POSITION PAPER

Biomarkers for monitoring clinical efficacy of allergen immunotherapy for allergic rhinoconjunctivitis and allergic asthma: an EAACI Position Paper

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Abstract

Background: Allergen immunotherapy (AIT) is an effective treatment for allergic rhinoconjunctivitis (AR) with or without asthma. It is important to note that due to the complex interaction between patient, allergy triggers, symptomatology and vaccines used for AIT, some patients do not respond optimally to the treatment. Furthermore, there are no validated or generally accepted candidate biomarkers that are predictive of the clinical response to AIT. Clinical management of patients receiving AIT and efficacy in randomised controlled trials for drug development could be enhanced by predictive biomarkers.

Method: The EAACI taskforce reviewed all candidate biomarkers used in clinical trials of AR patients with/without asthma in a literature review. Biomarkers were grouped into seven domains: (i) IgE (total IgE, specific IgE and sIgE/Total IgE ratio), (ii) IgG-subclasses (sIgG1, sIgG4 including SIgE/IgG4 ratio), (iii) Serum inhibitory activity for IgE (IgE-FAB and IgE-BF), (iv) Basophil activation, (v) Cytokines and Chemokines, (vi) Cellular markers (T regulatory cells, B regulatory cells and dendritic cells) and (vii) *In vivo* biomarkers (including provocation tests?).

Results: All biomarkers were reviewed in the light of their potential advantages as well as their respective drawbacks. Unmet needs and specific recommendations on all seven domains were addressed.

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Conclusions: It is recommended to explore the use of allergen-specific IgG4 as a biomarker for compliance. sIgE/tIgE and IgE-FAB are considered as potential surrogate candidate biomarkers. Cytokine/chemokines and cellular reponses provided insight into the mechanisms of AIT. More studies for confirmation and interpretation of the possible association with the clinical response to AIT are needed.

Allergen immunotherapy (AIT) is an effective treatment for allergic rhinoconjunctivitis (AR) with or without asthma (1–12). Allergen immunotherapy has disease-modifying properties and confers long-term clinical benefit after cessation of treatment (6, 7, 13–17). Allergen immunotherapy is routinely used in daily practice and can be administered either subcutaneously (SCIT) or sublingually (SLIT) (3–12). Although AIT is effective, the degree of remission strongly varies depending on the complex interaction between patient, allergy, symptoms and vaccines used for AIT (3–9). Clinical management of patients receiving AIT and efficacy in randomized controlled trials for drug development could be significantly enhanced if there were means to identify those who are most likely to respond, when to stop treatment, how to predict relapse and when to perform booster AIT. Furthermore, biomarkers in AIT can play a central role in personalized medicine (18).

Although recommendations for the standardization of clinical outcomes used in AIT trials for AR have recently been defined (1, 19–21), to date there is no consensus on candidate surrogate biomarkers of efficacy or biomarker combinations that would be prognostic, predictive and/or surrogate of the clinical response to AIT (22). In the sense of personalized medicine, biomarkers can be utilized to assist patient selection, identification of responders, target intervention at those who will benefit and to exclude those who are less likely to respond to treatment, thus meeting the criteria of personalized medicine. Additionally, they can be of major importance for the development of novel vaccines and for the optimization of existing therapeutic regimes. According to International Conference on Harmonization (ICH) E15 guidance on 'Definitions for Genomic Biomarkers, Pharmacogenomics, Pharmacogenetics, Genomic Data and Sample Coding Categories', biomarkers are 'indicators of normal biologic processes, pathogenic processes and/or response to therapeutic or other interventions' (22). Biomarkers can be applied in the context of controlled clinical trials for regulatory approval as well as in a clinical practice. Criteria for evaluating and selecting candidate biomarkers are provided by ICH E16 guideline 'Biomarkers Related to Drug or Biotechnology Product Developments: Context, structure and format of Qualification Submissions' (23). Per candidate biomarker, an overview containing the strengths and the limitations, design of the studies supporting its utility should be reported. The 'Guideline on the Clinical Development of Products for Specific Immunotherapy for the Treatment of Allergic Diseases' by the European Medicines Agency (EMA) published in 2008 advises to include immunological changes (e.g. changes in allergen-specific IgG levels, T-cell responses and/or cytokine production) and/or modifications of the end-organ specific response (e.g. provocation tests) in pharmacokinetic and dynamic studies in order to show the effect of AIT on the immune system (24). However, since 2008 several novel immunological markers in AIT have become available and may potentially be used as surrogate/predictive biomarkers for AIT. In this context, laboratory techniques should be reproducible, robust, sensitive and specific following the ICH guidelines for validation of analytical procedures 'Validation of Analytical Procedures: Methodology' (25).

The European Academy of Allergy and Clinical Immunology (EAACI) Immunotherapy Interest Group (IT IG) has conducted a task force (TF) on 'Biomarkers for monitoring the clinical efficacy of allergen Immunotherapy'.

The aim was (i) to collect and evaluate surrogate immunological and clinical biomarker data on the effects of AIT for AR with and without asthma obtained from clinical trials of AIT and (ii) to recommend a consensus position on candidate biomarkers for monitoring AIT and how these biomarkers could be used and implemented in future clinical trials of AIT and daily practice.

Methodology

Taskforce

After two initial meetings (Copenhagen June 2014, London October 2014), the primary objectives of the TF were confirmed: (i) to collect and review surrogate/predictive immunological and clinical biomarker data on the effects of AIT for AR with and without asthma, (ii) to identify surrogate candidate biomarkers that correlate with immunological and clinical effect of AIT, (iii) to identify surrogate/predictive clinical and immunological candidate biomarkers to monitor the effects of AIT in the target organ and systemically during the early and late allergic responses following allergen exposure, (iv) to identify surrogate cellular, humoral and molecular candidate biomarkers to monitor the effects of AIT during and after discontinuation of treatment, (v) to confirm (or reject) the candidate biomarkers for monitoring AIT. Subgroups of the TF drafted these sections on the background, advantages, disadvantages and current critical issues as well as on the unmet needs and recommendations for possible outcomes. During the third TF meeting which was held in London (United Kingdom) in August 2015, individual sections were thoroughly discussed and revised. Following this consensus meeting, the TF committee was responsible for drafting the EAACI TF position paper (PP) in a final draft, which was circulated once again to all TF members for critical review.

