Molecular biomarkers for grass pollen immunotherapy

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Abstract

Grass pollen allergy represents a significant cause of allergic morbidity worldwide. Component-resolved diagnosis biomarkers are increasingly used in allergy practice in order to evaluate the sensitization to grass pollen allergens, allowing the clinician to confirm genuine sensitization to the corresponding allergen plant sources and supporting an accurate prescription of allergy immunotherapy (AIT), an important approach in many regions of the world with great plant biodiversity and/or where pollen seasons may overlap. The search for candidate predictive biomarkers for grass pollen immunotherapy (tolerogenic dendritic cells and regulatory T cells biomarkers, serum blocking antibodies biomarkers, especially functional ones, immune activation and immune tolerance soluble biomarkers and apoptosis biomarkers) opens new opportunities for the early detection of clinical responders for AIT, for the follow-up of these patients and for the development of new allergy vaccines.

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Key words: Biomarkers; Molecular allergy; Grass pollen immunotherapy

Core tip: A concomitant approach of the component-resolved diagnosis biomarkers used to guide prescription of grass pollen immunotherapy, particularly important in regions of the world where grass pollen seasons temporal overlap with other types of pollen, together with candidate predictive biomarkers of clinical efficacy for this type of immunotherapy, classified as tolerogenic dendritic cells and regulatory T cells biomarkers, antibodies biomarkers, especially functional ones, immune activation and immune tolerance soluble biomarkers and apoptosis biomarkers, represents a methodological original presentation with an important educational role in the field molecular allergy considered imperative for clinical practice.

INTRODUCTION

Molecular biomarkers are indicators of biological or pathogenic processes, or responde to therapeutic interventions, which possess properties that allow their objective (reliable and accurate) measurements in biological samples, and include nucleic acid-based biomarkers, gene expression products, metabolites, polysaccharides and other molecules. These non-imaging markers, with an important role in the development of personalized medicine, can be classified (Table 1) into disease-related and therapy-related biomarkers[1][11]. Such biomarkers can be exploratory, probably valid or valid, according to differences in their scientific proposals, consensus in the medical community and acceptance by regulatory agencies.

The role of biomarkers has become increasingly important in molecular diagnostics and in guiding decisions related to drug development, clinical trials and modern
Table 1  General classification of molecular biomarkers

| Biomarkers | Definitions, comments |
|------------|----------------------|
| Disease-related genomic and proteomic biomarkers | |
| Disease risk biomarkers | Biomarkers associated with the risk of a disease |
| Diagnostic biomarkers | Indicators of the presence of a disease in an individual, including molecular diagnosis, early disease detection and screening biomarkers |
| Disease staging biomarkers | Biomarkers for assessing disease severity |
| Disease prognostic biomarkers | Indicators of the likely course/outcome of a disease for an individual; originally defined as markers that indicate the likely natural course of a disease in an untreated individual, also used to define the baseline risk that suggest the likely outcome of a disease independent of treatment |
| Drug-related biomarkers (provide information about a patient’s response to a therapeutic intervention) | |
| Pharmacogenomic biomarkers | Defining a DNA or RNA characteristic that is indicator of a response to a therapeutic intervention, facilitate the combination of therapeutics with diagnostics through pharmacogenomics (the study of genetic influence on drug response) and pharmacogenomics (the study of how genomic variation influences drug response) |
| Proof-of-mechanism biomarkers | Asses, in clinical trials, whether a drug has impacted its target |
| Drug activity biomarkers | Track the effect of a therapeutic intervention in accordance with its mechanism of action |
| Pharmacodynamic biomarkers | Measure the effect of a drug on the disease and determine the most effective dose for the patient, as efficacy biomarkers |
| Toxicity biomarkers | Determine the underlying susceptibility of a patient for a particular side effect or group of side effects |
| Surrogate biomarkers | Intended to substitute a clinical endpoint in clinical trials and expected to predict clinical benefit |
| Integral biomarkers | Used in clinical trials for eligibility, stratification, or treatment assignment |
| Integrated biomarkers | Intended to be used in clinical trials for hypothesis generation or testing, without impact on the treatment |
| Predictive biomarkers | Pretreatment or baseline measurements used to predict the patient response to a particular treatment |

personalized therapy. Significant progress has been made in the scientific research of oncology and neurological biomarkers, and also in the field of inflammatory and immunological biomarkers.[13-15]

Allergen-driven inflammation is the key pathogenic mechanism in respiratory allergies. Standard treatments, such as receptor agonists (glucocorticosteroids, beta-agonists), inverse agonists or antagonists (nonsedating H1 antihistamines, CysLT: leukotriene receptor antagonists) are used to treat symptoms, without eliminating the cause of allergy. Because conventional pharmacotherapy fails to restore dysregulated immune responses and, in some patients, to totally control clinical manifestations of allergy, there is a need for new treatment strategies. Although therapeutic tools for manipulation of gene expression in allergic diseases has received increased attention in the emerging era of functional genomics[16], only allergy immunotherapy (AIT) that aims to induce immune tolerance to allergens has reached a good level of robustness as an evidence-based therapy and is currently the only treatment with long-lasting clinical effects with the potential to modify the natural course of the disease. For allergic rhinitis and asthma, AIT is effective in reducing symptom scores and medication use, improving quality of life, and inducing favorable changes in specific immunological markers[17]. The diagnosis of respiratory allergy is usually based on skin prick tests and/or the measurement of allergen-specific IgE in serum. Currently, two types of AIT are in clinical practice: subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT). SLIT is a valid non-invasive and better tolerated alternative to SCIT. Special indications of this local type of immunotherapy exist in patients uncontrolled with optimal pharmacotherapy, in whom pharmacotherapy induces undesirable side effects, those who do not want to be on long-term pharmacotherapy or refusing injections[18].

SLIT tablets, with the convenience of self-administration, fulfill the requirements from the regulatory agencies that make mandatory pharmaceutical quality and are authorized as drugs available for grass pollen allergy[19]. Intra-nasal and intrabronchial immunotherapies are not commonly used because of administration-associated local symptoms[20]. New routes for grass AIT are under evaluation in clinical trials (intralympathic into inguinal lymph node under ultrasound control, epicutaneous via patch type epidermal delivery system)[20-21]. Second generation AIT vaccines based upon recombinant allergens (combined with mucoadhesive vector systems in sublingual products) are being developed as an alternative to conventional allergen extracts[22]. A mixture of different wild-type recombinant grass-specific allergen components of Timothy grass, adsorbed onto aluminium hydroxide, was studied as SCIT in grass pollen allergy, some of them being strong candidates for use as therapeutic vaccines[23]. Recombinant allergens for AIT aim to overcome the problems of natural extracts as they can be produced in unlimited amounts with exact physicochemical and immunological properties[24].

Currently, molecular diagnostic biomarkers can be used to guide AIT in the frame of component-resolved management of allergic diseases[25]. Identification and validation of biomarkers that are predictive of AIT clinical response are still unmet needs[16]. Recent advances in molecular biotechnology are destined to revolutionize immunotherapy treatments[26].

The major global health problem represented by respiratory allergies is due to their high prevalence, significant influence on quality of life and strong impact on work and school performance, productivity and economic burden. Allergic rhinitis is estimated to affect some 1.4 billion people globally and asthma is estimated to affect 300 million individuals worldwide. Respiratory allergies
affect all age groups and frequently coexist in the same subjects\textsuperscript{28-31}.

Pollen allergy is a public health threat of pandemic proportions. The most common outdoor allergens responsible for respiratory allergies are the pollen grains of anemophilous plants (wind-pollinated plants), such as of grasses, trees and weeds, each with specific seasons. Exposure to pollen grains depends of the plant type, wild spreading or cultivation, geographic area, altitude, air currents, temperature, precipitation and other weather events. Grass pollen is an important cause of pollinosis with a remarkable clinical impact all over the world. Its frequency differs regionally, but in many parts of the world, grass-induced respiratory allergy is the most common pollen allergy\textsuperscript{32,33,34}.

