epithelium-derived cytokines^{80,81} and PGD2 and cysLTs⁸² in AR patients, agents targeting the ILC2s and the mediators activating these cells have become targets for therapy. Rittchen and Heinemann have recently reviewed the central role of hematopoietic PGD2 synthase in allergic inflammation and indicated that PGD2 signaling might be a promising therapeutic target for AR, as PGD2 can activate Th2 cells, eosinophils and basophils.⁹³ Indeed, a randomized controlled phase II clinical trial has recently demonstrated that ONO-4053, a novel prostaglandin D receptor 1 antagonist, was more effective than pranlukast, a leukotriene receptor antagonist, in treating patients with seasonal AR.⁹⁴ Most recently, emerging studies have focused on biologics for treating allergic diseases; especially severe, uncontrolled asthma and AD, as well as AR.^{95,96} To date a high number of specific biologics targeting markers of Th1/2/17 inflammation have been introduced; with more under development.^{95,97} In particular, targeting IgE by omalizumab, a recombinant humanized anti-IgE antibody, has been shown to significantly improve symptoms in patients with inadequately controlled AR.⁹⁸ Furthermore, combining omalizumab with sub-cutaneous immune therapy (SCIT) in patients with SAR and comorbid seasonal allergic asthma has been shown to lead to greater clinical improvements in AR and lung function than SCIT alone.⁹⁹ Similarly, dupilumab, a biologic which targets IL-4R α to block the activity of both IL-4 and IL-13, has been shown to provide nasal symptom relief in patients with uncontrolled asthma and comorbid AR.¹⁰⁰

Biomarkers of viral infections in exacerbation of allergic rhinitis and asthma

Text box: Respiratory viral infections may exacerbate chronic airway inflammatory diseases, including allergic inflammation through both Type 2 (e.g., IL-25, IL-33 and TSLP) and non-type 2 (e.g., IFN types I and III, RIP3, OSM, MCIDAS) mechanisms.

Over the past decade, our understanding of immunological mechanisms underlying allergic diseases such as AR has substantially increased through the discovery of T helper (Th) subsets and their importance in allergic inflammation. Emerging data now provide new insights on the type 2 immune response that is an immune response to allergens and involves Th2 cells, type 2 B cells, ILC2s, type 2 macrophages, a small fraction of IL-4-secreting NK cells, IL-4-secreting NK-T cells, basophils, eosinophils and mast cells.¹⁰¹ At the same time, it has also been established that viral infection synergizes with allergic inflammation causing more severe exacerbations and symptoms compared to both conditions alone.^{102,103} There is increasing evidences that most respiratory viral infections could trigger or exacerbate chronic type 2 inflammatory responses via excessive release of chemokines and cytokines into the airways.¹⁰⁴⁻¹⁰⁶ While much of these studies focuses on lower airway inflammatory diseases instead of AR; insights from these studies can be applied to ongoing studies of viral induced AR exacerbations and the search for its associated markers.

Like other chronic airway inflammatory diseases, AR patients also suffer from altered responses and potentially increased susceptibility towards viral infection.¹⁰⁷⁻¹⁰⁹ This is similarly due to the reduced type III interferon response, which is crucial against incoming viral infection in the upper airway.¹⁰⁷⁻¹⁰⁹ Hence, markers for virus induced AR exacerbation may have significant overlap with findings from other inflammatory airway diseases. Proinflammatory cytokines such as TNF- α , IL-4, IL-5, IL-13, RANTES, Eotaxin, TSLP,

IL-25 and IL-33 are usually expressed at higher concentrations in chronically inflamed airways, some of which are also found in AR.^{101,110,111} These cytokines can be further triggered directly or indirectly by viral induced IFNs, cytokines and chemokines. Infections such as RSV can even further shunt antiviral responses towards a more type 2 centric response.¹¹²⁻¹¹⁶ In addition, the discovery of ILC2s, a group of lymphoid cells, further emphasized the role of epithelial alarmins IL-25, IL-33 and TSLP in viral induced exacerbation.¹¹⁷ During viral infection, these three cytokines were secreted in response to epithelial injury.¹¹⁸⁻¹²⁰ They then activate ILC2s to further secrete type 2 cytokines IL-4, IL-5 and IL-13, aggravating type 2 inflammation in the airway, resulting in acute viral exacerbation. Interestingly, these factors are not released readily and do not activate ILC2s in virus-infected healthy individuals, but effectively augment type 2 responses in chronically inflamed airways (Figure 4).^{121,122}

In addition, respiratory viral infections may also exacerbate chronic airway inflammatory diseases, including allergic inflammation through other non-type 2 mechanisms, in which other markers can also be used as an indicator of these exacerbations. Viral infections can lead to the destruction of epithelial barrier and disruption of mucociliary function due in part to cell death in the virus infected epithelium. Hence, markers for cell death (e.g. RIP3) and mucociliary dysfunction (e.g. MCIDAS) constitute part of the viral exacerbation repertoire.¹¹² Viral infection also causes increase in factors such as OSM and ANGPTL4 which disrupts tight junctions leading to increased allergen invasion and their contact with immune cells in the sub-epithelium region, thereby exacerbating allergic symptoms.^{123,124} In addition, miRNAs are increasingly implicated in the mis-regulation of inflammatory responses and several of them are found to be dysregulated in an inflamed airway. For example, expressional changes of miRNAs such as miR-21 may coincide with viral infection responses and hence linked to viral induced exacerbations.¹²⁵ Finally, an emerging field of bioenergetics and mitochondrial function may also contribute to the mechanism of viral induced exacerbation in AR. Oxidative stress and mitochondrial dysfunction from viral infection may induce increased inflammation and thus ROS and its associated markers may potentially serve as key markers for viral exacerbation.^{126,127}

Biomarkers in chronic rhinosinusitis

CRS, like asthma, can be divided into different pheno- and endotypes. The mostly used phenotype is the division into CRS with and without nasal polyps (CRSwNP and CRSsNP), although many other pheno- and endotypes are known.^{28,128} However, recently, the options to treat with biologicals have put more emphasis on markers of Th2 disease irrespective of the presence of nasal polyps. The first type 2 targeting biologic anti-IL4R α (Dupilumab) has entered the market for CRSwNP patients and others like, anti-IgE, anti-IL5 and anti-IL5R α may follow shortly.¹²⁹⁻¹³¹ Cluster analysis of CRS has shown that CRSsNP and CRSwNP are not dichotomous but instead have overlapping inflammatory signatures with type 2 inflammation as the predominant endotype mainly in CRSwNP but also CRSsNP especially in western parts of the world. Interestingly, some patients with CRS express a mixture of two or more inflammatory endotypes.^{22,132,133} The recently published EPOS2020 proposes a new clinical classification based on the disease being localized (often unilateral) or diffuse (always bilateral). Both these groups can be further divided into Th2 or non-Th2 disease.⁴

In the very near future, it may be possible to offer personalized medicine for CRS patients where treatment is based on molecular biomarkers for the endotype or sub-endotype activated in an individual patient.^{27,134} The major challenge is to find reliable biomarkers that define Th2 inflammation and predict reaction to treatment. Ideally, these biomarkers should be supported by a body of evidence clarifying the biological significance, be quantifiable in a cost-efficient way and be easily measurable, preferably in blood or nasal secretion.¹⁴ Potential biomarkers could be eosinophils, neutrophils,^{135,136} IgE,¹³⁷ Th2 cytokines,¹³⁸ innate (epithelial) cytokines,^{111,137,139} but also phenotypical phenomena like smell loss,¹⁴⁰ asthma and response to systemic corticosteroids.¹³⁴ Contrary to FeNO in asthma, nasal NO has not been shown to be helpful to identify the T2 endotype because the main source of production of nasal NO are the sinuses that are closed off when CRS occurs.¹⁴¹ The main biomarkers used at the moment to define Th2 disease are eosinophils, IgE levels, and in some more specialized centres periostin and/or IL-5. There is quite some evidence showing that tissue and blood eosinophils are a reasonable surrogate marker for Th2 disease, and that blood

eosinophils are a reasonable biomarker to predict eosinophilic CRS with or without nasal polyps.¹³³ On the other hand, low tissue and serum eosinophilia, and absence of tissue squamous metaplasia may predict a CRS phenotype suitable for a trial of long-term macrolide therapy when surgery and topical therapy has failed.¹⁴² Unfortunately, recent large studies with monoclonal antibodies directed to Type 2 endotypes have not found reliable biomarkers to predict response to treatment.^{129,130,143-145} As in asthma,⁸ we need large, maybe real-life studies to find better predictors to identify responders to biological treatments. For now, our treatment decisions still heavily rely on phenotypical characteristics like smell loss, asthma, and response to surgery and systemic corticosteroids.^{4,27}