The TF report consists of recommendations for seven domains elaborated by the workshop participants (Fig. 1): (i)

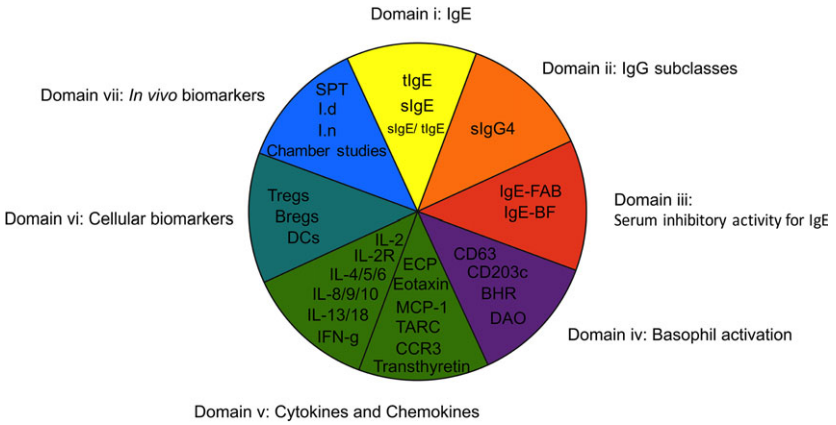

Figure 1 Seven domains: (i) IgE (total IgE, specific IgE, and sIgE/Total IgE ratio), (ii) IgG-subtypes (sIgG1, sIgG4 including SIgE/IgG4 ratio), (iii) Serum inhibitory activity for IgE (IgE-FAB and IgE-BF), (iv) Basophil activation, (v) Cytokines and Chemokines, (vi) Cellular markers (T regulatory cells, B regulatory cells, and dendritic cells), and (vii) *In vivo* biomarkers (including nasal and chamber provocation tests?).

IgE (total IgE, specific IgE and sIgE/total IgE ratio), (ii) IgG subtypes (sIgG1, sIgG4 including SIgE/IgG4 ratio), (iii) serum inhibitory activity for IgE (IgE-FAB and IgE-BF), (iv) basophil activation, (v) cytokines and chemokines, (vi) cellular markers (T regulatory cells, B regulatory cells and dendritic cells) and (vii) *in vivo* biomarkers (including nasal and chamber provocation tests). Health economic outcomes were not considered within the scope of this paper. Representatives from regulatory bodies, pharmaceuticals and biotech companies were invited to join the TF as observers and contribute to the discussion of the PP. EAACI is solely responsible for this PP, which does not represent an official document of any governmental agency such as the Paul-Ehrlich-Institute or the EMA.

Efficacy and biomarkers

The gold standard of efficacy of AIT is the evaluation of clinical symptoms and rescue medications during natural allergen exposure, as defined by the EAACI task force (1) following regulatory guidelines (24). The recommended primary outcome measure is the daily combined symptom and medication scores in AR.

According to the Biomarker Definition Working Group, biomarkers are quantitative measurements that allow clinicians to (i) diagnose, (ii) assess the disease stage, (iii) predict clinical outcomes and (iv) monitor the treatment effects (26).

Table 1 Introduced levels of evidence

Level of evidence	Study type
A	Randomized double-blinded placebo control
	Nonrandomized double-blinded placebo control
B	Nonrandomized open placebo control
C	Untreated control
	Cross-sectional
D	Retrospective
	Responders *vs* nonresponder

In this report, biomarkers are considered in the context of clinical trials as well as monitoring the response of patients in clinical practice.

Review of literature and level of evidence

Literature was reviewed by a PubMed search using the following MESH terms: immunotherapy, allergic rhinitis, desensitization, biomarkers, allergy. Additional articles were identified by reviewing the reference lists of relevant papers. Limitations were only studies published in English language and no older than 20 years (>1995) and available on PubMed. No limitation was set on the vaccines used. Only studies with a placebo or untreated allergic control group were included. We introduced the following levels of evidence (Table 1): (randomized) double-blinded placebo control (level A), nonrandomized open placebo control (level B), untreated control, cross-sectional (level C), retrospective, responders *vs* nonresponders (level D).

Recently, new administration forms such as intralymphatic (27, 28) or epidermal (29) routes have been advanced, but the overall clinical efficacy of these treatments is still debated and the treatments are not generally available, so these forms are not included in the present paper.

Results

Literature review

Tables S1 and S2 presents an overview of the results per biomarker and a summary of the included studies (Table S2).

Domain i: IgE

Background and study analyses

Elevated serum specific IgE levels and symptoms on exposure to the sensitizing allergen are currently the sole standard for allergy diagnosis and inclusion criteria for starting AIT (19, 20, 30). Several pollen AIT studies have reported that the

levels of specific IgE (sIgE) are transiently increased during treatment (Table S1) and followed by blunting of the seasonal increases. No functional or clinical relevance, for example severe allergic reactions, has been associated with this transient increase in sIgE. In long-term AIT studies, the levels of sIgE have been shown to be decreased over time, for example (31, 32). A heterogeneity in total IgE (tIgE) response during AIT (Table S1) has also been shown. Many studies confirmed no change, while others reported an increase or decrease in the levels of tIgE. These trends seem to depend mainly on the duration of the study or the time of sampling. Like sIgE, an initial increase in tIgE is followed by a decrease (Table S1).

