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REVIEW ARTICLE

Basophil activation test: Mechanisms and considerations for use in clinical trials and clinical practice

Alexandra F. Santos[1,2,3,4] | Oral Alpan[5] | Hans-Jürgen Hoffmann[6,7]

[1]Department of Women and Children's Health (Pediatric Allergy), School of Life Course Sciences, Faculty of Life Sciences and Medicine, King's College London, London, UK

[2]Peter Gorer Department of Immunobiology, School of Immunology and Microbial Sciences, King's College London, London, UK

[3]Asthma UK Centre in Allergic Mechanisms of Asthma, London, UK

[4]Children's Allergy Service, Evelina London Children's Hospital, Guy's and St Thomas' Hospital, London, UK

[5]Amerimmune, Fairfax, VA, USA

[6]Department of Clinical Medicine, Aarhus University, Aarhus, Denmark

[7]Department of Respiratory Diseases and Allergy, Aarhus University Hospital, Aarhus, Denmark

Correspondence
Alexandra F. Santos, Department of Paediatric Allergy, 2nd floor, South Wing, St Thomas' Hospital, SE1 7EH London, UK.
Email: alexandra.santos@kcl.ac.uk

Abstract

The basophil activation test (BAT) is a functional assay that measures the degree of degranulation following stimulation with allergen or controls by flow cytometry. It correlates directly with histamine release. From the dose-response curve resulting from BAT in allergic patients, basophil reactivity (%CD63$^+$ basophils) and basophil sensitivity (EC_{50} or similar) are the main outcomes of the test. BAT takes into account all characteristics of IgE and allergen and thus can be more specific than sensitization tests in the diagnosis of allergic disease. BAT reduces the need for in vivo procedures, such as intradermal tests and allergen challenges, which can cause allergic reactions of unpredictable severity. As it closely reflects the patients' phenotype in most cases, it may be used to support the diagnosis of food, venom and drug allergies and chronic urticaria, to monitor the natural resolution of food allergies and to predict and monitor clinical the response to immunomodulatory treatments, such as allergen-specific immunotherapy and biologicals. Clinical application of BAT requires analytical validation, clinical validation, standardization of procedures and quality assurance to ensure reproducibility and reliability of results. Currently, efforts are ongoing to establish a platform that could be used by laboratories in Europe and in the USA for quality assurance and certification.

KEYWORDS
allergy, basophil activation test, CD63, diagnosis, immunotherapy

1 | INTRODUCTION

The basophil activation test (BAT) is a flow cytometry laboratory assay which measures the expression of activation markers on the surface of blood basophils. CD63 was discovered by Edward Knol in 1991[1] and, since then, BAT has progressively gained importance in the diagnosis and monitoring of allergic diseases (Figure 1). In this review, we will cover the state-of-the-art BAT technology to explore immune mechanisms and to clinically assess patients with suspected IgE-mediated allergic disease. As a functional assay stimulating live cells in fresh whole blood with allergen, BAT assesses IgE cross-linking and is a more precise allergic readout than measuring the concentration of allergen-specific IgE.[2,3] When compared to a provocation test, BAT is less invasive, more comfortable and less expensive. BAT can be used if routine clinical (skin prick test) and laboratory (sIgE) analyses are ambiguous or discordant with the anamnesis, are to risky or if no reagents are available to perform them. Furthermore, as a laboratory test, BAT avoids exposure of patients to the allergen being investigated, thus making the diagnostic process safer and more comfortable for patients and their families.