Biomarkers in food allergy

Apart from clinical determinants of food allergy and the respective gold standard, the oral food challenge, biomarkers represent an area of extensive research. In food allergy the focus is on genetic risk factors, allergen-specific and non-specific humoral and cellular biomarkers.¹⁴⁶⁻¹⁴⁸

Although genetic markers for food allergy are not yet at the level of clinical relevance, genes linked to HLA-genes, and more importantly to epithelial integrity and consequently reduced barrier function like filaggrin, SPINK5,¹⁴⁹ and SERPINB7, are linked to eczema development and also food allergy.¹⁵⁰⁻¹⁵⁶ Given the importance of the exposome in allergy development, epigenetics may even play a more important role. Promising results in the context of peanut allergy await replication in larger cohorts.^{157,158} Regulation at another level has been linked to the microRNA 193a-5p. It is involved in the post-transcriptional regulation of IL-4 and downregulated in PBMCs from milk allergic children.¹⁵⁹ Due to the importance of barrier (dys)function in atopic diseases¹⁶⁰⁻¹⁶² measurement of skin integrity may be a very important tool to identify high risk populations. Electrical impedance spectroscopy, successfully tested in rodents, may be capable of assessing this biomarker also in humans.¹⁶³

Allergen extract-based testing methodologies like skin prick test (SPT) and/or specific IgE (sIgE) tend to over-diagnose food allergy. Thus, more specific approaches focusing on specific allergens (see Section on allergens) and epitope-specific antibody response patterns are explored.¹⁶⁴ Diversity of IgE-binding to linear epitopes correlated with the severity of peanut and milk allergy¹⁶⁵⁻¹⁶⁷ and IgG4 and IgE antibody binding to specific milk epitopes was stronger and more diverse in children who do not outgrow their milk allergy.¹⁶⁶ By measuring IgE and IgG4 responses with bead-immobilized milk epitopes and applying machine learning approaches, non-reactivity to baked milk could be predicted twice as successful as by conventional approaches.¹⁶⁸⁻¹⁷⁰ The soluble high affinity IgE receptor (FcεRI) may also act as a biomarker for IgE mediated pathologies in a less allergen independent way.¹⁷¹

Although allergen specific T-cells are extremely rare, they display a pronounced Th2 type in allergic individuals.^{172,173} A subset of allergen-specific memory Th2 cells called T_H2a cells, which are CD45⁺CD27⁻CD45RB⁻CRTH2⁺CD161⁺CD49⁺, has been discovered. They are almost exclusively found in allergic individuals, secrete IL-5 and IL-9 and within that group the percentage of type 2 cytokine double, triple, or quadruple positive cells is higher compared to Th2 cells. Moreover, mRNA expression of IL-25-, IL-33- and TSLP-receptors is higher.¹⁷⁴

Our understanding of B-cell regulation has significantly evolved over the last few years.¹⁷⁵ Evidence is pointing towards an extreme rarity of IgE memory B-cells in peripheral blood of allergic individuals which may be absent in non-peanut allergic individuals.¹⁷⁶ New therapeutic and diagnostic options opened up from data on allergen specific monoclonal antibodies that were generated via single-cell sorting of allergen-specific memory B-cells.^{177,178} B-cell IgE antibody mutational maturation has been associated with barrier dysfunction.¹⁷⁹ Recently, the co-emergence of short lived IgE plasmablasts and IgG memory B-cells early in grass AIT in the absence of memory IgE⁺ B-cells has been reported.^{180,181} Both subsets shared clonotypes supporting the existence of pools of specific B-cell subsets e.g. from IgG1 positive allergen specific B-cells upon switch factors and stimulation as demonstrated in mice.^{180,182} Yet many questions on the emergence of IgE producing cells and their regulation have to be answered and new biomarkers in this context will arise.

Functional tests that simulate allergen exposure *in vitro* like the basophil activation test (BAT) offer the

possibility to assess allergen induced IgE crosslinking. The BAT suggests adding significant diagnostic value to IgE based test methods.¹⁸³⁻¹⁸⁵ Promising results on the usage of passive sensitization strategies, mast-cell lines¹⁸⁶ or pre-cursors¹⁸⁷ have been reported.

There is still a significant need to develop biomarkers to diagnose and predict anaphylaxis to prevent near fatalities and fatalities.^{188,189} Beyond tryptase, which can be a very good marker in the emergency setting when baseline values exist^{76,190} predictors of life threatening reactions which can be measured on a routine basis or in multi-center trials are still missing.

There is the hope that the expanding array of novel mechanistic and diagnostic biomarkers provide the toolkit to develop algorithms or machine learning approaches to diagnose food allergy and predict treatment outcomes (Figure 5).

Biomarkers in drug hypersensitivity

Drug hypersensitivity reactions include those mediated by a specific immunological mechanism and those non-specific immune mediated (Figure 6).¹⁹¹

Immunologically-mediated specific reactions

These reactions are classified into immediate reaction (IR) and non-immediate reactions (NIRs) depending on whether they occur within 1-6 hours or later after the drug intake. The first group is mostly associated with an IgE-mediated mechanism and the latter with a T-cell dependent type.^{191,192}

Skin tests (STs) are useful biomarkers for IRs to beta-lactam (BL).^{14,191-194} However, their sensitivity based on the classical antigenic determinants has decreased over the last decades¹⁹⁵ possibly due to the changing patterns of consumption (e.g. amoxicillin-clavulanic acid is replacing penicillin).¹⁹³ Interestingly, one antigenic determinant recognized by most patients with confirmed reactions to clavulanic acid has been recently identified.¹⁹⁶ Therefore, amoxicillin and any suspected BL must be included when performing STs.^{192,195,197,198} For other drugs and for NIRs, the value of STs is very limited.^{14,192,193,199}

Regarding *in vitro* tests, during the acute phase of the reaction, serum tryptase is the most valuable biomarker for confirming mast cell activation in IR.¹⁹¹ The expression of granzyme B and granzysin in blood cells may be useful for detecting lymphocyte activation in severe NIR.²⁰⁰ At the resolution phase, immunoassays are used in IRs to determine sIgE, although the sensitivity is lower than for STs^{191,201,202} and only commercially available for limited drugs. For BLs, it shows a variable sensitivity (0-50%),^{191,192,201} with the possibility of inducing false positive results when testing for Penicillin-V.²⁰³ The value of BAT in IR has been proven for BL and quinolones, giving a sensitivity up to 55%^{204,205} and 83%,^{199,206} respectively. The sensitivity of both sIgE and BAT correlates with severity reaction²⁰¹, decreases with time,^{205,207} and depends on the activation basophil marker assessed, e.g. the detection of CD203c increases BAT sensitivity although remaining low (36.4%).^{206,208}

The value of lymphocyte transformation test (LTT) in NIRs has shown to be unsatisfactory.^{14,191} The Tim3/galectin-9 axis functions as a checkpoint inhibitor for Th1 cells. Interestingly, Th1 cells and dendritic cells of patients with drug-induced maculopapular exanthema expressed lower levels of Tim3/galectin-9 at baseline compared with tolerant individuals.²⁰⁹ This observation might help identify subjects at risk of NIRs.