The ratio of sIgE to total IgE (sIgE/tIgE ratio) as a predictive marker has been evaluated in a group of patients who received grass pollen or house dust mite (HDM) AIT for 4 years. The study involved both SCIT and SLIT treatment (33). Clinical outcome was measured using visual analogue scores. A cut-off value of 16.2% of IgE ratio predicted the successful outcome of AIT revealing a sensitivity of 97.2% and specificity of 88.1%. A randomized controlled open-label study of limited size could not replicate these results, while other studies did show a similar correlation between IgE ratio and clinical outcome of AIT (Table S1) (33–37).

Advantages
- Serum-based biomarker and a gold standard for selection of patients for AIT.
- Increases during updosing, maintenance and following AIT reflect immunogenicity and allergen exposure.
- Some studies show that an elevated sIgE/tIgE ratio is a potential positive predictive marker for AIT.

Disadvantages and critical issues
- sIgE shows a clear rise in early phase of SCIT and SLIT (more pronounced in SLIT) without clinical and functional relevance.
- The utility of the sIgE/tIgE ratio has not been properly evaluated and validated in Randomized double-blinded placebo control trial. One Randomized double-blinded placebo control trial of limited size did not replicate the relationship between clinical outcome and IgE ratio.
- A variety of assay platforms could be used to measure sIgE/tIgE ratio; however, equivalence between tIgE units IU/ml (kU/l) and sIgE units (kUa/l) has only been demonstrated for one singleplex IgE assay platform (38).

Unmet needs and recommendations
- More data are needed that assess the relationship between sIgE/tIgE and clinical outcome in responders *vs* nonresponders.
- We recommend that baseline sIgE/tIgE ratio is evaluated as a biomarker in AIT in controlled clinical trials in order to validate cut-off values for (to predict likelihood of response/nonresponse to treatment) sensitivity and specificity.
- For comparability, there is a need for using standardized assay platforms and established reference ranges and cut-off values.
- The role of locally produced sIgE/tIgE may provide an additional option to capture symptomatic relevance of the biomarker.

Domain ii: IgG subtypes

Background and study analyses

Analysis of the regulation of IgG subtypes following AIT has resulted in specific increases in the range of 10- to 100-fold in the concentrations of IgG1 and particularly of sIgG4, for example (39, 40). A correlation between allergen sIgG4 and clinical outcomes has been reported in some but not all studies (41–44), in a long-term follow-up study until 6 years after termination of AIT no correlation was found (45). In a withdrawal study of AIT, levels of sIgG4 were increased in a time-dependent fashion during treatment followed by a near 90% decline of sIgG4 levels which were still elevated compared to pretreatment levels. sIgG4 is considered to compete with allergen binding of sIgE bound to Fcε receptors of mast cells and basophils, and thus acts as a blocking antibody that prevents the activation and degranulation of effector cells (34, 46). Recent data show that immuno-solid-phase allergy chip (ISAC) can be used to determine the increased blocking of sIgG4 in AIT patients (47, 48). One study demonstrated an association with clinical outcome parameters (49). Immuno-solid-phase allergy chip may also be applicable to monitor the induction of sIgG4 in the updosing phase of SCIT while it shows the application of ISAC in the updosing phase of AIT (50).

In addition, some distinct features of sIgG4 suggest that it may have an anti-inflammatory role. IgG4 antibodies are dynamic molecules that exchange Fab arms by swapping heavy–light chain pairs between IgG4 molecules with different specificities (51). This process results in the production of bispecific antibodies with a substantially decreased capacity for cross-linking, because they are functionally monomeric (52). In addition, serum 'blocking' IgG4 antibodies have the capacity to suppress both allergen-triggered basophil histamine release and the binding of IgE–allergen complexes to B cells. A validated flow cytometry-based assay (IgE-FAB) has been developed as a surrogate for IgE-facilitated antigen presentation and activation of T cells during AIT (53).

It has been known for many years that sIgG levels in allergic individuals are elevated in nasal lavage (54). More recently, it was demonstrated that IgG1 and IgG4 appeared in mucosal fluids after AIT with genetically modified allergens. The increase in IgG4 levels was significantly associated with reduction in nasal sensitivity (40). Furthermore, the ratio of IgE to sIgG4 was shown to be decreased in several SLIT studies and was correlated with a decrease in late-phase skin reaction (55–58). This finding has not consistently been reproduced (59, 60).

Advantages
- Serum-based biomarker.
- Consistent results of elevated serum concentrations of sIgG4 are published in SCIT and SLIT studies.

- sIgG4 indicates allergen exposure and can be informative in combination with functional assay.
- Immuno-solid-phase allergy chip can be used to determine sIgG4-blocking activity.
- Data on local antibody levels are available, and further studies are needed.

Disadvantages and critical issues
- A firm relationship between quantitative levels of sIgG4 antibodies and clinical efficacy such as combined symptoms and rescue medication scores (CSMS) during SCIT and SLIT is missing.

Unmet needs and recommendations
- Low sIgG4 is a potential negative predictive marker.
- Failure in IgG4 induction may also be indicative for inadequate compliance.
- We recommend to use specific IgG4 rather than total IgG as a biomarker for evaluating immunological response to AIT in clinical research and drug development.
- Limited data are available on local antibody levels and activities. More studies, especially comparing local effects to peripheral effects, are needed in order to draw firm conclusions.
- More data are needed to evaluate the role of other IgG subsets, IgD and IgA.

Domain iii: Serum inhibitory activity for IgE

Background and study analyses

In the mid-1930s, Cooke et al. (61) reported on the induction of serum inhibitory antibody activity following AIT; later, this proved to be serum inhibitory activity for IgE. Mainly antibodies in the IgA and IgG fraction of the serum caused this effect (54, 62). Serum inhibitory effect for IgE includes the prevention of allergen binding to IgE (IgE-BF), the binding of IgE–allergen complexes to B cells and the inhibition of basophils. The latter will be discussed in a separate domain (iv) on basophil activation.