In the search for genomic biomarkers, some researchers tried to identify genetic variants associated with pollen sensitization. In studies performed more than a decade ago, susceptibility to grass allergy was associated with an increased frequency of HLA-DQB1*0301 when compared with the control population\textsuperscript{35}, while by both non-parametric and parametric statistical methods, scientists found significant associations between specific IgE to ryegrass group 1 and 2 allergens with HLA-DR3\textsuperscript{36} and specific IgE to ryegrass group 3 allergens with HLA-DR3 and DR5\textsuperscript{37}. A recent genome-wide meta-analysis revealed genetic variants associated with grass pollen sensitization in European adults. The HLA variant rs7775228 (6p21.32), which cis-regulates HLA-DRB4, was strongly associated with grass sensitization ($p_{\text{genom}} = 1.6 \times 10^{-9}$). Single nucleotide polymorphism (SNP) rs2155219, located at 11q13.5, upstream of chromosome 11 open reading frame 30 and downstream of leucine-rich repeat containing 32, was also strongly and consistently associated ($p_{\text{genom}} = 9.4 \times 10^{-8}$). The third-strongest association ($p_{\text{genom}} = 1.2 \times 10^{-3}$) was for rs17513503 located at the 5q22.1 locus near transmembrane protein 232 and solute carrier family 25, member 46. SNP rs1898671 from thymic stromal lymphopoietin gene showed weak association with grass sensitization ($p_{\text{genom}} = 9 \times 10^{-3}$)\textsuperscript{37}. In a Japanese study on matrix metalloproteinase 9 gene SNPs and pollen allergy in children, a haplotype associated with -1590T and 668Q revealed a significant association with cedar pollinosis and orchard grass pollen allergy\textsuperscript{38}. Although findings from such studies could enhance the understanding of immunological mechanisms involved in the pathogenesis of pollen allergy, with possible implications for prevention and treatment, additional scientific data are needed to evaluate genetic determinants, not only for IgE sensitization, but also for potential circulating biomarkers.

Currently, component-resolved diagnosis (CRD) biomarkers can be used to evaluate sensitization to grass pollen allergens. In patients with multi-sensitization, sensitization to cross-reactive panallergen biomarkers, specific IgE to profilins and/or polcalcin, may reduce the anticipated response to pollen AIT. In patients with mono-/oligo-sensitization profiles, major species-specific non-glycosylated allergen biomarkers, specific IgE to Poa- and Pooidea-specific molecules, suggest suitability for AIT\textsuperscript{39}. A better understanding of the AIT mechanisms of action to induce peripheral tolerance to allergens is useful to identify proper candidate predictive biomarkers for AIT efficacy: biomarkers of tolerogenic dendritic cells (DCs), T cell biomarkers, antibody biomarkers, immune activation and immune tolerance soluble biomarkers, and apoptosis biomarkers\textsuperscript{39,40}.

Molecular biomarkers for grass pollen immunotherapy are summarized in Table 2.

\section*{COMPONENT-RESOLVED DIAGNOSTIC BIOMARKERS}

\textbf{Used to guide prescription of grass pollen immunotherapy}

Recognition of disease-causing allergen components involved in pollen allergy, using the specific IgE against recombinant allergen components as molecular biomarkers, is of utmost importance, especially in patients with multiple sensitizations to different pollen types from plants, with total or partial, temporal and spatial overlap of significant airborne pollen concentration periods. This is particularly imperative in patients with a clinical suboptimally informative history, in regions of the world with great anemophilous plant biodiversity and/or areas where unrelated plants have pollination seasons which are at least partially concomitant in some months of the year\textsuperscript{41}. Retrospective symptom assessment is not a reliable method as grass pollen symptoms interfere with the recollection of symptoms induced by other pollen\textsuperscript{42}.

There is general consensus that AIT should be indicated in patients presenting with established clinical relevance for an allergen source. When seasonal symptoms point to grass pollen allergy, \textit{in vivo} and/or \textit{in vitro} testing typically confirm the presence of specific IgE to this type of pollen. In cases of IgE-sensitization to more than one pollen source from grasses, trees or weeds, it is essential to identify the clinically significant pollen types and exclude any source that may appear involved due to cross-reactivity, thus misrecognizing the primary sensitizing source, and compromising the expected immunological responses to AIT\textsuperscript{43}.

Grasses are universally distributed. Grass pollen grains are produced by wild or cultivated herbaceous plants (Table 3) belonging to \textit{Liliopsida} class, \textit{Pfleder} order, \textit{Poaceae} family (\textit{Gramineae}).

The most abundant allergenic grass pollen in many temperate regions originates from tall grasses (up to 1.4 m tall), such as \textit{Pileum pratense, Dactylis glomerata} and \textit{Arhenatherum elatius}. Cultivated rye also has a remarkably high pollen production. Allergic cross-reactivity between the members of the \textit{Pooidea} subfamily grasses of temperate regions (\textit{Lolium perenne, Pileum pratense, Poa pratensis}) is extensive, but it is limited with other tropical or...
Table 2  Molecular biomarkers summarized for grass pollen allergy immunotherapy[24,39-44]

| Biomarkers | Description, comments |
|------------|-----------------------|
| Serum specific IgE antibodies to rPhl p 1, rPhl p 2, rPhl p 5, rPhl p 6 | Molecular specific biomarkers of genuine sensitization to Poaceae grass pollen |
| Serum specific IgE antibodies to nCyn d 1 | Molecular specific biomarkers of genuine sensitization to Chloridioideae grass pollen |
| Serum specific IgE antibodies to CCDs | Molecular biomarkers of sensitization to CCDs involved in specific IgE assays cross-reactivity |
| Serum specific IgE antibodies to rPhl p 7 | Molecular biomarkers of sensitization to pollen polcalcin panallergens cross-reactive with pollen from most plants |
| Serum specific IgE antibodies to rPhl p 12 | Molecular biomarkers of sensitization to pollen profilin panallergens cross-reactive with pollen, some plant-derived foods and latex |
| Predictive candidate biomarkers of AIT clinical efficacy | Intrapleural markers of tolerogenic dendritic cells |
| Stabilin-1 (intracellular scavenger receptor), CIQ complement component expression | Surface cell biomarker of tolerogenic antigen presenting cells |
| Coregulatory PD-L1 (B7-H1, CD274) expression | Regulatory T cell biomarker |
| Peripheral IL-10/Fosq3 cells proportion among CD3+ CD4+ leukocytes | Allergen-specific antibodies biomarkers |
| Serum allergen-specific IgE to total IgE ratio | Allergen-specific antibodies biomarkers |
| Serum allergen-specific IgG, IgG1 and IgA | Functional biomarkers of serum IgG-associated inhibitory activity |
| Inhibition of CD23-dependent IgE-FAB to B cells, serum specific IgE-BF competing with IgE for allergen binding | Molecular markers of T cell mediated immune activation |
| Serum neopterin and kynurenine-tryptophan ratio | Non-classical MHC class 1 immune tolerance molecular biomarker |
| Serum sHLA-G | TRAIL biomarker |

CRD: Component-resolved diagnostic; CCDs: Carbohydrate cross-reactive determinants; IgE-FAB: IgE-facilitated allergen binding; IgE-BF: IgE-blocking factor; sHLA-G: Soluble HLA-G; sTRAIL: Soluble tumor necrosis factor-related apoptosis-inducing ligand; AIT: Allergy immunotherapy; PD-L1: Programmed death ligand-1; MHC: Major histocompatibility complex.