Immunologically-mediated non-specific reactions

The most important group in this type of reactions is cross-intolerance to NSAIDs (CI),^{210,211} in which patients react to NSAIDs from different pharmacological groups related to its COX-1 inhibitory activity.^{191,210} CI has been classically divided into phenotypes with exclusive skin involvement (NSAIDs-exacerbated cutaneous disease (NECD) and NSAIDs-induced urticarial-angioedema (NIUA)) or with exclusive respiratory involvement (NSAIDs-exacerbated respiratory disease, NERD).²⁸ Nevertheless, novel data indicate that almost 30% of patients with CI can experience blended reactions, especially involving skin and airways.²¹² Genetic predisposition might account for the burden of some CI phenotypes (e.g. variants of *GNAI2* in NIUA) (Figure 7).²¹³

As the underlying mechanism is related to arachidonic acid metabolism, potential biomarkers focus on determining leukotriene and prostaglandin metabolites. Therefore, urinary LTE4 has been reported as a useful biomarker to distinguish between different sub-phenotypes in NERD,²¹⁴ between NERD and aspirin-tolerant asthma²¹⁴ and phenotypes with skin involvement (NIUA and NECD).²¹⁵ Sensitivity and specificity of urinary LTE4 for identifying NERD has been reported to range from 0.55-0.81 and 0.77-0.82, respectively, which depends on the detection method used.²¹⁶ Serum LTE4 and LTE4/PGF2 ratio have also been reported as potential *in vitro* biomarkers for NERD²¹⁷ and urinary PGF2 for NIUA and NECD (Table 1).²¹⁵

Table 1

Biomarker	Disease	Reference
LTE4	Aspirin-sensitivity in asthma	216,217
LTE4/PGF2 ratio	Aspirin-sensitivity in asthma	217
LTE4 and PGF2	NIUA and NECD	21
Tryptase	Acute phase of IgE mediated reactions	191,201
Skin tests	Immediate and Non-immediate reactions to drugs	193
Specific IgE	Immediate reactions to betalactams	202
Basophil activation	Immediate reactions to betalactams Immediate reactions to FQ	204,206
lymphocyte transformation test	Non immediate reactions	201
granzyme B and granulysin	Stevens-Johnson syndrome	200

Biomarkers in immune monitoring of allergen-specific immunotherapy

Allergen immunotherapy (AIT) is the only therapy that can alter the natural course of immune responses to specific allergens, directing it towards desensitization and perhaps, towards tolerance. Routes of AIT include subcutaneous (SCIT), sublingual (SLIT), oral (OIT), or epicutaneous (EPIT).^{218 219} Licensed products are available for therapy of AR, allergic asthma, stinging insect hypersensitivity, AD.²²⁰⁻²²⁵ AIT for food allergy is currently investigational, however, phase 3 trials are promising.²²⁶ Current evidence indicates that immunological changes associated with AIT differ from permanent tolerance as in healthy non-allergic individuals. AIT-acquired desensitization is often temporary, waning with time, but can be maintained with regular exposure to the allergen.²¹⁹

Biomarkers that can identify responders, monitor treatment, predict durability of desensitization, and determine adverse event risk would aid clinical decisions and delivery of targeted and effective treatment. With the advent of data-driven “-omic” technologies, more potential biomarkers have been identified.²²⁷ Here, we briefly review the more promising candidates that are being evaluated for immune monitoring with an expanded number in Figure 8.²²⁸

Specific IgE and IgG

With the discovery of IgE in the 1960s, measurements of IgE have been a first step in diagnosis for atopic diseases. Both allergen-specific IgE (sIgE) and total IgE levels increase during the initial stages of AIT and subsequently decrease,²²⁹ however, decreases may not accompany a positive clinical outcome.²³⁰ Numerous studies indicate IgG1 and IgG4 levels increase with therapy but do not always differentiate between responders and non-responders. IgG4 increases during AIT may reflect compliance not clinical efficacy.²³⁰ sIgG4 is purported to be a blocking antibody by competing for allergen binding with IgE bound to Fcε receptors on mast cells and basophils, preventing degranulation. IgA and other subclasses of IgG may have similar blocking function.²³¹ Recent data support skewing towards IgG2 and IgG4 subclasses after SLIT for

temperate grass pollen.¹⁸¹ The IgG4/IgE ratio may monitor AIT progress and outcome, but has demonstrated conflicting utility.²³²⁻²³⁴ Flow cytometry-based assay (IgE-FAB) and a solid-phase assay enzyme-linked immunosorbent-facilitated antigen binding (ELIFAB) assay can determine IgE-inhibitory activity. Although robust with good clinical efficacy correlation for AIT, these technologies are complex with usage limited to specialized centers.^{230,235,236}

Immunophenotyping of immune cells

Allergic sensitization is associated with multiple changes in blood immune cells,¹⁷⁵ and high dimensional immunophenotyping using flow- and mass spectrometry (i.e. Cytometry Time of Flight; CyTOF) has contributed greatly to this identification.²³⁷ A study on bee venom AIT provided detailed characterization of allergen-specific B cells before and after bee venom tolerance in allergic patients and bee-keepers with Api m 1-specific B cells showing increased CCR5 expression after high-dose allergen exposure.²³⁸ In a study of OIT for milk allergy, researchers found, using flow cytometry, a significant increase in blood invariant natural killer cells and a shift from a type 2 T-helper (Th-2; i.e. IL-4, IL-13) to a Th-1 (ie IFN- γ) cytokine profile.²³⁹ Similar studies in other immunotherapy models can assist with determining potential biomarkers for monitoring AIT.

Recent evidence shows that AIT modulates the balance between circulating T follicular helper (Tfh) and regulatory cells (Tfr), with Tfr as a potential biomarker for AIT efficacy.^{240,241} Upregulation of the activated allergen-specific Tregs and downregulation of dysfunctional allergen-specific Treg cell subset, associated with improved clinical response, were recently described.²⁴² Responder status was shown to be associated with increased frequency of IgA- and IgG4-expressing allergen specific B cells, plasmablasts, and IL-10+ and/or IL-1RA+ Breg cells.²⁴³

AIT-induced T regulatory cells secreting IL-35 (iTR35) cells promote production of IL-10 from CD19+ B cells, Breg subsets and Tfr cells.²⁴⁴ Circulating Tfr cells share properties of memory cells and are distinct from their lymph nodes (LN) counterpart as they suppress B and Tfh cells with a much lower capacity, while circulating memory-like Tfh cells are more potent than LN effector Tfh cells.²⁴⁵ In addition, circulating memory-like Tfr cells persist for long periods, thus they could support the long-term immunomodulatory effect of peptide AIT. Functional evaluation of T regulatory cells via expression of Glycoprotein A repetitions predominant (GARP) and Special AT-rich sequence binding protein 1 (SATB1) is also interesting as a future potential biomarker. Tregs uniquely express GARP on their cell surface and GARP functions as a delivery system for latent TGF- β , which might augment the immunosuppressive role of Tregs on effector cells.²⁴⁶ GARP expression was described as an activation marker of parasitic infection induced Tregs that strongly suppress allergic inflammation, thus is a novel potential mechanistic pathway for AIT. SATB1 is a genome organizer protein expressed in a lineage specific manner in CD4+ T-cells. During the early Th2 cell differentiation, IL-5 expression is repressed through direct binding of SATB1 to the IL-5 promoter. Thus, SATB1 modulation might expand the impact of AIT on eosinophilic inflammation. In addition, SATB1-dependent Treg-cell specific super-enhancers activation is crucial for Treg cell lineage specification in the thymus

Basophil activation test

Blood basophils express high levels of Fc ϵ RI that have bound IgE. To determine sensitization, basophils are incubated *in vitro* with allergen, followed by examination of degranulation. Fusion of granules to the cell membrane leads to upregulation of surface markers CD63 and CD203c, which are indicative of specific IgE functional activity. In patients with AR undergoing SLIT, basophil activation decreased with clinical efficacy.²⁴⁷ Similar decreases occurred in patients undergoing peanut immunotherapy.²³⁴ However, in grass pollen SLIT, basophil activation did not correlate with clinical efficacy (Table 2).²⁴⁸