IgE-blocking factor (IgE-BF) is the extent to which several factors can hinder IgE from binding to its allergen and thus preventing a pro-allergic response and clinical symptoms (34, 63, 64). To examine this effect, a solid-phase assay is available (65). Several studies confirmed an increase in IgE-BF following AIT (Table S1), associated with clinical outcome in clinical trials. The IgE-BF assay is operated on an Advia Centaur instrument that has limited availability as it is no longer produced, or an alternative reverse-type IgE assay platform.

IgE-FAB is a highly reproducible flow cytometry-based bioassay that was developed to detect the binding of allergen–IgE complexes to B cells that express surface low-affinity IgE receptor FcεRII (CD23). This IgE-facilitated allergen presentation via CD23 to B cells has been used as a surrogate rate-limiting step for the subsequent processing of allergen and HLA class II-dependent presentation of allergen peptides by B cells to specific T-cell clones. For example, it has been demonstrated that serum obtained post-birch immunotherapy inhibited allergen–IgE binding to B cells that correlated closely with inhibition of IgE-facilitated presentation to specific T-cell clones (34, 46, 66). Furthermore, serum obtained from patients that received grass pollen AIT could inhibit IgE-facilitated allergen presentation to a grass-specific T-cell clone (67). Specific IgG4 within postimmunotherapy serum appears to play a key role in inhibiting this mechanism (31, 68). IgE-FAB has been shown to decrease after AIT and modestly correlates with clinical response to grass and birch AIT (Table S1) (46, 67). One study showed that increases in serum inhibitory activity for IgE-FAB persisted for 2 years (63). An inverse correlation has been found between symptom scores, rescue medication scores and IgE-FAB (6, 69). To date, there are no data available on the relationship between levels of serum inhibitory activity for IgE-FAB in responders *vs* nonresponders to immunotherapy.

Although the assay is reproducible, it is complex and might be limited to specialized centres or laboratories. Recently, an alternative less complex test has become available (65) – the enzyme-linked immunosorbent-facilitated antigen binding (ELIFAB) assay, a cell-free assay that substitutes EBV-transformed B-cell lines with soluble CD23 monomers bound to a solid surface. The assay follows the basic principles of a standard ELISA protocol using a 96-well plate.

Advantages
- An association between symptom scores, rescue medication scores and IgE-BF has been demonstrated in several studies.
- The IgE-FAB assay is a serum-based assay that is highly reproducible.
- Enzyme-linked immunosorbent-facilitated antigen binding is commercially available, as an alternative test that may be applicable in both research and clinical settings.
- An association between IgE-FAB and symptom and rescue medication scores has been demonstrated in some studies.

Disadvantages and critical issues
- IgE-BF has limited availability, as the Advia Centaur instrument is no longer produced.
- No data on responders *vs* nonresponders in relation to IgE-FAB have been published.
- There are only limited data exploring the correlation between IgE-FAB and the clinical response to AIT.

Unmet needs and recommendations
- IgE-BF is not a candidate biomarker for clinical use due to the limited availability.
- We recommend strongly that more data be collected on the relationship between IgE-FAB and responders/nonresponders.
- We recommend that IgE-FAB is further evaluated as a surrogate/predictive biomarker for AIT.

Domain iv: Basophil activation

Background and study analyses

Basophils represent 1% of leucocytes in peripheral blood and contain cytoplasmic secretory granules. They are

considered as easily accessible cells that share functional characteristics with mast cells and have their own role in systemic allergic responses (70, 71). After allergen cross-linking of specific IgE on basophils, degranulation is induced with release of histamine, leukotrienes and other mediators of the allergic inflammatory response (72, 73). Allergen immunotherapy has been associated with inhibition of basophil activation, and this is achieved via allergen-specific IgG antibodies. Allergen-specific IgG has potential to compete with IgE for allergen binding, thereby preventing allergen–IgE receptor cross-linking on basophils. Alternatively, allergen–IgG complexes may act by triggering basophil surface inhibitory IgG receptors FcγRIIb adjacent to IgE receptors, thereby inhibiting downstream IgE receptor activation (46, 67, 74).

A number of assays to monitor basophil activation are available and are important for allergy diagnosis, particularly in drug hypersensitivity (75, 76). Determination of basophil activation by measuring histamine release or other mediators such as leukotrienes and platelet-activating factor can be complex and time-consuming. Multicolour flow cytometry of basophil surface markers in whole blood enables the evaluation of basophil activation in the presence of potential inhibitory factors including allergen-specific IgGs. CD63 is most commonly used: it detects degranulation of basophils as the epitope is localized within granular membranes and becomes surface exposed upon fusion of the granules with the basophil surface membrane (77). CD203c is an alternative surface marker that not only detects basophil degranulation, but is also a highly selective marker for basophils in peripheral blood. CD203c is located directly underneath the plasma membrane and is induced rapidly on the outside of the plasma membrane after activation (78). Other less frequently used markers for basophil activation are CD13, CD107a and CD164. CD13 and CD164 follow a CD203c pattern, whereas CD107a follows more closely a CD63 pattern of detection by flow cytometry (79). A recently developed reverse staining technique for basophil activation involves measurement by flow cytometry of intracellular phycoerythrin-conjugated diamine oxidase (DAO) that detects intracellular histamine, the natural substrate of DAO. Degranulation of basophils results in a decrease in intracellular DAO corresponding to release of histamine from the cell (80).

The results obtained with basophil activation during AIT in placebo-controlled studies are conflicting. Some authors describe reduction in basophil activation following AIT that is possibly due to serological factors (69, 81–83). Other studies failed to demonstrate suppression of basophil activation in successful trials of AIT (37, 41). These contrasting findings may be partly explained by the route of immunotherapy, with SLIT being possibly less effective in inhibiting basophils than SCIT. Several studies show that basophil activation decreases after AIT, not only at the level of CD63 or CD203c, but also as measured by decreased DAO and increased CD107a (84–88). One study using DAO has shown persistent basophil suppression in four subjects 12–24 months after stopping AIT (84).