Table 3  Grasses (Poaceae family) which are sources of the most allergenic pollen grains[31,32,47]

| Subfamily | Tribe | Species (common names) |
|-----------|-------|-----------------------|
| Poaceae   | Hordeum vulgare (barley) | Hordeum vulgare (barley) |
|           | Secale cereale (rye) | Secale cereale (rye) |
|           | Triticum aestivum sp. vulgare (cultivated bread wheat) | Triticum aestivum sp. vulgare (cultivated bread wheat) |
| Chloridoideae | Cynodon dactylon (Bermuda grass, Bahama grass, Devil grass) | Cynodon dactylon (Bermuda grass, Bahama grass, Devil grass) |
| Panicoideae | Sorghum bicolor (Johnson grass) | Sorghum bicolor (Johnson grass) |

Monitoring pollen in the air, carried out by various gravimetric, impaction and suction sampling devices, may be used for the management of pollen allergy, and for biomedical and biological research. The Hirrst trap and later modified Burkard or Lanzoni traps are widely used samplers. Counting and identifying pollen grains is performed by optical microscopy. Pollen calendars are created based on differences in airborne pollen recorded in time[45]. Although pollen is routinely monitored, it is unknown whether pollen counts represent allergen exposure because pollen grains can vary substantially in allergen release, even although they are morphologically identical. There is a switch of importance from pollen pollen to pollen potency in the modern molecular era of aerobiology[9,10]. Phenological studies reveal that airborne grass pollen results from both local and distant sources, although the pollen airborne concentration peaks usually appear when such local herbaceous plants are shedding the greatest amounts of pollen. Although there is an association between flowering phenology and airborne pollen records for some of the tree and weed pollen types, for Poaceae the flowering and airborne pollen peaks usually do not coincide, with up to one week difference in phase[32]. Moreover, diurnal variations, climate and weather changes impact pollen exposure. Meteorological factors (temperature, wind speed, humidity, rain, thunderstorms) along with climatological regimes (warm or cold anomalies, dry and wet periods) influence pollen distribution. Human activities increase atmospheric greenhouse gases, such as carbon dioxide, and induce changes in global climate. Over the last decades, high

subtropical grasses, such as Cynodon dactylon and Paspalum notatum[26,39-44].

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Grass pollinating season peaks from October to February (longer grass flowering); in subtropical regions, grasses of the subfamily Panicoideae produce the only allergenic pollen with ubiquitous types within this context. Patients with multiple sensitizations to different pollen binant allergen components, are especially important in biomarkers represented by specific IgE against recom

Table 4 Grass pollen seasons timing and temporal overlap in Europe

| Regions                        | Grass pollen seasons timing and temporal overlap with other types of pollen                                                                 |
|--------------------------------|-------------------------------------------------------------------------------------------------------------------------------------|
| Northern, Western, Central and Eastern Europe | Grass flowering period starts at the beginning of May, finishes at the end of July  
Some grass plants are in flower even in August, even September (e.g., Poland)  
Air concentration of grass pollen usually peaks in June; pollination occurs about two-three weeks earlier at sea level and thereafter in the mountain regions  
Birch (Betula spp): Western Europe flowering period starts at the end of March, Central and eastern Europe from early April until May (2-8 wk), Northern Europe from late April to late May  
Agrimony (Eupatorium spp): In Central and South-Eastern Europe flowering period may partially overlap (April to May) with grass pollen season  
Artemisia spp, such as ragweed Ambrosia artemisiifolia var. elatior and mugwort Artemisia vulgaris, pollen season in Central and Eastern Europe may last from July to August-September  
Plantain Plantago spp: Pollen season from May to September in Eastern Europe                                                                 |
| Mediterranean regions of Europe | Different grasses are flowering between April and August  
Olive (Olea europea): Pollen season lasts from April to June, in regions of Greece, Spain, and southern Italy, overlapping grass pollen season  
Plane (Platanus spp): Pollen season partially overlapping with grass season, from April to May, in Southern France or Spain  
Pellitory Parietaria spp: Pollen has a long persistence in the atmosphere in the Mediterranean region, from April to October, even longer (perennial)  
Artemisia spp: Pollinate from August to October, similar to Chenopodiumae/Amaranthaceae pollen from salt-tolerant weeds significant also for semi-arid areas  
Plantain Plantago lanceolata: Pollen season from April to July in Northern Spain  
Cupressaceae (Cypress) and Chenopodiaceae (goosefoot) are most important weeds with pollen seasons in late summer and autumn, overlapping with the grass pollen season |
| European islands with special climate characteristics | Icelandic (cold-temperate oceanic country): Some grass species and sorrel (Rumex spp) flower in June, both with peaks in July; a second peak of grass-pollen is possible in some years in August; pollen season tails off in September; birch pollen season is short, starting in the second part of May until the beginning of June  
Canary Islands (Spanish archipelago with subtropical climate): Long-range transport of Paeonia and Amaranthaceae/Chenopodiaceae pollen from southern Iberian Peninsula and Morocco (mixed with Oleaceae tree pollen) and from the African Saharan sector and Sahara |

Table 5 Grass pollen seasons timing and temporal overlap in Africa

| Regions                        | Grass pollen seasons timing and temporal overlap with other types of pollen                                                                 |
|--------------------------------|-------------------------------------------------------------------------------------------------------------------------------------|
| Morocco (North Africa)         | Extremely variable pollen seasons exist due to great differences in plant distribution  
Mediterranean region: grass pollen season starts in April and has highest air pollen concentration in May and June, overlapping olive pollen season  
Date palm (Phoenix dactylifera) from Arecaceae family is distributed not only in the Mediterranean areas of Morocco, but also Middle East and central Africa  
Nigeria (West Africa)            | Grass pollinating season peaks from October to February (longer grass flowering); in tropical regions, grasses of the subfamily Panicoideae are predominant: Cynodon dactylon (kikuyu) and Stenotaphrum secundatum (buffalo grass)  
South Africa_Cupressaceae (cypress) trees start flowering in June, followed by Quercus robus (oak) in late July, Platanus (plane) in September and Olea europaea subsp. africana (olive) in January, and because their pollen season duration lasts three to four months it overlaps the grass pollen season  
Plantago lanceolata (English plantain) and Chenopodiaceae (goosefoot) are most important weeds with pollen seasons in late summer and autumn, overlapping with the grass pollen season |

temperatures and atmospheric carbon dioxide concentration have impacted plant and pollen distribution and induced changes in quantitative production and dispersion of pollen, pollen seasons and allergen content of pollen grains, which are region and species-specific.[32,33,34]

Grass pollen seasons timing and temporal overlap with other types of pollen must be discussed for different regions in the world (Tables 4-8). Diagnostic molecular biomarkers represented by specific IgE against recombinant allergen components, are especially important in patients with multiple sensitizations to different pollen types within this context.

As is inferred from the presented data, grass species produce the only allergenic pollen with ubiquitous representation and clinical significance across the globe. In many regions, grass pollen seasons overlap other pollination periods of other anemophilous plants (trees and weeds); therefore, commercially marketed CRD assays for inhalant sources include grass pollen allergens.[26]

Serum levels of specific IgE: to recombinant and native allergen components (specific and cross-reactive pollen allergen components) can be measured in vitro using two types of tests. Singleplex diagnostic tests (one result for a single serum specimen) are the same immunoassays as those used for the IgE determinations for allergenic extracts, the difference being that the antigen is a highly purified molecule, either natural or recombinant. Multiplex diagnostic tests (several results for a single speci-
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Table 6 Grass pollen seasons timing and temporal overlap in Asia\(^{[32,66-84]}\)