Soluble cytokines

With immunotherapy, there is redirection of Th2 phenotype towards a Th1 and Treg phenotype with decreases in Th2 cytokines (eg, IL-4, IL-19 13, IL-9) and upregulation of Th1 (eg, IFN γ), and regulatory

cytokines (eg, TGF β and IL-10).^{181,249} However, serum cytokine measurements are challenging due to low levels, which are often below the detection limit of current methods.²³⁰ Changes in cytokine production by CD4 T cells following AIT are assessable through *in vitro* stimulation of PBMC from patients using allergen extracts, both at the protein and the transcript level. In T-cell of patients with HDM allergy, high levels of *IL10* transcripts predicted immunotherapy success.²⁵⁰

Epigenetic biomarkers

Beyond cells, proteins and transcripts, epigenetic biomarkers may suggest prognosis.^{251,252} DNA methylation of promoter regions is associated with gene silencing, and *FOXP3* gene expression is in part controlled through this. FoxP3 is the master regulator of Treg cells, and Syed et al found that peanut OIT resulted in hypomethylation of *FOXP3*, indicating increased gene expression with immunotherapy.²⁵³

In conclusion, technological advances inform novel methods for monitoring immune responses with increased sensitivity and specificity. Studies indicate dysregulation of a number of molecular markers with AIT. Detection method optimization continues and should facilitate precision immunotherapy.

Table 2

Immune cell type	Biomarkers	Assay	References
Basophils	Decrease in basophil activation post allergen-specific OIT (CD63, CD203, histamine release)	BAT, iBAT	254-256
B cells	Induction of Breg cells on AIT, Serological readouts: food, and allergen-specific IgE, IgG4 Epitope-specific IgE and IgG4	Flow cytometry, ImmunoCAP, Eptiope mapping	170,234,243,257
T cells	Functional changes in tetramer positive/CFSElo/CD69+ CD40L+ allergen-specific CD4+ T cells, Tracking Th2A cells Decrease in T-cell derived type 2 cytokines Epigenetic changes in T cell subsets (Tregs)	Flow and mass cytometry, Luminex/ELISA, pyrosequencing	258-263
Dendritic cells	Changes in costimulatory potential (CD80, CD86)	Flow cytometry, DC-T cell co-culture	253,264

sIgE as biomarker in food allergy

Accurate diagnoses are essential for the management of food allergies.¹⁴⁷ They depend on detailed clinical history, objective markers of sensitization and double-blind placebo-controlled food challenges (DBPCFC).²⁶⁵ These are time-consuming, require specialized medical facilities, and side effects may occur. Consequently, molecular allergy diagnosis aims to reveal significant associations between sIgE and clinical phenotypes.

Peanut

A retrospective study of 205 peanut-challenged Danish patients found the best correlation between sIgE and clinical thresholds for the 2S albumin Ara h 2.²⁶⁶ A diagnostic model for peanut allergy predicted the food challenge outcome with 100% accuracy in 59% versus Ara h 2 in 50% of 100 Danish peanut-allergic patients.²⁶⁷ Co-sensitization to Ara h 2 and 6 was associated with severe allergy in peanut-challenged Finnish patients.²⁶⁸ A French study of 48 peanut allergic children found that Ara h 2 sIgE titers could predict the risk of anaphylaxis.²⁶⁹

Soy

The cupins Gly m 5 and 6 were suggested as markers for severe allergic reactions in 30 soy-allergic European patients.²⁷⁰ A high diagnostic value of the 2S albumin Gly m 8 was reported in soybean-sensitized Japanese children with and without symptoms.²⁷¹ Gly m 8 had an AUC=0.75 for soy allergy while the values for Gly m 5 and 6 were 0.69 and 0.64, respectively. In a study on soy allergy diagnostics, Gly m 8 had the highest AUC (0.79), comparable to skin prick test (0.76) and sIgE to soy extract (0.77).²⁷² In this study, the cupins Gly m 5 and 6 were related to mild symptoms.

Hazelnut

sIgE to the cupin Cor a 9 and the 2S albumin Cor a 14 was strongly associated with clinical symptoms in 161 Dutch hazelnut-sensitized patients.²⁷³ sIgE to Cor a 9 and 14 allowed correct diagnosis of 90% of severely hazelnut-allergic Belgian children.²⁷⁴ In 423 hazelnut-allergic patients, Cor a 9 and 14 were associated with severe symptoms (AUC=0.70).²⁷⁵ A model combining clinical symptoms and sIgE to Cor a 14 and walnut increased the AUC to 0.91. In a prospective multicenter study of 90 peanut and 44 hazelnut-allergic German children, a 90% probability for a positive food challenge was calculated for Ara h 2-specific IgE at 14.4 kU/L and for Cor a 14-specific IgE at 47.8 kU/L.²⁷⁶

Walnut

sIgE to the 2S albumin Jug r 1, the cupin Jug r 2, and the nsLTP1 Jug r 3 (AUC=0.79, 0.70, 0.62, resp.) predicted anaphylaxis in 45 walnut-allergic children.²⁷⁷ In 91 walnut-allergic subjects from Switzerland, Germany and Spain, severe reactions correlated with higher sIgE levels to Jug r 1 and the cupin Jug r 4.²⁷⁸ sIgE to Jug r 1 (AUC=0.79) from 32 walnut-allergic Korean children better discriminated clinical walnut allergy from tolerance than sIgE to walnut extract (AUC=0.56).²⁷⁹ In 34 peanut-, hazelnut- or walnut-allergic Italian children, sIgE to Ara h 1 and Ara h 2, Cor a 9 and particularly Cor a 14, or Jug r 1, 2 and 3 was associated with anaphylaxis.²⁸⁰

Cashew

In 63 cashew-allergic Greek children, sIgE to the 2S albumin Ana o 3 (AUC=0.97) performed better than extracts for predicting cashew allergy.²⁸¹ A markedly greater risk of a positive food challenge was observed for higher levels of sIgE to the cupins Ana o 1 and 2, and to Ana o 3 in 173 Dutch children with suspected cashew nut allergy.²⁸² Ana o 3 discriminated between allergic and tolerant children better than extract-specific IgE with an AUC=0.94 versus 0.78. A 95% probability for a positive cashew challenge was estimated for Ana o 3-sIgE at 2.0 kU/L.²⁸³

Egg and shrimp

sIgE to the ovomucoid Gal d 1 correlated with an increased risk of persistent egg allergy.²⁸⁴ Sensitization to tropomyosin and sarcoplasmic calcium-binding protein was associated with clinical reactivity in 58 shrimp-allergic patients.²⁸⁵

Non-food

Studies associating sIgE to allergens from multi-allergen non-food sources to clinical phenotypes are less in number. A study on *Artemisia* pollen allergy of 240 Chinese patients found that patients sensitized to three or four allergens had a higher risk of allergic asthma (56.4%) than patients sensitized to two or one (31.1%).²⁸⁶ Distinct sensitization patterns of 159 patients to dog, cat and horse allergens were associated with

specific respiratory symptoms.²⁸⁷ Polysensitization to several house-dust mite allergens predicted current mite-related allergic rhinitis and current or future asthma.²⁸⁸

In conclusion, severe reactions to legume seeds and tree nuts are predominantly caused by sensitization to storage proteins rather than by pollen-related allergens such as Bet v 1 or profilin homologues, or nsLTPs (Table 3).