Advantages

- *Ex vivo* basophil activation with the sensitizing allergen reflects the FcγRI-mediated *in vivo* response.
- Requires small amount of blood (<2 ml) to perform the test.

Disadvantages and critical issues

- Basophil responses after AIT are variable with inhibition being shown in some but not all studies.
- Only a limited number of studies of basophil activation are yet available.
- Handling viable basophil cells is technically more challenging than determination of factors in serum.
- Dose–response curves are needed for accurate interpretation of results.
- Five to ten percent of population show no basophil response to IgE cross-linking.

Unmet needs and recommendations

- There is a need to understand the mechanism of allergen-induced basophil hyporesponsiveness during AIT.
- Standardized assays are needed. This applies to markers for accurate selection of basophil selection as well as markers of activation and histamine release.

Domain v: Cytokines and chemokines

Background and study analyses

One postulated mechanism of long-term clinical tolerance following AIT is a shift from a dominant Th2 response towards a Th1 response (13, 15). Hence, from the current state of the art, one would expect down-regulation of Th2 cytokines (IL-4, IL-13, IL-9), of inflammatory cytokines and chemokines such as IL-17, eotaxin or TNF-α, and up-regulation of Th1 (IFN-γ, IL-12) and regulatory cytokines (IL-10, TGF-β). In reality, some studies report increases in Th1 cytokines and chemokines, paralleled by an up-regulation of Th1 (IFN-γ, IL-12) and regulatory cytokines (IL-10, TGF-β), for example (89–94); others report no changes (95, 96). Furthermore, no clear relationship between serum cytokines and clinical outcome of AIT has been demonstrated. Besides addressing interleukins, numerous studies during AIT investigated chemokines CCR3 (unchanged) and CCR4 (97) (increased) and other original serum markers, like adiponectin (98) (unchanged), apolipoprotein A-IV (99) (increased), beta thromboglobulin (100, 101) (unchanged), complement factors C3a and 5a (102, 103) (decreased), C4a (99) (increased), ECP (104) (unchanged), eotaxin (97, 105, 106) (increased/decreased), soluble HLA molecules (107) (unchanged), leptin (98) (unchanged or increased), signalling lymphocytic activation molecule (108) (increased), thymus and activation-regulated chemokine (TARC) (97) (increased), TRAIL (109) (reduced), transthyretin (110) (increased) to tryptase (105) (unchanged). Importantly, none of these markers showed any correlation with the clinical response. It is thus likely that the changes in serum cytokines and chemokines are immunological paraphenomena of AIT that do not directly correlate with clinical outcome.

Local rather than serum levels of cytokines may be more indicative of immunological and clinical effects of AIT, but few studies of local cytokines have been performed (105, 111). For example, a cross-sectional study demonstrated lower concentrations of Th2 cytokines (IL-4, 5, 9 and 13) and chemokines (eotaxin) in local nasal fluid at 2–8 h after nasal allergen provocation following successful AIT compared to untreated controls (105).

Advantages

- These assays explore mechanisms of AIT.
- These assays may be useful for proof of concept at early stages of drug development.

Disadvantages and critical issues

- The low frequency of allergen-specific T cells dilutes the cytokine signal in the pool of cytokines secreted from T cells with other specificities.
- So far, no cytokines or chemokines have been identified that predict the clinical outcome in individual patients before the onset of AIT.
- Results are inconsistent: further studies of local nasal cytokines during AIT are required.

Unmet needs and recommendations

- At this stage, cytokines and chemokines are not applicable as a biomarker. However, nasal cytokines can serve as a marker of the immunological response and be used for proof of concept in drug development.
- Local cytokine production and secretion following allergen challenge may provide increased treatment-associated signals.
- Cytokines secreted from epithelial cells may reflect more closely the condition at the site of inflammation.

Domain vi: Cellular markers

Allergen immunotherapy has been associated with the induction of cellular responses within regulatory T cells (Tregs), regulatory B cells (Bregs) and dendritic cells (DCs). Immunological tolerance induction has been shown to be characterized by the up-regulation of peripheral and local allergen-specific regulatory T (Treg) cells (16, 93, 112–115). Tregs can be grouped into two subsets: (i) Foxp3+ regulatory T cells (nTregs) and (ii) inducible regulatory T cells (iTregs) that produce regulatory cytokines such as IL-10, TGF-β and IL-35 (93, 116, 117). Several studies have reported the immunomodulating properties of both allergen-specific nTregs and iTregs, suggesting that there is an overlap between these subsets of Tregs (118). The early induction of Tregs during AIT has been associated with delayed immune deviation from a Th2-pattern response to a Th1-type response. The association of increased numbers of Tregs in the nasal mucosa after AIT with clinical efficacy and the suppression of seasonal allergic inflammation supports the concept of a role for Tregs in the induction of allergen-specific tolerance (114, 115). A recent study investigating the epigenetic modification of memory Tregs during dual house dust mite (HDM) and grass pollen SLIT indicated that methylation of the FOXP3 locus might be involved in the mechanism of allergy tolerance after AIT (119).

B cells contribute to immune responses through antigen presentation to T cells, secretion of cytokines and production of antibodies after differentiation to plasma cells. Following receipt of the appropriate signals, plasma cells can reside for many years in the bone marrow and continuously produce antibodies independent of exposure to antigen. Upon activation, IgM+IgD+ naïve B cells undergo class switch recombination (CSR) leading to the expression of IgA, IgG or IgE antibodies. IL-10 suppresses antigen presentation through down-regulation of class II major histocompatibility complex molecules and costimulatory molecules on antigen-presenting cells. Furthermore, IL-10 suppresses the production of pro-inflammatory chemokines and cytokines. In parallel, IL-10 enhances the survival, proliferation, differentiation and isotype switching of human B cells. IL-10 augments IgG_4 production, and along with IFN-γ, it inhibits IL-4-induced IgE CSR (118). IL-10-mediated immunosuppressive functions of B cells have been described in murine models of autoimmunity, infection and cancer. Bregs expressing IL-10 suppress immune responses and the lack or loss of Bregs leads to exacerbated symptoms in several experimental autoimmune diseases (121). In addition, IL-10-overexpressing B cells produced less IgE and show a general ability to suppress T cells and DCs (122).