| Regions          | Grass pollen seasons timing and temporal overlap with other types of pollen |
|------------------|--------------------------------------------------------------------------------|
| Western Asia,    | Desert and semi-desert countries: Chenopodiaceae pollen season overlapping with grass pollen season, such as for *Cynodon dactylon* Indigineous trees/shrubs, such as mesquite (*Prosopis juliflora*) and date palm (*Phoenix dactylifera*) pollen seasons from March to May, also in Egypt.                                                                 |
| Middle East      | Israel: *Cupressaceae* (cypress family) and *Poaceae* pollen seasons throughout the year, grasses especially in spring (March-May), cypresses February and April; *Olea europaea* flowering begins in late March till July-August; *Paritaria takaoka* pollen highly allergenic in northern Israel.                                                                 |
| South Asia       | Turkey Mediterranean coastal area (Antalya): grass pollen frequently detected between April-May and October-November and found in the atmosphere in high concentrations during May to July. *Pinus* pollen airborne between March and June; *Cupressaceae* pollen, in high levels in February, usually present until May; *Chenopodiaceae/Amaranthaceae* pollen grains found in air during June to October, all overlapping with the grass pollen season. |
|                  | Beijing and different provinces of the People’s Republic of China, such as Guangdong, Yunnan and Hebei: pollen season lasting from August to October is due to weed pollen from different plant families, *Chenopodiaceae*, *Asteraceae* (mugwort *Artemisia* spp), and *Chenopodiaceae* (*Amaranthus*, *Chenopodium* spp), and of trees (*Prosopis juliflora*, *Cocos* and *Eucalptus* spp). |
|                  | Pakistan: paper mulberry (*Broussonetia papyrifera*, family *Moraceae*) pollen season from March to April overlaps with the grass pollen season.                                                                                                                                                                                                                   |
|                  | India: spring (February-April), autumn (September-October), winter (November-January) pollen seasons include periods of flowering of grasses (*Cynodon dactylon*, *Paspalum distichum*, *Serjania vulgaris*, *Poa annua*), of weeds *Cannabis* (Cannabis sativa), *Asteraceae* (*Parthenium*, *Artemisia* spp), and *Chenopodiaceae* (*Amaranthus*, *Chenopodium* spp), and of trees (*Prosopis juliflora*, *Cocos* and *Eucalptus* spp). |
|                  | East Asia |
|                  | Beijing and different provinces of the People’s Republic of China, such as Guangdong, Yunnan and Hebei: pollen season lasting from August to October is due to weed pollen from different plant families, *Chenopodiaceae*, *Asteraceae* (mugwort *Artemisia* spp), *Cannabaceae* (hemp *Humulus* spp), but also to grass pollen. |
|                  | South Korea: grass pollen airborne between end of April and November, especially Korean lawn grass, Timothy grass, Bermuda grass, and orchard grass; Pollen seasons of trees (pine, birch, oak) and weeds (mugwort, ragweed, Japanese hop) overlap with grass pollen period in the first, respectively last part of it. |
|                  | Japan: pollen season for Japanese cypress/hinoki (*Chamaecyparis obtusa*) lasts from March to May, while for orchard grass (*Dactylis glomerata*), in May-June to August, and for weed yomogi *Artemisia*, from August to October. |
|                  | Olive pollen from May to June overlaps with orchard grass season in Shodoshima. |
|                  | *Plantago lanceolata* pollen dispersed from mid-May to early September, in Sapporo. |

Table 7 Grass pollen seasons timing and temporal overlap in America\(^{[28,85-92]}\)

| Regions                  | Grass pollen seasons timing and temporal overlap with other types of pollen |
|--------------------------|--------------------------------------------------------------------------------|
| United States of America | Temperate regions: tree pollen predominates in spring, grasses in late spring and early summer, and weeds from summer until fall, with variable overlap periods.                                                                                                                                                                                                 |
| and Canada (North America)| Ragweed (*Ambrosia* spp) pollen season starts in July and peaks between August and October, warming by latitude being associated with increased length of pollen season in central North America.                                                                                                    |
|                          | Mountain cedar (*Juniperus ashei*) pollen season, in Oklahoma, Arkansas, central Texas, lasts from December to February, not overlapping with grass pollen period.                                                                                                                                                                                                 |
|                          | Southern Texas, on the western Gulf Coast: airborne grass pollen concentrations have two peaks, one in May (due to cool temperate grass species) and one in September and October (due to temperate and subtropical species), long distance dispersal of grass pollen is possible also out of season. |
| Mexico, Central and      | Subtropical regions in Mexico: similar to Southern California and Florida. |
| South America (Latin      | Mesquite (*Prosopis* sp) pollen in Northern Mexico and Southwestern United States. |
| America)                 | Tropical regions: grass pollen grains airborne throughout the year, overlapping with the pollination periods of trees, such as *Asteraceae*, *Cupressaceae*, and weeds, such as *Amaranthaceae*, *Asteraceae* and *Euphorbiaceae* spp. |
|                          | Maule region of Chile: *Platanus acerifolia*, *Olea europaea*, *Cupressus* spp pollen and grass pollen detected in August through November until end of January; *Plantago* spp, *Rumex* and *Chenopodium* spp pollen present from October to April. |

Table 8 Grass pollen seasons timing and temporal overlap in Australasia\(^{[28,94-98]}\)

| Regions              | Grass pollen seasons timing and temporal overlap with other types of pollen |
|----------------------|--------------------------------------------------------------------------------|
| Australia            | Subtropical northern regions (Brisbane, Queensland): grass pollen season, such as for *Paspalum notatum*, *Sorghum halepense* and *Cynodon dactylon*, from summer to autumn months, December to April, overlaps with the pollen season of groundsel bush (*Baccharis halimifolia*, *Asteraceae* family). |
|                      | Oceanic southern regions (Melbourne, Victoria): temperate grasses pollinate especially in spring, from September to November, overlapping with trees *Cupressus* and *Itelis* spp pollen season. |
| New Zealand          | Temperate grasses form the major component of atmospheric pollen levels during spring and summer (October to February) and *Plantago* spp pollen season overlap. |
men) are immuno solid-phase allergen chip based on multiplex microarray-based technology, multiparameter immunoblot test system based on single purified allergen components, and a multiplex flow cytometry allergenic molecule-based micro-bead array system[45,99-103].

In contrast to traditional specific IgE biomarkers, CRD in allergy does not rely upon whole extract preparations from native allergen sources, but on quantification of specific IgE antibodies to single protein components, purified from natural sources (native allergen components) or obtained using recombinant techniques (recombinant allergen components). These modern diagnostically useful are for a detailed CRD of the sensitization and cross-reactivity profiles, discriminating between clinically significant and irrelevant specific IgE, reduce the need for provocation testing and improve the prescription and specificity of AIT[26,33,106-124].

**Molecular specific biomarkers of genuine sensitization to grass pollen**

Molecular and biochemical characterization of grass pollen reveals several important specific allergen components. Timothy grass (*Phleum pratense*), also known as Herd's grass, meadow cat's-tail or common cat's tail, belongs to the *Poaceae* subfamily and it is one of the most significant source of grass pollen allergens in temperate regions. Bermuda grass (*Cynodon dactylon*), also known as Scutch grass, Bahama grass, Devil grass, belongs to the *Chloridoideae* subfamily, and it is an important grass which typically grows in warm temperate, subtropical and tropical climates areas of the world.

Specific IgE antibodies to recombinant temperate grass-specific pollen allergen components, rPhl p 1, rPhl p 2, rPhl p 5 and rPhl p 6, are biomarkers of genuine sensitization to *Poaceae* pollen. From references[45,99-103], these specific components and correspondent antibody biomarkers are discussed below:

- **Phl p 1** belongs to the group 1 grass pollen allergens, acidic glycoproteins with molecular mass of 31-35 kDa, a family of major allergens present in all grass species (*Poaceae* family-specific marker). More than 90%-95% of grass pollen allergic patients, adults or children, have specific IgE to group 1 grass pollen allergens. Group 1 grass pollen allergens are glycosylated proteins that show 60%-70% sequence identity to beta-expansin family of cell wall-loosening proteins with a role in pollen tube penetration into the style and pollen tube growth. A major IgE-reactive domain of Phl p 1 exhibits significant sequence identity of 43% with the family of immunoglobulin domain-like group 2/3 grass pollen allergens. Recombinant Phl p 1, rPhl p 1 (27 kDa) is not glycosylated and resembles native Phl p 1 (nPhl p 1) closely binding to IgE in about 90% of patients with grass pollen allergy, revealing that rPhl p 1 shares many of the IgE epitopes with natural grass allergens of the group 1. Sensitization to rPhl p 1 seems to appear earlier in life in comparison with other allergen components. Group 1 grass pollen allergens with great sequence identities and homologies include, besides Phl p 1, other important allergen components from important grass pollen grains: *Anthocanthon odoratum* (Ant o 1), *Dactylis glomerata* (Dac g 1), *Holcus lanatus* (Hol l 1), *Lolium perenne* (Lol p 1), *Poa pratensis* (Poa p 1). There is a partial cross-reactivity between Phl p 1 and Cyn d 1, the group 1 major allergen in Bermuda grass (*Cynodon dactylon*), thus Phl p 1 is only partially specific for the *Poaceae* grass subfamily.