2 | BASIC PRINCIPLES OF THE BASOPHIL ACTIVATION TEST

The BAT focuses on the basophil population at a single cell level using flow cytometry and assesses the activation state of these cells before and after stimulation with allergens or controls. BAT is usually performed using whole blood. Basophils have low side scatter, intermediate between lymphocytes and monocytes and can be identified through a number of near-unique selection markers: $CD193^+$ (also expressed on SSC^{high} eosinophils), $CD123^+$ (also expressed on HLA-DR^+ plasmacytoid dendritic cells) and CD203c and FcεRI (are also expressed on pluripotent progenitors of mast cells). Common methods of identifying basophils are as SSC^{low} $CD193^+$, SSC^{low} $CD193^+CD203c^+$, SSC^{low} $CD203c^+$ $CD123^+HLA\text{-}DR^-$, SSC^{low} $CD123^+$HLA-DR-, SSC^{low} $CD203c^+$ or SSC^{low} $CD193^+CD123^+$.[4–6] FcεRI and IgE, when used as selection markers in isolation, have the disadvantages of varying with plasma concentration of IgE and of inducing activation of the IgE-mediated pathway leading to degranulation.[7,8] Figure 2 shows examples of gating strategies currently used in assays used clinically and for research purposes.

Activation of basophils can be detected through upregulation of selected surface proteins; of which CD63 is the most commonly used activation marker[1] and is the focus of this review. CD203c is already expressed on resting cells, is upregulated slightly earlier than CD63, and can be upregulated by IL-3.[9,10] CD107a and CD107b co-localize with CD63 in secretory lysozymes, whereas CD164 and CD13 co-localize with CD203c in vesicles distinct from these. Upregulation of CD18/CD11b[1] and CD45 can also be detected on basophils, but it is not nearly as dichotomous as the upregulation of CD63. The tetraspanin CD63 is located in the membrane of secretory lysosomes inside basophils[1] and mast cells.[11] It is a 4-transmembrane protein that may be associated with reorganization of the cell membrane[12] and with exosome formation.[13] Its role in these processes is not yet well understood, but it is very useful as a biomarker of basophil activation. The expression of CD63 on the surface of basophils is directly and strongly correlated with histamine released into the cell supernatant.[1,14,15]

2.1 | Basophil signalling in IgE-mediated basophil degranulation

Crosslinking of IgE bound to FcεRI, the high affinity IgE receptor on blood basophils, results in increased phosphorylation of ITAMs of the FcεRIβγ subunits and of the SH2-domains of kinases Syk and Lyn,[16] which are under constant counter-regulation by dephosphorylation through CD45.[17] Net phosphorylation of FceRIβγ and Syk leads to massive amplification of the initial signal, similar to that of neuronal signalling and regulated exocytosis of secretory lysosomes that stain with basic dyes as they contain histamine, histidine decarboxylase, heparin and proteases.[18] IgE-mediated activation is an example of a bi- or multivalent activation mechanism through adaptive immune signalling. Immune-regulated exocytosis uses SNAP23 and VAMP8, whereas SNAP25 and VAMP1 and VAMP2 are used in neuronal signalling.[19] Degranulation has been studied mainly in murine mast cells and the RBL cell line, as these can be cultured in sufficient quantities and in high purity.[20] The use of wortmannin-sensitive kinases PI3 K and MAPK can confirm the IgE-mediated origin of the activating signal.[1,21] The fusion of secretory lysosomes with the cell membrane in basophil and mast cells may also be activated through G-coupled protein receptors linked to receptors for univalent exogenous substances like fMLP and ligands for MRGPRX2[22] and may be modulated by receptors for endogenous univalent substances like PAF, IL8 and C5a.[18]

2.2 | The dose-response curve

The typical BAT result in allergic patients is a dose-response curve for the %CD63-positive basophils with increasing concentrations of allergen, plateauing above baseline (Figure 3). As antigen-specific IgE-FcεRI complex causes a receptor aggregation reaction that depends on the affinity of IgE for the allergen and on the valency of the allergen, a dose-response curve is often bell-shaped reaching a plateau at higher concentrations. However, the complexity of antigens and the relative affinity of different allergen epitopes for profiles of epitope-specific IgE (bound to the cell) of different patients results in dose-response curves that vary in form. As can be seen from the variability shown by the different dose-response curves, tests with single concentrations of antigen can be misleading. There are a number of factors that can impact the dose-response curves of basophil surface activation markers such as affinity of the antigen for the IgE, epitope diversity of the IgE antibody, the density of the epitope-specific IgE on the cell surface and an intrinsic characteristic of the basophil itself. The combination of these factors determines the optimal allergen concentration for basophil activation, which varies significantly among subjects and between different allergens in the same subject.[23,24] Therefore, it is preferable to include a broad range of allergen concentrations to better appreciate the effect of the allergen on basophil response.