Dendritic cells are specialized antigen-presenting cells capable to of integrating a variety of incoming signals and subsequently orchestrating adaptive immune responses. Dendritic cells can either initiate and sustain allergic inflammation, or support tolerance induction. Molecular markers associated with polarized monocyte-derived DCs supporting the differentiation of either effector Th1, Th2, Th17 or regulatory $CD4^+$ T cells (termed DC1, DC2, DC17 and DCreg, respectively) have been identified by comparative transcriptomic and proteomic analyses (123, 124). Using such markers, recent AIT studies have documented a significant impact of SLIT on blood DCs. Specifically, 4 months of SLIT has been shown to up-regulate C1Q and stabilin DCreg markers while down-regulating DC2-associated markers such as CD141 in PBMCs from grass pollen-allergic patients (123). Importantly, such molecular alterations were found only in PBMCs from patients exhibiting a significant decrease in rhinoconjunctivitis symptoms, providing further corroboration at the level of the innate immune system for the paradigm that a reorientation of immune responses from a Th2 to a regulatory profile is critical to the success of AIT (123). Interestingly, the C1Q molecule itself, which can be secreted or expressed at the surface of monocyte-derived cells, was shown to be a strong inhibitor of Th2 responses in a murine asthma model. The latter observation suggests that DCregs induced during AIT not only support the differentiation of Tregs, but also mediate a direct anti-inflammatory activity by themselves.

In agreement with these findings, SLIT for 1 year in HDM-allergic children resulted in a decrease in the capacity of monocyte-derived DCs to mature in the presence of

Lipopolysachariden, with a blunted expression of CD86, a low production of IL-12 and an increased IL-10 secretion, consistent with their acquisition of a tolerogenic phenotype (125). Another study reported induction of PD-L1 (programmed cell death ligand 1) and IL-10 in parallel with a reduction in CD80 and CD86 expression on antigen-presenting cells during SLIT in ragweed-allergic patients (126). Also, DCs retrieved from the blood of peanut-allergic patients after 2 years of oral immunotherapy significantly down-regulated Foxp3 CpG methylation when cultured with T lymphocytes, suggesting the induction of DCregs (127).

Collectively, the latter studies confirm a significant and persisting impact of AIT on blood DCs, and suggest that changes in markers associated with DCreg and DC2 cells can be used to detect the early onset of AIT in grass pollen-allergic patients.

Advantages

- Tregs appear to play a key role in the immunological processes of AIT, mainly skewing the Th2 to Th1 immune response.
- It appears that a change in allergen-specific B cells in the direction of Breg cells is one of the major alterations in the course of AIT.
- Markers associated with DC polarization have been identified, which can be monitored in blood by quantitative PCR.
- Changes in such markers could represent an early signature within the innate immune system of the subsequent orientation of adaptive immune responses.
- In contrast with circulating $CD4^+$ T cells, which are constantly migrating to tissues, DCs might provide a more persistent signature in the blood of a transition from a Th2 to a regulatory immune response during AIT.

Disadvantages and critical issues

- No specific marker exists for Tregs – this means they are difficult to detect without sophisticated experimental approaches (and this cannot be performed routinely).
- There are not enough data to link the appearance or function of Tregs with clinical efficacy.
- Tregs appear very early in the treatment long before it is possible to evaluate clinical efficacy, so it remains difficult to use Tregs as a predictive biomarker in AIT in the absence of analysis of responders *vs* nonresponders.
- The frequency of allergen-specific T and B cells is very low: it is technically challenging and currently impossible to use this in clinical practice.
- Although Bregs can be characterized, this requires sophisticated experimental approaches and cannot be performed routinely.
- Dendritic cell-associated candidate markers of efficacy have been identified in a single short-term SLIT study which requires corroboration before it could be adopted in practice.
- The expression of some of DC-associated markers is shared with other leucocyte subsets (e.g. T or NK cells).
- Changes during AIT have been documented at the level of monocytes and monocyte-derived DCs. No information is available regarding the impact of AIT on myeloid and plasmacytoid DCs.

Unmet needs and recommendations

- At this stage, neither Tregs nor Bregs can serve as biomarkers for monitoring AIT. However, they may be useful in drug development as a marker of immunological response. Future determination of AIT-responsive phenotypes and analysis windows is critical for the practicability of cell-based biomarkers.
- Results remain to be validated in field studies, in the context of natural allergen exposure, in large cohorts of patients allergic to grass, tree pollens or HDM.
- We recommend further study of the impact of AIT on myeloid and plasmacytoid DCs in blood as well as tissues.

Domain vii: *In vivo* biomarkers

Allergen provocation tests are frequently used in clinical practice to evaluate patients' allergen-specific reactivity in disease-affected organs in order to demonstrate the clinical relevance of the underlying IgE-mediated sensitization (4). However, these tests can also be used as *in vivo* methods for stratification of patients for clinical trials as well as for investigating the effects of therapeutic interventions in AIT trials (4).

In the current European Medicinal Agency (EMA) guideline, provocation tests are recommended for proof of concept or phase II dose-finding trials in AIT (24). Therefore, several AIT trials have already included these models as primary objectives (examples in Refs 7, 128–131; reviewed in Refs 1, 132). Allergen provocation tests include skin prick tests, intradermal tests (ID) and direct evaluation of target organ responses with conjunctival provocation tests, nasal provocation tests (NPT) and environmental exposure chambers (EEC). Several protocols have been published using different challenge models (133). As outlined in the EAACI PP on 'Recommendations for the standardization of clinical outcomes used in AIT trials for allergic rhinoconjunctivitis', there is a clear unmet need for thorough harmonization and further validation of the different provocation models (1).