- Phl p 5 is another major allergen from Timothy grass pollen and is one of the most reactive of the group 5 allergens, ribonucleases generally restricted to the *Poaceae* subfamily of grass pollen. Between 65%-90% of grass pollen allergic patients in temperate climate areas are sensitized against group 5 grass pollen allergens components. Grass pollen grains in ambient air is not quantitatively correlated with the airborne Phl p 5 concentration. Rainfall contributes to an increase in respirable particles containing group 5 allergens, which bursts the pollen grains. Moreover, exposure of pollen to gaseous pollutants induces a decrease in Phl p 5 detection in pollen extracts due to a mechanical loss of allergens from the altered pollen grains and/or post-translational modifications, such as ozone acidification. Phl p 5b, a smaller isoform (32 kDa), contains at least one more IgE antibody binding epitope than Phl p 5a isoform. rPhl p 5 is very similar to nPhl p 5 and reacts with serum IgE antibodies in a great part of grass pollen-allergic patients. rPhl p 5 is cross-reactive with similar group 5 allergen components: Dac g 5, Lol p 5, Poa p 5, Ant o 5. Because group 5 allergens are restricted to the *Poaceae* subfamily, there is a limited cross-reactivity between the pollen of temperate-type *Poaceae* subfamily grasses and pollen from warm temperate/subtropical-type grasses belonging to *Chloridoideae* (*Cynodon dactylon*) and *Panicoidae* (*Paspalum notatum*) subfamilies. Common reed (*Phragmites communis*), a grass from the *Arundinoideae* subfamily with a low phylogenetic affinity to *Poaceae* plants, produces pollen in late summer to autumn with a very low degree of cross-reactivity to group 5 allergens. There is a dissociation of the major IgE and T-cell-reactive peptide domains in Phl p 5. Specific IgE antibodies against Phl p 1 and Phl p 5 might be used as a reliable biomarker of allergy to *Poaceae* pollen. These major allergen components are defined on the basis of both frequency (prevalence of specific IgE antibodies) and potency (average level of specific IgE antibodies). Mono-sensitization to rPhl p 1 seems important in patients with lower IgE against Timothy grass pollen extract levels, while sensitization to rPhl p 5 is rarely found as the only sensitizing allergen.

Other grass-specific pollen allergen components must be discussed. IgE to rPhl p 2 (13 kDa) may also be regarded as a fairly specific biomarker for patients sensitized to grass species of the *Poaceae* subfamily. Immunologically significant group 5 and group 2 allergens seem to be absent in non-*Poaceae* grass pollen grains. Phl p 6 (a group 6 acidic, nonglycosylated protein of 15 kDa, for which N-terminal sequencing reveals homology to an internal region of group 5 allergens), along with Phl p 5,
do not exhibit significant serological cross-reactivity to pollen allergens outside the Pooidae subfamily. rPhl p 6, with the same reactivity with serum IgE antibodies as the native molecule, can be used for in vitro diagnosis of grass pollen allergy.

In conclusion, specific IgE against rPhl p 1 is a Poaceae family-specific biomarker for genuine sensitization to grass pollen and specific IgE antibodies against rPhl p 2, rPhl p5 and rPhl p 6 are Pooidae subfamily-specific biomarkers for true sensitization to temperate grass pollen. rPhl p 1, rPhl p 5 and natural Timothy extract are used to identify grass pollen allergy. Mono/oligo-sensitized patients with specific IgE to non-glycosylated major species-specific allergen markers (Phl p 1, Phl p 5) are suitable for Pooidae grass-specific AIT.

Specific IgE antibodies to nCyn d 1, a warm climate grass-specific native pollen allergen component, represent biomarkers of genuine sensitization to Chloridoideae subfamily grass pollen, as discussed below.[26,125-129] Cyn d 1 is a major allergen most abundant in Bermuda grass pollen, representing 15% of the whole-pollen extract. The frequency of sensitization to Cyn d 1 in Bermuda grass-allergic individuals is between 70% and 100%. Cyn d 1 belongs to Group 1 grass pollen allergens, including highly cross-reactive pollen allergens from other Chloridoideae subfamily grasses, such as Bou g 1 from the pollen of the North American Grama grass (Bouteloua gracilis). Cyn d 1 is to some extent immunologically distinct from Phl p 1 from Timothy grass and therefore a suitable marker for sensitization to Cynodon dactylon. Partial cross-reactivity between Phl p 1 and Cyn d 1 may impede the identification of the sensitizing allergenic source. When testing for rPhl p 5 as a Pooidae-specific molecular biomarker is negative, relatively higher levels of IgE specific to nCyn d 1 than to rPhl p 1 have been suggested to be indicative of primary sensitization to Bermuda grass pollen, an AIT extract containing Cynodon dactylon pollen might be suitable. If testing for IgE, anti-rPhl p 5 is positive and specific IgE against nCyn d 1 higher than to rPhl p 1, there is a true double sensitization. Finally, if antibodies against Pooidae-specific molecules, such as rPhl p 5, are positive and specific IgE levels against rPhl p 1 have higher levels than those to nCyn d 1, the case is most probably primary sensitization to Pooidae grasses and Cynodon dactylon pollen representation can be omitted from the AIT regimen.

Specific IgE antibodies to recombinant and native specific allergen components from tree and weed pollen are important to differentiate the true sensitization profile in patients with multiple sensitizations, including grasses, as described below.[26,7,129-130] When testing for these specific pollen components is negative and testing for IgE against specific and cross-reactive grass allergen components, then IgE sensitization is to grass pollen. If testing for IgE against recombinant specific grass pollen components is positive and specific IgE against specific tree or weed components are also significant, the condition is a true double or multiple sensitization.

Tree pollen-specific allergen components are described for the anemophilous plants belonging to the Betulaceae family: rBet v 1, a 17 kDa pathogenesis-related protein PR-10 with ribonuclease activity from the pollen of silver birch Betula pendula or Betula verrucosa, cross-reactive with other Betulaceae pollen PR-10 components with about 70% identity to Bet v 1 (black alder Alnus glutinosa rAln g 1, hazel Corylus avellana rCox a 1.0101); Oleaceae family: rOle e 1 and rOle e 1, a 19-20 kDa trypsin inhibitor from the pollen of olive Olea europea, Platanaeae family: rPla a 1, a 18 kDa invertase inhibitor, and nPla a 2, a 43 kDa polygalacturonase, from the pollen of plane tree Platanus acerifolia; Cupressaceae family: nCop a 1, a 43 kDa pectate lyase from the pollen of Arizona cypress Cupressus arizonica, cross-reactive with other Cupressaceae pollen pectate lyase components (Japanese cedar Cryptomeria japonica rCry j).

Major native or recombinant weed pollen-specific allergen components are described for herbaceous weeds belonging to the Asteraceae (Compositae) family: nArt v 1, a 28 kDa defensin from the pollen of mugwort Artemisia vulgaris and nAmb a 1, a 38 kDa pectate lyase from the pollen of short ragweed Ambrosia artemisiifolia var. elatior, family Plantaginaceae. rPla l 1, a 17 kDa Ole e 1-like trypsin inhibitor from the pollen of plantain Plantago lanceolata, family Urticaceae. rTar j 2, a 14 kDa lipid transfer protein, member of the PR-14 protein family, from the pollen of wall pellitory Paritaria judaica, family Amaranthaceae/Che nopenoideae. rChe a 1, a 24 kDa trypsin inhibitor from the pollen of goosefoot Chenopodium album and nSal k 1, a 43 kDa protein belonging to the pectin methylesterase family from the pollen of saltwort Salsola kali.