Table 3 : Specific IgE to these allergens is associated with severe symptoms

Allergen source	2S albumin	Cupin *vicilin-type **legumin-type	nsLTP1	Ovomucoid	Tropo-myosin	References
Peanut	Ara h 2 Ara h 6	Ara h 1*				266-269,280
Soy	Gly m 8	Gly m 5* Gly m 6**				270-272
Hazelnut	Cora 14	Cor a 9**				273-276,280
Walnut	Jug r 1	Jug r 2* Jug r 4**	Jug r 3			277,278,280
Cashew	Ana o 3	Ana o 1* Ana o 2**				281-283
Egg				Gal d 1		284
Shrimp					Lit v 4	285

Biomarkers of the epithelial barrier

Environmental factors, microbiome, epithelial cells and immune cells show a dynamic crosstalk at the skin and mucosal barriers in the development of AD, AR, CRS, eosinophilic esophagitis and asthma.^{162,289-294} Studies on the pathogenesis of these diseases have clearly demonstrated a barrier defect in the skin and involved mucosas and a systemic inflammation.²⁹³⁻²⁹⁶ Defects in the epithelial barriers, caused by both environmental risk factors and a genetic predisposition, may represent the starting point of a chronic inflammation and allergen sensitization.²⁹³⁻²⁹⁶ A significant number of studies have reported that environmental factors directly affect the barrier function of epithelium.^{150,297,298} In addition, T helper 2 cells, ILC2s and their cytokine IL-13 damage skin and lung barriers.²⁹³⁻²⁹⁹ In addition mast cells and their enzyme chymase damage the epithelial barrier.³⁰⁰⁻²⁹⁰ The effects of environmental factors may, at least in part, be mediated by epigenetic mechanisms. Histone deacetylase activation by type 2 immune response has a major effect on leaky barriers and blocking of histone deacetylase activity corrects the defective barrier in human air-liquid interface cultures and mouse models of allergic asthma with rhinitis.^{95,293,301} The assessment of the barrier function of the skin and mucosas *in vivo* has an extremely high value in the clinics to identify barrier leakiness for an individual patient and requires the discovery of biomarkers.

To date, there were a few noninvasive methods to assess the skin epidermal barrier function *in vivo*. The quantification of transepidermal water loss (TEWL) in the skin across the stratum corneum had received some interest for early prediction of atopy prone children and detection of skin barrier.³⁰² Although TEWL increases in proportion to the level of damage, it is also affected by environmental factors such as humidity, temperature, season and moisture content of the skin.³⁰³ The noninvasive and rapid measurement of natural moisturizing factor by Raman Spectroscopy provides a method suitable for use in children. The association of natural moisturizing factor, filaggrin null mutations and AD suggest Raman Spectroscopy as a promising approach for stratification of endotypes in AD in the clinics. Other noninvasive methods currently used include assessment of the stratum corneum hydration, colorimetry, skin surface pH and sebumetry, but they only provide information on different characteristics and/or the condition of the skin and do not directly

measure the barrier function.³⁰⁴

Recently, electrical impedance spectroscopy (EIS) has been identified as an effective and stable tool for the detection of epidermal barrier.¹⁶³ This method works by transmitting a harmless electrical signal through the skin at several depths and frequencies and measuring the electrical resistance and impedance response of the tissue. EIS reflects particularly the tissue barrier status by collecting the electric impedance information from extracellular and intracellular tissue.³⁰⁵ Recently, our group has studied the impairment of the epidermal barrier in mice by the epicutaneous administration of barrier damaging proteases, such as papain, trypsin, Vibrio cholera toxin or by tape stripping. We were able to show the barrier damaging effects of these substances which correlates with the electric conductivity of the skin, causing a decrease of electrical impedance. According to these results, EIS shows a broad range of possible clinical applications in AD and atopic march, including early prediction of atopy prone children, early diagnosis of the disease, stratification and endotyping of patients, evaluation of the overall therapy response as well as single lesions and assessment of disease severity.

A critical feature of the gastrointestinal epithelium is intestinal barrier permeability as it must allow an efficient passage of nutrients while restricting the entry of larger molecules to avoid food allergy development. Multiple autoimmune diseases have been identified to arise or be exacerbated by a leaky gut, such as in inflammatory bowel disease, celiac disease, type 1 diabetes, systemic lupus erythematosus, multiple sclerosis.³⁰⁶⁻³⁰⁹ Recent studies point to a leaky gut as the initiator of type 1 diabetes because a subclinical intestinal barrier dysfunction was already detected before clinical onset of type 1 diabetes.³¹⁰ Zonulin is a prehaptoglobulin protein and a biomarker for gut barrier leakiness that downregulates TJ function and it has been proposed to play a role in several autoimmune diseases.³¹¹

Translocation of bacterial endotoxin (lipopolysaccharide, LPS) from the gut microbiota to blood circulation stimulates systemic inflammatory responses.³¹² Measurement of intestinal permeability is often used in the examination of inflammatory gastrointestinal disorders. It can be assessed by measurement of urinary recovery of ingested non-metabolizable lactulose and mannitol. Urine L/M ratio measured by ¹H NMR spectroscopy showed high correlation with the standard measurement of the urinary recoveries by enzymatic assays. In conclusion, NMR metabolomics enables simultaneous intestinal permeability testing and discovery of biomarkers associated with an impaired intestinal permeability.³¹³ In conclusion, identification of clinically reliable biomarkers for skin, respiratory and intestinal barrier measurements represent an important future research area.

In vivo biomarkers in AIT development programs: European implications

An increasing number of clinical trials in AIT have been published underlining both efficacy and safety of AIT as the only disease-modifying treatment option for patients with IgE-mediated respiratory allergic diseases.³¹⁴ With country-specific exceptions, AIT products are regulated by Authorities such as the European Medicines Agency³¹⁵ and others on the basis of methodological guidelines.

In its guideline “*Clinical Development of Products for Specific Immunotherapy for The Treatment of Allergic Diseases*”³¹⁵ the European regulatory authority, (European Medicine Agency (EMA)), has provided strict guidance for designing and performing clinical development programs in AIT.^{101,316} In Germany, these principles were followed in the “*Therapy Allergen Ordinance (TAV)*” for future registration of allergens and allergen-mixtures (derived from grass pollen, early flowering trees pollen, house dust mites, and bee and wasp venom) based on the main prevalent respiratory allergies in Germany.³¹⁷⁻³¹⁹ First registrations have been granted fulfilling the TAV demands by the German Paul Ehrlich Institut (PEI).³²⁰ Besides, a sufficient body of evidence exists in the clinical documentation of both SLIT and SCIT AIT products.³²¹⁻³²³

An important unmet need for further harmonization of methodological principles in AIT study design³²⁴ has been followed in a series of Task-force initiatives of the European Academy of Allergy and Clinical Immunology (EAACI)³²⁵ as overviewed in the 2019 report of our group.¹⁰¹ As such, position papers and guidelines on allergen-challenge procedures including clear standardization of procedures (SOPs) through the nasal³²⁶ or conjunctival³²⁷ route has been provided by the EAACI.

In addition, the combined symptom and medication score (CSMS) as defined by the EAACI as standard primary endpoint for future (pivotal) trials in AIT³²⁸ has been recently used recently in an increasing number of key trials in AIT.³²⁹⁻³³¹ However, further formal validation and amendments especially for the pediatric population are needed.^{320,332} As another example a further Task Force initiative aimed to provide clear clinically and aerobiologically justified definitions of pollen-counts for onset, peak and duration of pollen-seasons³³³ and those have been confirmed to be robust³³⁴ and clinically relevant as reflecting patients' symptom load in different countries in Europe in recent reports.^{334,335} Recently, the EAACI has published a Position Paper reporting the impact of the placebo effect in AIT from different methodological perspectives and outlining possible strategies to minimize this bias in clinical trials.³³⁶

Taken together, further emphasis should be put on international collaborations of clinical experts, methodologists and regulatory authorities to optimize methodological standards for AIT clinical development programs aimed to increase the level of evidence of AIT as the only disease-modifying therapy available.^{320,323,337}

Biomarkers on the clinical usage of biologicals in the EAACI

The EAACI recently published the Guidelines on the use of biologicals in severe asthma. Recommendations were formulated following the GRADE approach³³⁸⁻³⁴⁰ for each biological (benralizumab, dupilumab, mepolizumab, omalizumab, reslizumab) and each outcome (decrease in asthma exacerbations, oral and inhaled corticosteroids and rescue medication use; improvement in asthma control, quality of life and lung function; and, safety).³³ The recommendations were informed by 3 systematic reviews.³⁴¹⁻³⁴³ FeNO, sputum and blood eosinophils were scored as outcomes with low importance, however the evidence was analysed for subgroups based on biomarkers and co-morbidities. The higher the blood eosinophils the higher the expected impact of benralizumab, dupilumab and mepolizumab in reducing severe asthma exacerbations. The improvement in asthma control was significantly better in the high eosinophil subgroup for benralizumab and reslizumab. Lung function increased significantly more in the high eosinophil subgroup for benralizumab, dupilumab and reslizumab. Benralizumab improved QoL better in the high eosinophil subgroup. The effect of omalizumab on exacerbations did not depend on blood eosinophils. Neither the atopic status or total IgE predict the magnitude of effect of benralizumab and serum IgE thresholds (within regulatory limits) did not influence the response to omalizumab (Table 4).