During NPT, subjective nasal symptom scores can be supplemented by objective measurements of peak nasal inspiratory flow (134). Nasal early responses have been shown to be inhibited (135) following intralymphatic cat and epicutaneous grass AIT (136). Nasal provocation test allow evaluation of local cells, mediators and cytokines in nasal fluid. Creticos showed suppression of nasal eosinophil numbers (135, 137, 138) and inflammatory mediators (histamine, TAME esterase during nasal provocation after ragweed immunotherapy). Suppression of the late nasal response by grass pollen (135) and ragweed AIT was associated with decreases in IL-4 mRNA+ cells and increases in IFN-γ mRNA+ cells in nasal biopsies. Recent advances include the

use of more precise nasal allergen delivery devices (139) and the availability of synthetic materials for filters, sponges, etc., that enable collection of neat or minimally diluted nasal fluid directly from the nasal mucosa (139, 140). The parallel development of miniaturized assay systems has allowed the reproducible measurement of multiple mediators, cytokines and antibodies in nasal fluid volumes as low as 20–50 μl. Nasal provocation has been shown to result in early increases in local nasal fluid tryptase (at 5–30 min) and later increases in chemokines and Th2 cytokines (eotaxin, IL-4, 5, 9 and 13) and innate lymphoid 2 cells (ILC2s) that parallel the late nasal response. Grass pollen AIT was associated with blunted increases in tryptase, eotaxin and Th2 cytokines (IL-4, 5, 9 and 13) in nasal fluid compared to untreated allergic controls (105). In addition to local suppression of type 2 allergic responses (204), grass pollen AIT has been associated with suppression of systemic basophil activation (84) following nasal provocation, as shown by a decrease in surface CD63 and intracellular DAO, compared to untreated allergic controls.

The EEC represents a recent alternative to NPT that more closely simulates natural exposure. Several recent studies have utilized the EEC to evaluate time-of-onset studies of AIT (141, 142). One study showed a correlation between symptoms provoked in the EEC compared to natural seasonal exposure (143).

For (pivotal) phase III trials, the EMA guideline (24) highlights that 'Provocation tests in allergen chambers [are] deemed to be a promising tool for the evaluation of efficacy; however, the results of such provocations have to be validated (...)'. An increasing number of EEC have been thoroughly investigated and validated regarding stability and reproducibility of allergen exposure under standardized environmental conditions (144). For AIT, there is a clear unmet need for further validation of treatment effect size as evaluated in EEC challenges to be correlated with effect sizes found under natural exposure in field trials (144). Pending the results, EEC models may become promising candidates in evaluating '*in vivo*' biomarkers in adjunct to natural exposure (1).

Advantages

- Provocation tests (i.e. titrated mucosal challenges) may indicate a change in responsiveness to allergen and/or a change in allergen sensitivity following AIT.
- Provocation tests permit more standardized procedures and the ability to control environmental factors (temperature, humidity) and avoid the variability caused by seasonal variations in pollen exposure.
- Provocation tests have been used as surrogate markers of clinical response to AIT. They are recommended for understanding mechanisms and permit biomarker discovery both at local level and in peripheral blood.
- They permit accurate time-course and dose–response studies, are less expensive to perform, require fewer participants than field studies and are often completed in a single centre, thereby reducing variability of outcome measures.
- European Medicines Agency recommends provocation methods as primary endpoints in proof-of-concept and dose-finding trials of AIT.

Disadvantages and critical issues

- Allergen provocation is not the same as natural exposure: standardization and validation vary for the different challenge protocols.
- Therefore, regulators do not accept replacement of natural allergen exposures by provocation challenges as primary endpoints in pivotal phase III trials.
- Conjunctival provocation test comprises mostly subjective outcomes, and there are no standardized/harmonized scoring methods.
- Different objective methods to measure nasal obstruction after NPT are currently used. Standardization is needed.
- Allergen products for provocation testing require regulatory approval and are not always available internationally for standardization purposes.
- Although environmental chamber studies are attractive, the procedure is expensive and standardization and confirmation of reproducibility within or between sites is required.
- Intradermal tests do not necessarily correlate with improvement of symptoms.

Unmet needs and recommendations

- Comparisons between provocation test results and symptoms evoked under natural exposure should be evaluated (204).
- In AIT, treatment effect sizes as evaluated in provocation tests should be compared with treatment effect sizes as evaluated under natural allergen exposure.
- Meanwhile, provocation tests provide proof of concept for novel approaches and are useful to assess time to onset of effect of AIT. The EMA recognizes their use for allergen dose-finding studies (phase II) before further investigation in (pivotal) phase III AIT trials.
- Provocation tests cannot substitute for assessing symptoms and requirements for rescue medication during natural allergen exposure in phase III trials.
- Pending standardization and clinical validation, EEC are likely to be an optional adjunct to natural exposure studies for phase III trials of AIT.
- Provocation tests should be tightly linked to the local and systemic biomarker assessments described above.

Discussion

Biomarkers, in the context of AIT, are defined as quantitative measurements that can predict clinical and immunological responses during treatment and could assist in patient selection, identification of responders, target intervention of those who will benefit and to exclude those who are less likely to respond to AIT as well as efficacy monitoring during intervention. Biomarkers for AIT would thus facilitate the introduction of personalized medicine in allergy.

Furthermore, they could be of assistance in clinical trials for the development of treatment modalities. An overview and recommendations for the standardization of clinical outcomes used in AIT are available (1), but to date there is no consensus on candidate biomarkers that are predictive of the clinical response to AIT (22). Although several biomarkers such as sIgG4, IgE-BF or IgE ratio have been included as secondary measurements in AIT studies, there are only very limited data on the relationship between biomarkers and clinical response *vs* nonresponse. This EAACI Task Force PP presents an overview of biomarkers tested in AIT trials in relation to clinical outcome. It emphasizes the pros and cons of different biomarkers and, finally, gives recommendations on the use of biomarkers in future research and AIT trials. It is important to note that currently available biomarkers are experimental and are confined to research and AIT trials. There is no evidence they may predict responses in individual patients in a clinical setting, but this is the ultimate goal of biomarker development.