**Molecular biomarkers of sensitization to carbohydrate cross-reactive determinants**

Carbohydrate cross-reactive determinants (CCDs) are carbohydrate moieties of glycoproteins that induce the production of highly cross-reactive IgE, as discussed below.[26,140] Many allergens are glycoproteins containing carbohydrate moieties called N-glycans or O-glycans, according to their site of attachment to the protein. N-glycans containing beta1,2-xylose and alpha1,3-fucose in many glycoproteins are more extensively studied. Markers of sensitization to CCDs are bromelain (nAna c 2) and MUXF3 (Ana c 2.0101) carbohydrate epitope, the purified N-glycan from Ananas comosus bromelain, able to detect IgE to N-glycans in most pollen sources. Anti-CCD IgE biomarkers indicate the presence in serum of IgE directed against carbohydrate epitopes. CCDs rarely cause allergic reactions, but may produce positive in vitro test results to CCD-containing allergens from pollen, plant foods, insects and venoms. Patients sensitized to grass pollen develop anti-CCD IgE that also binds to CCD monovalent peanut allergens, but does not induce any clinical symptoms. Approximately 20% of patients with multiple pollen allergies have IgE antibodies to pollen allergens with molecular masses higher than 30 kDa and a great part of their IgE-binding is dependent on CCDs.
a major cause of cross-reactivity for in vitro specific IgE assays. If testing for IgE against a specific native allergen component, such as nCyn d 1, is positive, because native components are CCD-containing natural purified glycoproteins, it is necessary to assess the epitope protein nature in multi-sensitized patients. In cases of positive in vitro results to a natural allergen component, negative IgE to CCD markers reveal the protein nature of IgE epitopes. Positive IgE to CCD markers should optimally be accompanied by assessment of biological activity, such as positive skin prick testing or nasal/conjunctival challenge with the allergen, important aspects in the AIT decision process.

Molecular biomarkers of sensitization to cross-reactive pollen panallergens

Panallergens, usually classified as minor allergens, are defined as homologous and structurally related proteins belonging to different biological sources and causing IgE cross-reactivity between evolutionary unrelated species. Among panallergen families, only profilins are distributed ubiquitously throughout the plant kingdom and are responsible for allergic reactions to a multitude of evolutionary unrelated pollen and food allergen sources. Occurring exclusively in pollen grains of plants, polcalcin is not involved in pollinosis-associated plant food allergies. Bet v 1 homologues represent major allergens in pollen of trees (including the Betulaceae and Fagaceae families) but can also be found in many allergenic foods belonging to the botanical families of Rosaceae (PR-10 proteins with 50%-60% identity to Bet v 1: apricot Pru ar 1, plum Pru c 1, peach Pru p 1, cherry Pru av 1, apple Mal d 1, pear Pyr c 1), Betulaceae (hazelnut Cor a 1.0101 with 50% identity to Bet v 1) and Apiaceae (PR-10 proteins with 40%-50% identity to Bet v 1: carrot Dau c 1, celery Api g 1), giving rise to many birch pollinosis-associated food allergies. Bet v 1-like allergens are not normally present in the pollen of grasses or weeds. Although AIT with the recombinant major birch pollen allergen Bet v 1 proved as efficient as purified native Bet v 1 or birch pollen extract, the presence of IgE-sensitization to minor allergen components acting as panallergens, profilins and/or polcalcin, would be expected to decrease the efficacy of pollen AIT, at least to some extent, especially in the absence of IgE to species-specific allergen components. Sensitization to both profilin and/or polcalcin typically follows previous cosensitization to other molecular allergens from the same pollen source, being recognized at a later stage, and it is associated with a longer duration of allergic disease and with resulting cosensitization to a larger number of species-specific allergen molecules. When molecular multi-sensitization is present, sometimes it is associated with the practical inability to administer a more appropriate, allergen-matching AIT extract. Even if the content in various pollen AIT extracts, at least for profilin, is remarkably low, if specific IgE antibodies against major allergens are present, AIT with extracts containing these allergens can be administered, especially as the clinical relevance of profilins and polcalcins is still arguable.

Only a limited number of pollen panallergens are available for routine use (grass profilin, rPhl p 7, and birch profilin, rBet v 2; grass polcalcin, rPhl p 7 and birch polcalcin, rBet v 4), but due to marked structural homology among allergenic species, these serve as efficient markers of IgE-mediated hypersensitivity to the entire group of homologous proteins, with the possible exception of profilins from pollen of wall pellitory (Parthenaria judaica (Par j 3) and cypress (Cupressus sempervirens (Cup s 8), the latter being cross-reactive with the goosefoot Chenopodium album profilin, Che a 2. The molecular biomarkers of sensitization to cross-reactive grass pollen panallergens are discussed below.

rPhl p 7, a 9 kDa calcium-binding protein, is used as a polcalcin marker. Phl p 7 is a minor allergen of Timothy grass pollen, recognizing serum IgE antibodies in 10%-15% of grass pollen-sensitized subjects. Phl p 7 is a polcalcin cross-reactive with other polcalcins contained in pollen grains of non-Proneaeridae Bermuda grass (Cyn d 7), trees, such as birch (Bet v 3), alder (Aln g 4), olive (Ole e 3), juniper (Jun o 4), and weeds, such as goosefoot (Che a 3). Unlike Bet v 3 which contains three typical calcium-binding motifs, Bet v 4 is a polcalcin which contains only two calcium-binding domains. rBet v 4, a 8 kDa calcium-binding protein, is also used as a polcalcin marker. Other weed pollen polcalcins are from Asteraceae family (Art v 5, Amb a 10). Polcalcin rPhl p 7 is therefore likely to cross-react with pollen proteins from most plants, in particular with other grass species, several weeds and trees.

rPhl p 12, a 14 kDa actin-binding protein, is used as a profilin marker. This acidic protein is involved in cytokkeleton dynamics by binding to actin. Phl p 12 is a minor allergen of Timothy grass pollen, binding IgE antibodies from approximately 15%-30% of grass pollen-allergic subjects with varying degrees in different geographical regions. Phl p 12 has more than 75% sequence identity with profilins from pollen, various plant-derived foods and latex. It is cross-reactive with pollen profilins from many plants, such as birch (Bet v 2), olive tree (Ole e 2), date palm (Pho d 2), Bermuda grass (Cyn d 12) and sunflower (Hel a 2). rBet v 2, a 15 kDa profilin, is also used as a cross-reactive marker. Other pollen profilins are those from ragweed (Amb a 8) and mugwort (Art v 4). Cross-reactivity between profilins of mugwort pollen (Art v 4) and Apiaceae foods, such as celery (Api g 4), carrot (Dau c 4) and spices, are involved in the pathogenesis of the celery-mugwort-spike syndrome. Cross-reactivity between profilins of ragweed pollen (Amb a 8) and fruits, such as melon (Cuc m 2) and banana (Mus xp 1), are involved in the pathogenesis of the ragweed-melon-banana association.

Molecular diagnosis biomarkers, together with clinical history data, can help clinicians make a better selection of the most appropriate patients and allergens for AIT. Moreover, application of the component-resolved diagnosis biomarkers may change the diagnosis and the
choice of AIT in some patients.[149]

Taken together, the CRD biomarkers are used to guide prescription of grass pollen AIT after an initial basic diagnostic discrimination between mono/oligo- and multi-sensitization, based on skin prick testing results and/or values of in vitro evaluation of specific IgE using common pollen extracts. The use of a panel of species-specific allergen molecular markers, representing the most common allergenic species in the region, along with the panallergen screening molecules from grass pollen (polcalcin rPh p 7 and profilin rPh p 12), may facilitate the selection of those AIT candidates with an increased probability of benefiting from this type of treatment.