Table 4. Current biologicals, biomarkers, asthma outcomes and GRADE recommendation

Biological				
Biological	Asthma-related outcome	Biomarkers	Recommendation as per GRADE methodology	Benralizumab Dupilumab R

Conclusions

In our discipline, a huge amount of omics research has been employed during recent years that needs to be analyzed in depth. Allergic diseases are a group of complex and heterogeneous diseases. Specific, sensitive and reliable (point-of-care) biomarkers are critical for selecting the proper treatment for the proper patient and enabling precision medicine. Precision endotyping is linking pathobiological mechanisms with visible properties via specific biomarkers. It is expected to be translated into pathway-specific diagnostic tests and opens a pathway to accurate disease classification and individualized targeted treatment options. We are expecting to change the diagnostic and therapeutic landscape in our specialty as well as of all medical disciplines. However, endotype-driven treatment still needs to face multiple challenges before its implementation in daily practice in allergic diseases and asthma. Most of the disease endotyping research is conducted in severe forms of allergic diseases nowadays. Over the past few years, the biomarker discovery field has gained great advances, which facilitates the identification of novel methods for disease diagnosis and therapy. With these fast-growing efforts, we can imagine that in the near future, we will be able to find better ways to treat allergic diseases, with a therapeutic approach targeted to the individual patient.

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Figure Legends

Figure 1: Treatment based on molecular biomarkers for endotypes in asthma. Asthma can be subdivided into type 2 (high) and non-type 2 (or type 2 low) endotypes based on their underlying inflammatory pathways. For type 2 high asthma, potential biomarkers could be serum specific IgE (sIgE), fractional exhaled nitric oxide (FeNO) and blood or sputum eosinophils, and in some more specialized centres periostin. Moreover, type 2 cytokines (IL-4, IL-5 and IL-13) and innate (epithelial) cytokines (IL-25, IL-33 and TSLP) can also be important biomarkers. The options to treat with biologicals emphasizing biomarkers of Type 2 high endotype have entered the market: IgE (Omalizumab), IL-5 (Mepolizumab, Reslizumab, Benralizumab) and IL-4/IL-13 (Dupilumab). In contrast, the diagnosis of type 2 low asthma is difficult to establish as generally based on increased sputum neutrophils or pauci-granulocytic with normal levels of other type 2 markers, and non-type 2 cytokines (IL-8 or IL-17). There are still some associated indicators including obesity, smoking habits and psychological aspects. Therefore, therapeutic strategies for patients with type 2 low asthma could be macrolides and bronchial thermoplasty.

Figure 2: Microbiome Biomarkers in Asthma. Alterations in the gut and airway microbiota during childhood have been associated with asthma risk. The higher relative abundance of *Veillonella* and *Prevotella* and a switch from a *Corynebacterium* and *Dolosigranulum* cluster to a *Moraxella* cluster in the upper-airways were associated with a higher risk of severe asthma exacerbation in children with asthma. The lower relative abundance of genera including *Lachnospira*, *Veillonella*, *Faecalibacterium*, *Rothia*, *Bifidobacterium* and *Akkermansia* in the gut during early life have been associated with the development of asthma. The increases in relative abundance of *Gemmiger*, *Escherichia*, *Candida* and *Rhodotorula* within the gut were also associated with the subsequent development of asthma.

Figure 3: Immune cells and mediators as biomarkers in allergic rhinitis (AR). AR is associated with abnormalities in epithelial barrier function which is caused by exposure to exogenous proteases from allergens bacteria and viruses. These changes in epithelial barrier could contribute to the allergen absorption and disruption of epithelial tight junction. Activated dendritic cells (DCs) present allergen peptides to naive T cells and drive them to differentiate into Th2 cells and also allergen specific Th2A cells. Damaged epithelial cells release a high level of alarmin (TSLP, IL-25, and IL-33), which activate the group 2 innate lymphoid cells (ILC2s) as well as pathogenic memory T helper (Th) 2 cells. All these cells produce large amounts of

proinflammatory mediators including IL-4, IL-5, IL-9, and IL-13. Besides, IL-4 and IL-13 are involved in IgE class-switch in B cells. IgE binding to mast cells can trigger the release of mast cell-associated mediators, such as prostaglandin D2 and leukotrienes, which could also activate the function of ILC2. PGD2 signaling could be a promising biomarker, as it can also activate eosinophils and basophils. Moreover, CD203c expression on basophils exhibits a time-of-day-dependent variation, which could partly be responsible for temporal symptomatic variations in AR. IgG4 increased during allergen immunotherapy (AIT) is purported to be a blocking antibody by competing for allergen binding with IgE bound to Fcε receptors on mast cells and basophils.cysLT, Leukotrienes; PGD2, prostaglandin D2.

Figure 4: Biomarkers of viral infections in the exacerbation of AR. After the epithelial cells are infected with viruses, the replicating virus can cause cell lysis and direct damage to the epithelium which causes deficiency in the production of antiviral interferon (IFN)-β and IFN-λ1. Together with the allergen induced cytokines IL-25, IL-33 and TSLP, ILC2s are activated and produce more type 2 cytokines. Subepithelial plasmacytoid dendritic cells (pDCs) recognize virus antigens and present them to CD4⁺ T cells and CD8⁺ T cells through MHC class II or I, and drive them towards a more type 2 centric response. Excessive release of chemokines and cytokines can be triggered by infections such as respiratory-syncytial virus (RSV). Together with type 2 cytokines, they could further promote the function of type 2 macrophages, a small fraction of IL-4-secreting NK cells, IL-4-secreting NK-T cells, neutrophils, eosinophils and mast cells, and augment type 2 responses in chronically inflamed airways. With the production of perforin and granzymes, CD8⁺ T cells can show cytotoxicity to virus-infected epithelial cells and induce apoptosis. The viral RNA is released and detected by airway smooth muscle cells and stimulates the production of prostaglandins (PGs) in an autocrine manner.

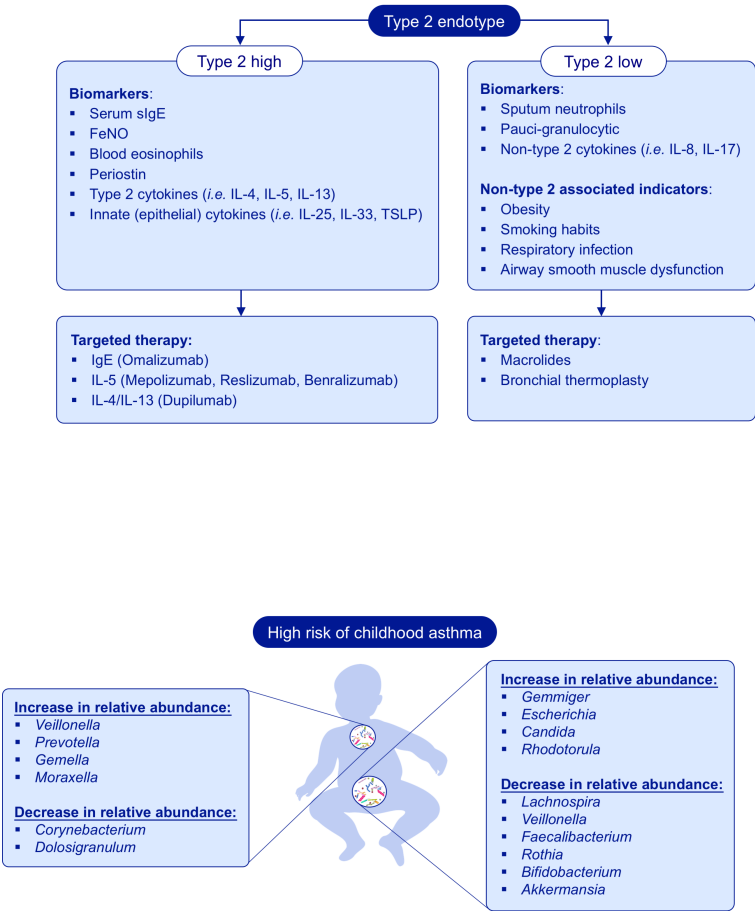
Figure 5: Biomarkers in food allergy diagnosis and treatment outcomes prediction. Conventional clinical approaches to diagnose food allergy include family history, skin integrity and the oral food challenge. Nowadays, expanded approaches focusing on genetic risk factors, allergen-specific and non-specific humoral and cellular biomarkers were explored. Genome, epigenome and mRNA linked to epithelial integrity and barrier (dys)function are linked to the development of food allergy. The measurement of IgE and IgG4 binding to linear or conformational epitopes could be more powerful to diagnose food allergy than conventional approaches. The soluble high-affinity IgE receptor (FcεRI) may also act as a biomarker for IgE mediated pathologies in a less allergen independent way. Moreover, allergen-specific Th2A cells and memory B cells have been discovered as new cellular biomarkers. Functional tests that simulate allergen exposure *in vitro* or *ex vivo* like the basophil activation test (BAT) and mast cell activation test (MAT) offer the possibility to assess allergen induced IgE crosslinking.