We applied the definition of biomarkers provided by ICH E15 guidance on 'Definitions for Genomic Biomarkers, Pharmacogenomics, Pharmacogenetics, Genomic Data and Sample Coding Categories', which states that a biomarker is 'an indicator of normal biologic processes, pathogenic processes and/or response to therapeutic or other interventions' (22). European Medicines Agency advises in the 2008 guidelines to include immunological changes (e.g. changes in allergen-specific IgG levels, T-cell responses and/or cytokine production) and/or modifications of the end-organ specific response (e.g. provocation tests) in pharmacokinetic and dynamic studies (24). No advice is provided on the use of immunological changes as a predictive biomarker.

Humoral changes are included in many AIT clinical trials as secondary outcome measures (e.g. IgE, IgG_4, IgA). Several pollen AIT studies have reported transient increase in the levels of specific IgE but no functional relevance or severe allergic reactions have been associated with this transient increase in sIgE (31, 32). The ratio of sIgE to total IgE (sIgE/tIgE ratio) is more promising as a predictive marker (33). With a cut-off value of 16.2%, the sIgE/tIgE ratio predicted the successful outcome of AIT revealing a sensitivity of 97.2% and specificity of 88.1%. A limited number of studies showed a similar correlation between sIgE/tIgE ratio and clinical outcome of AIT; one open-label study could not replicate these data (34–36). We therefore recommend that more studies, including prospective cohort studies, should include the sIgE/tIgE ratio and correlate with responders and nonresponders. For $sIgG_4$, a small number of studies demonstrate correlation between allergen $sIgG_4$ and clinical outcomes (41–44). Most studies, however, have shown an increase in IgG_4 soon after the initiation of AIT which is followed by a decrease after cessation of treatment. The increase in the IgG_4 levels after AIT is likely to indicate an immunological response following allergen exposure during treatment and could potentially be used as a marker of therapy compliance reflecting the standardized preparation of the vaccine and effective administration.

The serum inhibitory activity of IgE following AIT has been known for many years and includes IgE-BF and IgE-FAB (46, 61). Although several studies have confirmed that IgE-BF increases after AIT, the limited availability of this assay means that this surrogate biomarker cannot be widely used in clinical practice or clinical trials. Two tests are available for IgE-FAB, a flow cytometry-based bioassay and ELIFAB. Enzyme-linked immunosorbent-facilitated antigen binding can easily be used in clinical settings, whereas the IgE-FAB assay may be better for clinical trials. IgE-FAB has been shown to decrease after AIT and remains decreased even after discontinuation of AIT (46, 63, 67). We recommend that IgE-FAB is explored further as a biomarker in clinical trials and in prospective cohort studies.

Cellular responses following AIT, including cytokines and chemokines, have been reported in several studies. Investigating cellular responses requires highly technically skilled personnel to perform the assays. To date, no cellular marker or cell-derived serum marker has been identified that would be useful in clinical practice. However, studies on cellular responses are of utmost importance in our quest to understand the underlying mechanisms of AIT and identify novel biomarkers. We recommend that the use of cellular markers is limited to clinical trials and mechanistic studies of AIT.

Environmental chambers and provocation tests are included here as they can be combined with the evaluation of local cells, mediators or cytokines (4). The current EMA guidelines already recommend provocation tests as a proof of concept in phase II dose-finding trials (24). This resulted in several AIT trials that included provocation testing in analysing their primary objectives (7, 128–131). There is, however, a clear unmet need for thorough harmonization and further validation of different provocation models (1, 133). Some data are available on local cytokines in NPTs showing that Th2 cytokine responses are blunted after AIT (204). Data so far are very limited; we recommend that protocols of provocation tests are harmonized and that studies that include provocation tests also include serological biomarkers. For example, provocation tests could be combined with sIgE/tIgE ratios or IgE-FAB.

So far, EMA guidelines advise inclusion of immunological changes in pharmacokinetic and dynamic studies only in order to show the effect of AIT on the immune system and they recommend provocation tests for assessing proof of principle (24). There is an urgent need for standardizing the use of potential biomarkers that are related to clinical outcome and reflect the immunogenicity of vaccines in inducing clinical and immunological tolerance. We propose that measurements of $sIgG_4$, IgE-FAB, sIgE/tIgE ratio and local cellular responses are investigated and implemented in future guidelines for registration of novel vaccines.

Conclusions

To date, there are no validated and generally accepted candidate biomarkers that are predictive or indicative of the clinical

response to AIT. Although several studies include biomarkers as secondary outcomes, current guidelines do not include biomarkers in the recommendations for clinical trials or clinical response. Therefore, this PP on biomarkers in AIT, as proposed by the EAACI Immunotherapy Interest Group, has reviewed all of the candidate biomarkers used in clinical trials of AR patients with or without asthma and grouped them into seven related domains. All biomarkers have been reviewed in the light of their potential advantages as well as their respective drawbacks. Furthermore, unmet needs and specific recommendations in all seven domains have been addressed. In order to raise the evidence level for candidate biomarkers from each domain, it is critical to conduct biomarker studies with a novel approach in design (i.e. responders *vs* nonresponders) and determine their clinical relevance as surrogate or predictive markers of the efficacy of AIT. In the light of the evidence above, this EAACI PP recommends exploration of the use of allergen-specific sIgG4 as a biomarker for compliance. Candidate biomarkers for clinical outcome are sIgE/tIgE ratio and IgE-FAB: more studies are needed to confirm and to interpret their association with the clinical response to immunotherapy and how they relate to persistence of clinical benefit after discontinuation of immunotherapy.

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Conflicts of interest

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary of study results.

Table S2. Study overview.

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References 145–203 are cited in Supporting information.