**PREDICTIVE BIOMARKERS OF CLINICAL EFFICACY**

*In grass pollen immunotherapy*

Because very complex immunological mechanisms of action, both cellular and humoral, are involved in the AIT efficacy, its long-lasting effect and the way it changes the course of IgE-mediated allergic disease, candidate biomarkers of clinical efficacy or biomarker combinations remain to be validated in order to clearly distinguish between strong and weak or early and late AIT responders.[42]

The AIT mechanisms of action to induce peripheral tolerance to grass allergens may be useful to classify some candidate predictive biomarkers for AIT efficacy, especially those derived from the antigen presenting cell (APC)-regulatory T cell (Treg)-IgG antibody immunoregulatory loop.[150] These candidate biomarkers can be classified as tolerogenic DCs biomarkers, regulatory T cell biomarkers, serum blocking antibodies biomarkers, especially functional ones, immune activation and immune tolerance soluble biomarkers and apoptosis biomarkers.[150-154,46]

**Biomarkers of tolerogenic DCs**

Oral APCs are key players in SLIT: Langerhans cells, CD207+ cells (Langerin or CD207 being a C-type lectin receptor localized in Birbeck granules) located in the mucosa itself, with a FcγRI expression greater compared with similar cells in the skin,[151] and a predominant subpopulation of myeloid DCs located along the lamina propria, CD11b+CD11c+ monocyte-derived DCs (moDC), are critical in capturing allergen and processing it as small peptides presented in association with major histocompatibility complex (MHC) class I and class II molecules at the cell surface. DCs loaded with allergen-derived peptides migrate to the cervical lymph nodes within 12-24 h, where they interact with naive CD4+ T cells to support the differentiation of Treg cells within 2-5 d. These CD4+ T cells subsequently migrate through blood back to mucosal tissues, resulting in allergen tolerance associated with downregulation of Th1 responses.[152,153]

Intracellular and surface biomarkers of tolerogenic DCs are important to be presented.

Biomarkers of tolerogenic DCs (DCreg) are biomarkers of DCs driving differentiation of Treg cells, evidenced by differential gel electrophoresis and mass spectrometry.[154] Two such biomarkers must be discussed. Stabilin-1 (STAB1) is an intracellular scavenger receptor expressed by DCs and macrophages. Complement component 1 (C1Q) is the first component of complement which may be associated with arrest of moDC differentiation and may induce tolerogenic properties in developing DCs.[154,155]. Tolerogenic moDCs are the most prominent source of C1Q and STAB1 gene expression in the blood and are generated in vitro from peripheral blood mononuclear cells (PBMCs). Induction of DCreg biomarkers (DCs in vitro treatment with dexamethasone) in PBMCs (containing < 0.5%-1% DCs) of patients with grass pollen allergy treated four months with SLIT is indicative of clinical tolerance induced by AIT (short-term efficacy).[154]

Regarding surface biomarkers of tolerogenic DCs, SLIT downregulates APC functions by modulating the expression of costimulatory molecules. There is a recent role revealed for the programmed death-1 receptor (PD-1) and PD-1 ligand (PD-L1) pathway in regulating lymphocyte activation and promotion of Treg cell development and function.[157]. PD-L1 (B7-H1, CD274), the programmed death ligand-1, is a coregulatory molecule critical for Treg generation with important expression on tolerogenic APCs (upregulated by TLR4 ligand monophosphoryl lipid A). PD-L1 may play an important role in induction of T regulatory cells by SLIT.[158]. Pollen SLIT reduces the expression of CD86 on B cells (CD19+), and the expression of CD80 on monocytes (CD14+), and increases the expression of PD-L1 on APCs (CD14+, CD19+) evaluated by flow cytometry analysis. PD-L1 may be a major target of pre-seasonal pollen SLIT and that modulation of its expression could be used as a clinical efficacy marker.[158].

**Regulatory T cell biomarkers**

These biomarkers may also be important because multiple mechanisms are related to Treg cells in AIT. Treg cells directly and indirectly control the activity of effector cells of allergic inflammation, such as eosinophils, basophils and mast cells. AIT-induced Treg cells inhibit the FcεRI-dependent mast cell degranulation, OX40-OX40 ligand interaction playing an important role, decrease the thresholds for mast cell and basophil activation and reduce IgE-mediated histamine release.[159-163]. Both main subsets, naturally occurring forkhead box P3 (FoxP3) expressing CD4+CD25+ regulatory T cells and inducible IL-10-producing T regulatory type 1 (Tr1) cells, are decisive for the development of immune tolerance to allergens under AIT.[160]. Mucosal Treg cell induction in SLIT was revealed by immunofluorescence microscopy, FoxP3+ cells being increased in the oral epithelium of grass pollen SLIT.[164]. The induced Treg cell level defined as the proportion of IL-10+FoxP3+ cells among CD25+CD4+ leukocytes, analyzed in the peripheral blood by flow cytometry, may be a potential therapeutic biomarker for SLIT, as revealed in a preliminary report in Japanese cedar (Cryptomeria japonica) pollinosis.[165]. Allergen-specific CD4+ T cell responses in...
Peripheral blood do not predict the early onset of clinical efficacy during grass pollen SLIT, as revealed in a more recent study in which these peripheral allergen-specific CD4^+ T cells were assessed using pMHCII-tetramers or flow cytometry surface phenotyping, as CTLA-4^+ IL-10^+ or CD25^+ CD127 FoxP3^+ Treg cells. Moreover, transcription factors (GATA-3, FoxP3) and cytokines (TGF-beta) gene expression assessed by quantitative reverse transcriptase polymerase chain reaction in allergen-stimulated peripheral cells do not predict clinical efficacy in SLIT, and the downregulation of IL-4 or IL-10 gene expression, as well as IL-10 secretion, by allergen-stimulated T cells seems to be unrelated to clinical benefit^69.

**Antibodies biomarkers**

The candidate antibodies biomarkers for the prediction of efficacy and monitoring of grass AIT must be discussed correlated with the allergen-specific IgE and IgG4 responses during AIT.

**Serum allergen-specific IgE antibodies**

Although AIT rapidly induces peripheral T-cell tolerance, B-cell changes seem to appear at a relatively later phase. Serum allergen-specific IgE values are not generally considered appropriate biomarkers to assess STT efficacy. Sometimes they transiently increase early in SCIT, and then gradually decrease over months or years of continued treatment. In pollen-sensitive patients who have undergone AIT and become desensitized, these values do not increase during the pollen season. There is a blunting of seasonal increases in specific IgE antibodies by AIT. Very late in the course and after termination of AIT, a decrease of allergen-specific IgE values is possible, occurring one to three years after starting therapy. Changes in IgE levels cannot account for reduced responsiveness to specific allergens after AIT because the decrease in serum IgE levels is late, relatively small and poorly correlated with efficacy. The reason for the persistence of serum IgE despite clinical improvement may relate to long-lived bone-marrow-resident IgE producing plasma cells^64,81,163,167,168.

The ratio of allergen-specific IgE to total IgE (sIgE/tIgE) was proposed as a candidate prognostic biomarker for SLIT. Symptom-medication score in patients treated with pollen SLIT seems to be correlated with the sIgE/tIgE ratio before treatment, being significantly improved in patients with a low sIgE/tIgE ratio compared to that in patients with a high sIgE/tIgE ratio. The grass-specific IgE to total IgE ratio seems significantly higher in responders than in nonresponders following four years of pollen SLIT. Further validation studies are needed before this biomarker can be considered in the clinical management of SLIT^34,58,169.

**Serum allergen-specific IgG4 antibodies**

IgG4 blocking antibodies prevent allergen-induced IgE-mediated release of inflammatory mediators from basophils and mast cells, directly compete with IgE on mast cells and APCs, inhibit IgE-facilitated allergen presentation to T cells and allergen-induced IgE production during allergen exposure. There is also an IgG4-dependent blocking of IgE binding to B cells. IgG4 production is confined to human IL-10-producing regulatory B (BR1 cells or CD73^+ CD25^+ CD71^+ B cells)^60,163,170,171.