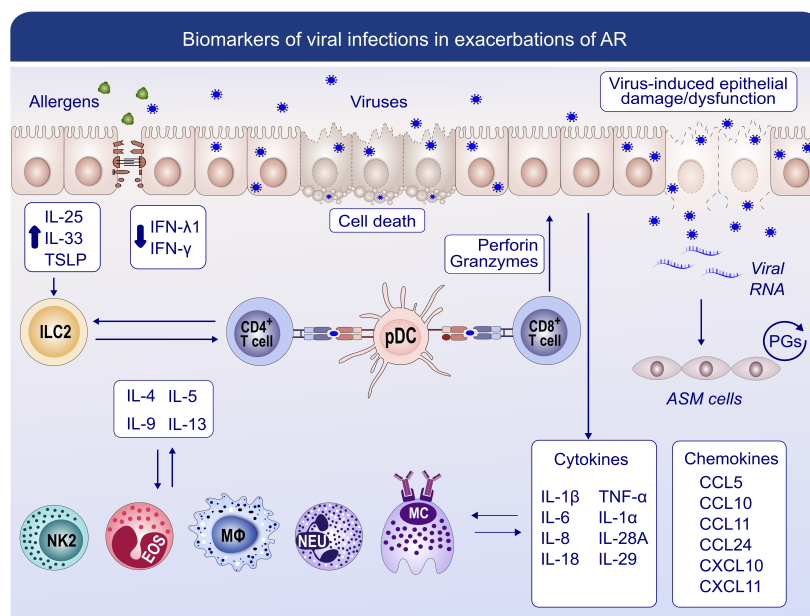
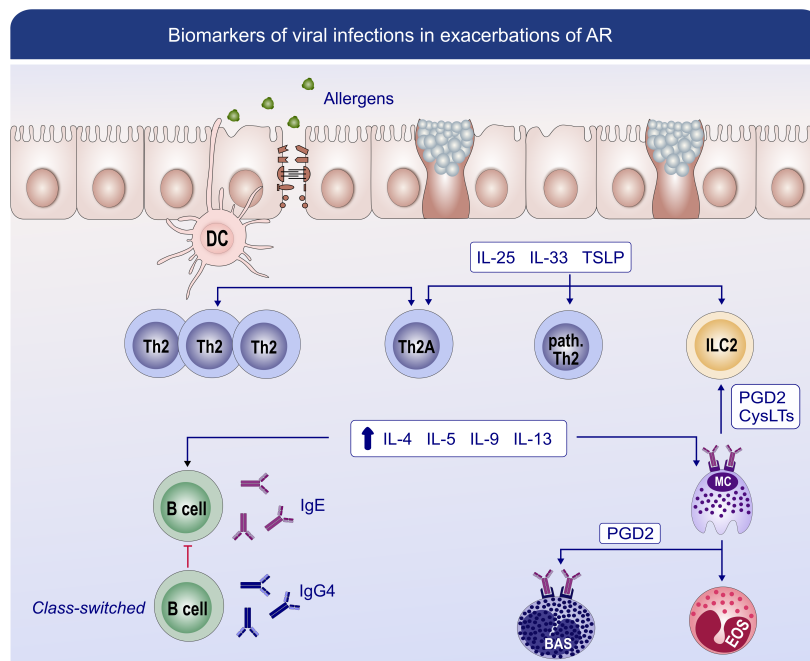
Figure 6: Mechanisms of immune-mediated reactions to drugs. These reactions encompass immediate reactions (mediated by IgE), and non-immediate reactions (mediated by T cells). In immediate reactions, drug-induced polarization of Th2 cells from Th0 cells, promote B cells to produce specific IgE (sIgE). These sIgE bind to the FcεRI receptor on mast cells. In subsequent drug contacts, the simultaneous recognition by at least two sIgE initiates the degranulation and release of mediators. Non-immediate reactions are generally characterized by a Th1 response with the increased secretion of IFN-γ from Th1 cells and granzysin from NK cells.

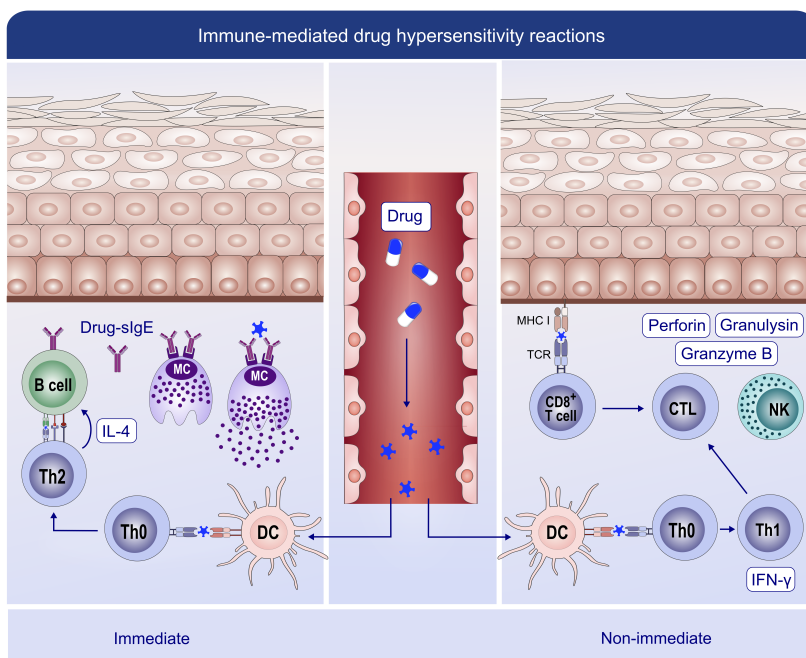
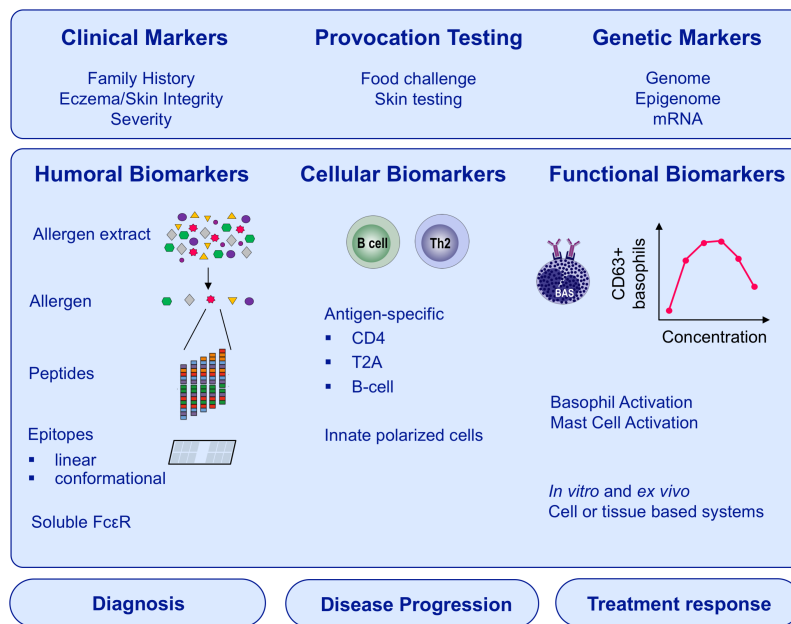
Figure 7: Mechanisms of cross-reactive hypersensitivity reactions to NSAIDs. NSAIDs induce reactions relying on their COX-1 inhibitory activity, *i.e.* activation of mast cells and other immune cells without involvement of adaptive immunity. During NSAID-exacerbated respiratory disease (NERD), the administration of NSAIDs permits strong 5-lipoxygenase (5LOX) activation and further generation of leukotriene E4 (LTE4). LTE4 induce the release of IL-33 and TSLP, and consequent mast-cell activation, with bronchoconstriction occurring as a result of the direct effects of leukotriene C4 (LTC4), prostaglandin D2 (PGD2), and other mast cell-derived products. PGD2 recruits effector cells such as Th2 cells, group 2 innate cells (ILC2s), basophils and eosinophils to the airway. Consistently, in NSAIDs-exacerbated cutaneous disease (NECD) and NSAIDs-induced urticarial-angioedema (NIUA), increased PGD2 can act on the skin epidermis. In addition, cross-reactive hypersensitivity to NSAIDs may involve additional sources of inflam-

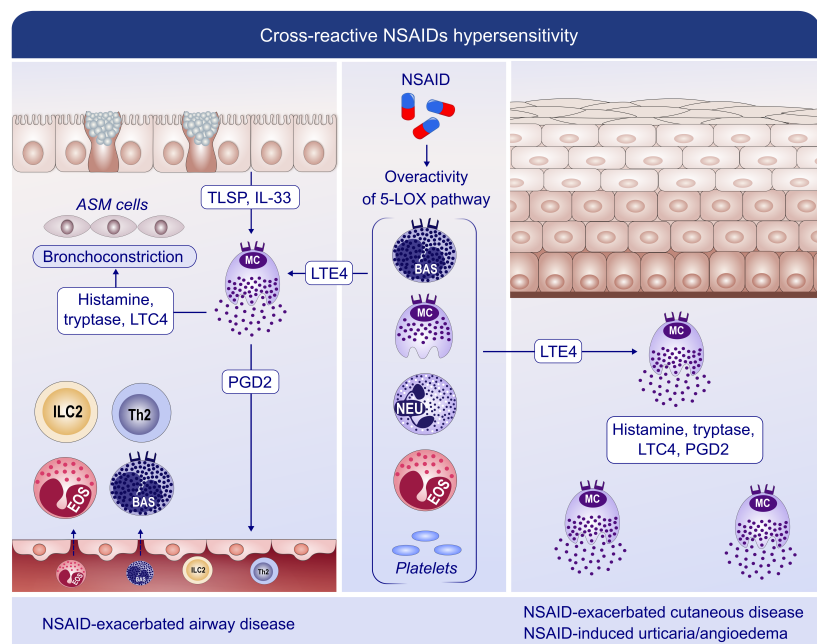
matory mediators, such as eosinophils and platelets. ILC2: innate lymphoid cells 2; LTE4/C4: leukotriene E4/C4; NSAID: Non-steroidal anti-inflammatory drugs; PGD2: prostaglandin D2; TSLP: thymic stromal lymphopoietin; 5LOX: 5-lipoxygenase.

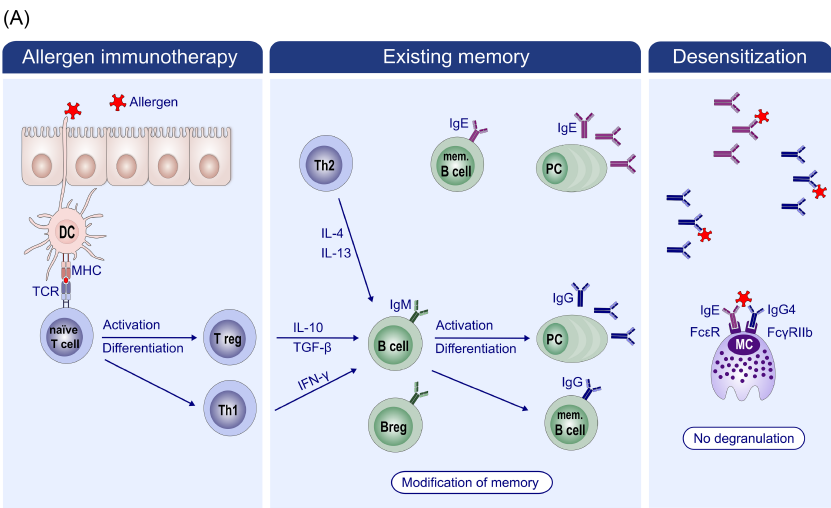
Figure 8: Current view on mechanism and biomarkers in use to monitor AIT. **A.** Scheme of immune modulation by AIT, where low dose, repeated exposure to allergen is thought to occur with limited to no inflammation. As a result, Th skewing is balanced towards Th1 and Treg, which subsequently modify the B-cell response. Especially the production of IL-10 is thought to drive IgG4 class switching. Thus, local and systemic memory is rebalanced, both in the T-cell and the B-cell compartment, and there is a strong increase in allergen-specific IgG4 antibodies. Upon allergen challenge, IgG4 and potentially other soluble factors are thought to inhibit IgE-mediated degranulation of target cells, i.e. desensitization. Together with the loss of Th2 skewing, this underlies the observed clinical tolerance. **B.** Laboratory biomarkers utilized in diagnostics and clinical trials for AIT (adapted from ²³⁰). Abbreviations: IgE-FAB, IgE-facilitated allergen binding; IgE-BF, IgE-blocking factor; BHR, basophil histamine release; DAO, diamine oxidase; Treg, regulatory T cell; Breg, regulatory B-cell; DC, dendritic cell. Figure reproduced from²²⁸.











(B)

Domain	Type of target	Assays	Strengths	Limitations
I: IgE	Serum protein	Total IgE Allergen-specific IgE	Serum markers; sIgE gold standard patient selection for AIT	No correlation of levels with therapy success
II: IgG subclasses	Serum protein	Allergen-specific IgG1 Allergen-specific IgG4	Serum markers; sIgG4 elevated by AIT	Failure in sIgG4 induction might reflect inadequate treatment compliance
III: Serum inhibitory activity for IgE	Serum protein	IgE-FAB IgE-BF	Serum-based assay; functional output	Laborious and requires specialized reagents/equipment
IV: Basophil activation	Cell activation	Flow cytometry of allergen-activated basophils (CD63, CD203c, BHR, DAO)	Requires small amount of blood	<i>In vitro</i> assay, which is difficult to optimize and standardize
V: Cytokines and chemokines	Serum protein	ELISA or Luminex for: IL-2, IL-2R, IL-4/5/6, IL-8/9/10, IL-13/18, IFN-γ, ECP, Eotaxin, MCP-1, TARC, transthyretin	Serum-based assay; high-throughput	Low sensitivity for many cytokines in serum
VI: Cellular biomarkers	Cell phenotyping / activation	Immunophenotyping of <i>ex vivo</i> or <i>in vitro</i> activated cells: CD4+ T cells, Treg, Breg, DC	Multiple cell subsets can be measured in single analysis from small blood sample	Lack of standardization in definitions and evaluation of most cell types. Antigen-specific cells can currently only be measured after <i>in vitro</i> culture (laborious)