Regarding serum allergen-specific IgG4 antibodies as biomarkers, only specific IgG4 antibodies with high affinity and avidity are functionally relevant. Pollen-specific IgG4 may be evaluated by fluoro-enzyme immunosassay. Serum allergen-specific IgG4 levels significant increase relatively early in SIT (weeks to months after AIT start), in an allergen-dose dependent manner (10-100-fold increase) and persist for up to two years after AIT discontinuation. Although this indicates a good immunological response to AIT, there are contradictory correlations with clinical improvement, there is no correlation with clinical outcomes (after up-dosing) and there is no common cutoff value for specific IgG4 antibodies^53,163,170,174.

Basophil activation evaluation may be used to detect IgG blocking activity in AIT. Allergen-IgG complexes bind to Fc epsilon RI (low affinity IgG receptor) containing a cytoplasmic immunotyrosine inhibitory motif that counters immunoreceptor tyrosine-based activation motif signals from Fc epsilon RI. Phosphorylated Fc epsilon RI binds mediates inhibition of Fc epsilon RI signaling, crossaggregation of Fc epsilon RI with Fc epsilon RIIB inhibits degradation, although there is a controversial role of Fc epsilon RIIB in mediated post-AIT serum inhibitory activity^173,174. Basophil activation test by flow cytometry evaluating CD203c expression, an ecto-nucleotide enzyme associated with basophil activation and piecemeal degranulation, may be a candidate biomarker for AIT monitoring, as suggested by a Japanese cedar pollen allergy study revealing a reduction in CD203c expression post-AIT^75.

Functional biomarkers of serum IgG-associated inhibitory activity in AIT may be more useful surrogates of clinical response than serum IgG1 levels.

The inhibition of CD23-dependent IgE-Facilitated Allergen Binding (IgE-FAB) to B cells assay evaluates the serum inhibitory activity for binding of allergen-IgE complexes on to B cells. It is performed incubating allergen-IgE complexes with an EBV-transformed B-cell line, complexes bound to CD23 on the surface of cells being detected by flow cytometry. Addition of serum from patients who have received AIT inhibits allergen-IgE complex binding to CD23 on B cells. The following formula may be used to calculate the percentage relative B cell binding: % relative allergen-IgE complex binding to B cells = (% IgE-FAB using indicator and immunotherapy serum/% IgE-FAB using indicator serum only) × 100. Pollen SCIT induces in grass allergic rhinitis patients time- and dose-dependent increases in antibody-associated serum inhibitory activity for IgE-FAB and increases in IgE-blocking factor (IgE-BF)^169.

Serum specific IgE-BF competing with IgE for allergen binding is determined using a wash assay, IgE measurement with a chemiluminescent immunosassay, and
The immunopathogenesis of pollen respiratory allergy includes a preponderance of Th2-type responses and the biochemical pathways triggered by Th1-type cytokine interferon-γ, such as tryptophan degradation by indoleamine 2,3-dioxygenase and neopterin production, might be altered [179]. Neopterin is a low molecular weight soluble biomarker of immune activation, synthesized from guanosine-triphosphate and produced preferentially by human monocytes/macrophages. Neopterin production and tryptophan catabolism through the kynurenine pathway, measured by the kynurenine-tryptophan ratio, are induced by interferon γ (IFN-γ) [180], thus both are considered markers of T cell mediated immune activation. Serum neopterin concentrations can be determined by an enzyme immunoassay technique. SLIT may reduce serum neopterin levels, this phenomenon being possible due to the Treg response able to induce IL-10 production, that may inhibit neopterin production. Thus, serum neopterin could be a serum biomarker of achieved immune tolerance toward the causal allergen in allergic patients successfully treated with SLIT [181,182]. Tryptophan and kynurenine serum concentrations seem to be higher in allergic rhinitis patients, especially out of pollen season. Simultaneous measurement of serum tryptophan and kynurenine may be performed by high performance liquid chromatography. Some authors suggested that non-responders to SCIT seem to have significantly higher tryptophan concentrations, higher tryptophan levels being a result of lower indoleamine 2,3-dioxygenase activity [179], and others revealed that serum tryptophan and kynurenine concentrations decrease after pollen SCIT, and a correlation between changes in tryptophan metabolism and neopterin concentrations was also possible after AIT [183].

The non-classical MHC class I molecule HLA-G plays important immunomodulatory activities. The differentiation of Tr1 cells by tolerogenic IL-10-producing human DCs requires the IL-10-dependent ILT4/HLA-G pathway [182]. Leukocyte immunoglobulin-like receptor B2 (LILRB2) or ILT 4 (CD85d) is a human inhibitory immune receptor that recognizes HLA-G with a higher affinity [184]. Soluble HLA-G (sHLA-G) has increased serum values in patients with pollen allergic rhinitis studied outside the pollen season [186]. These can be determined by ELISA, while cell production of IFN-γ is possible to be evaluated by enzyme-linked immunosorbent spot assay [185]. sHLA-G serum levels are reduced by pollen SLIT in allergic rhinitis patients and lowering of these levels and the increased IFN-γ production after SLIT in pollen allergic rhinitis are significantly related phenomena. Thus, sHLA-G might be considered as a candidate biomarker of response to SLIT [183].

**Apoptosis biomarkers**

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)/Apo2L is a type II transmembrane protein that was identified and cloned based on its sequence homology with members of the TNF ligand family. TRAIL-induced initiator caspase-8 and executioner caspase-3 cleavage is enhanced by IgE-dependent activation
of mast cells, which increases the expression of anti-
apototic molecules FLIP (Fas-associated death domain-
like IL-1 beta-converting enzyme-like inhibitory protease)
and myeloid cell leukemia 1 (MCL-1 belonging to the
bcl-2 family proteins), and a pro-apoptotic molecule
Bcl-2 interacting mediator (BIM of cell death), thus fine
modulating mast cell apoptosis[186]. Apoptosis of mast
cells may be also regulated by some IgG receptors, such as
FcγRRIIB[187]. TRAIL is also present in cells, eosino-
phils, fibroblasts and airway epithelial cells. The soluble
TRAIL (sTRAIL) is an apoptosis biomarker which can
be measured in the serum by a sandwich enzyme-linked
immunosorbent assay. sTRAIL levels may decrease after
SCIT to healthy levels and may be of use as a marker of
efficacy of immunotherapy in allergic rhinoconjunctivitis
patients[189]. The role of sTRAIL in AIT is poorly under-
stood and this makes the evaluation of the value of this
biomarker difficult.

CONCLUSION

CRD biomarkers have proven utility in the assessment of
sensitization to grass pollen allergens, allow the clinician
to confirm genuine sensitization to the corresponding
allergen plant sources and guide an accurate prescription
of AIT, important in many regions of the world with
great plant biodiversity and/or where pollen seasons
may overlap. These disease-related molecular biomark-
ers, important tools for the future in allergy diagnostics,
are hitherto available for the most important grass pol-
len allergens, although they have not currently replaced
the classical existing methods of in vivo/in vitro allergy
testing. Molecular diagnostic algorithms to guide pollen
immunotherapy in some European regions are already
designed[20].

It is difficult to estimate which of the presented
candidate predictive biomarkers for grass pollen AIT
will be validated in clinical practice, but those related to
tolerogenic regulatory cellular responses are most prom-
ising. Some answers to questions regarding the upcoming
guidelines for the use of predictive biomarkers for AIT
and the possible role of combined application of bio-
markers are not known and should be addressed as po-
tential issues in future research. The search for candidate
predictive biomarkers in AIT opens new opportunities
for the early detection of clinical responders during AIT,
for the follow-up of AIT patients and for the develop-
ment of new allergy vaccines.

Molecular allergy biomarkers represent a complex
area providing novel and relevant information for aller-
gists and educational programs on their use in clinical
practice are imperative[185].

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