2.3 | The importance of non-IgE and IgE-mediated controls and the enigma of non-responder basophils

It is important to document that the blood basophils are alive and capable of mounting a response to a non-IgE stimulus, confirming that the activation test is valid. The bacterial tripeptide fMLP that activates basophils through the G-protein coupled fMLP receptors, is often used as a non-IgE-mediated positive control.[1] Degranulation through fMLP occurs faster than the IgE-mediated response. It is insensitive to Staurosporine and Wortmannin, that inhibit IgE-mediated degranulation.[31] After confirming that blood basophils respond to fMLP, it is important to assess whether they respond to IgE-mediated controls, such as anti-IgE or anti-FcεRI. Basophils that

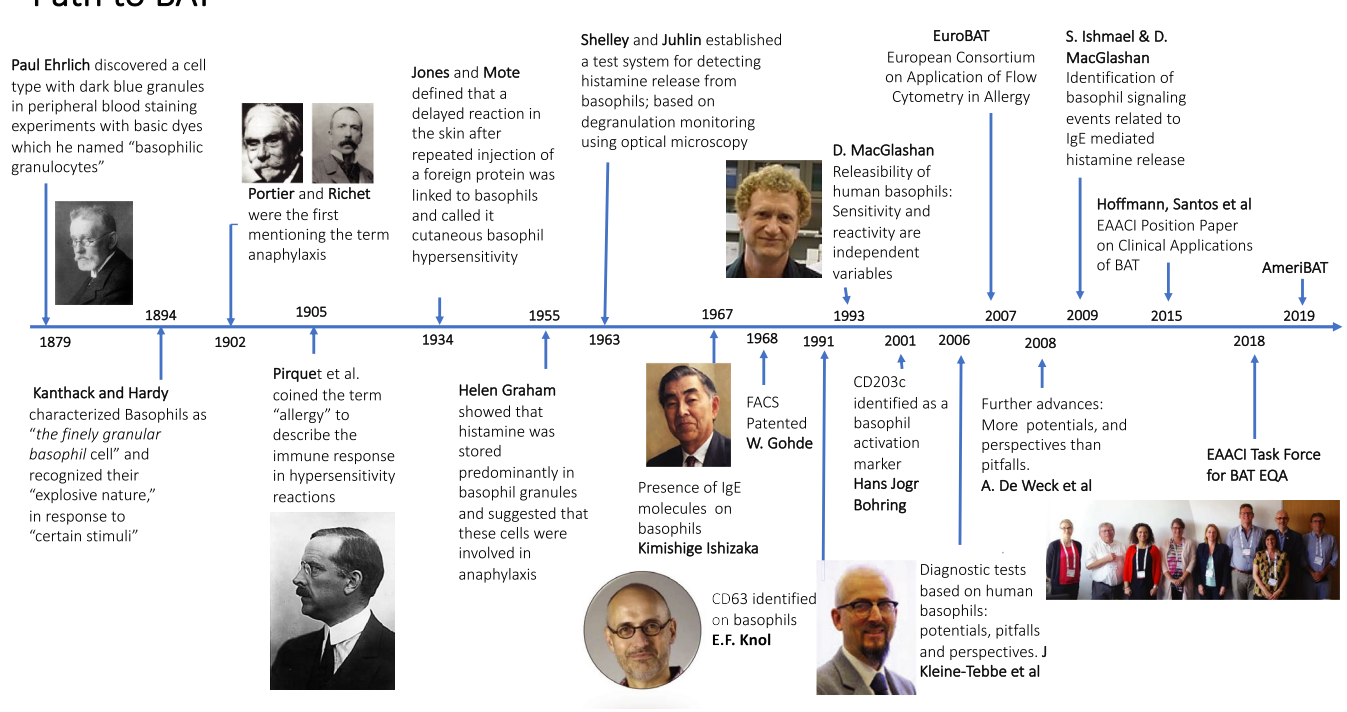

FIGURE 1 Historical timeline of the basophil activation test (BAT). EQA, external quality assurance

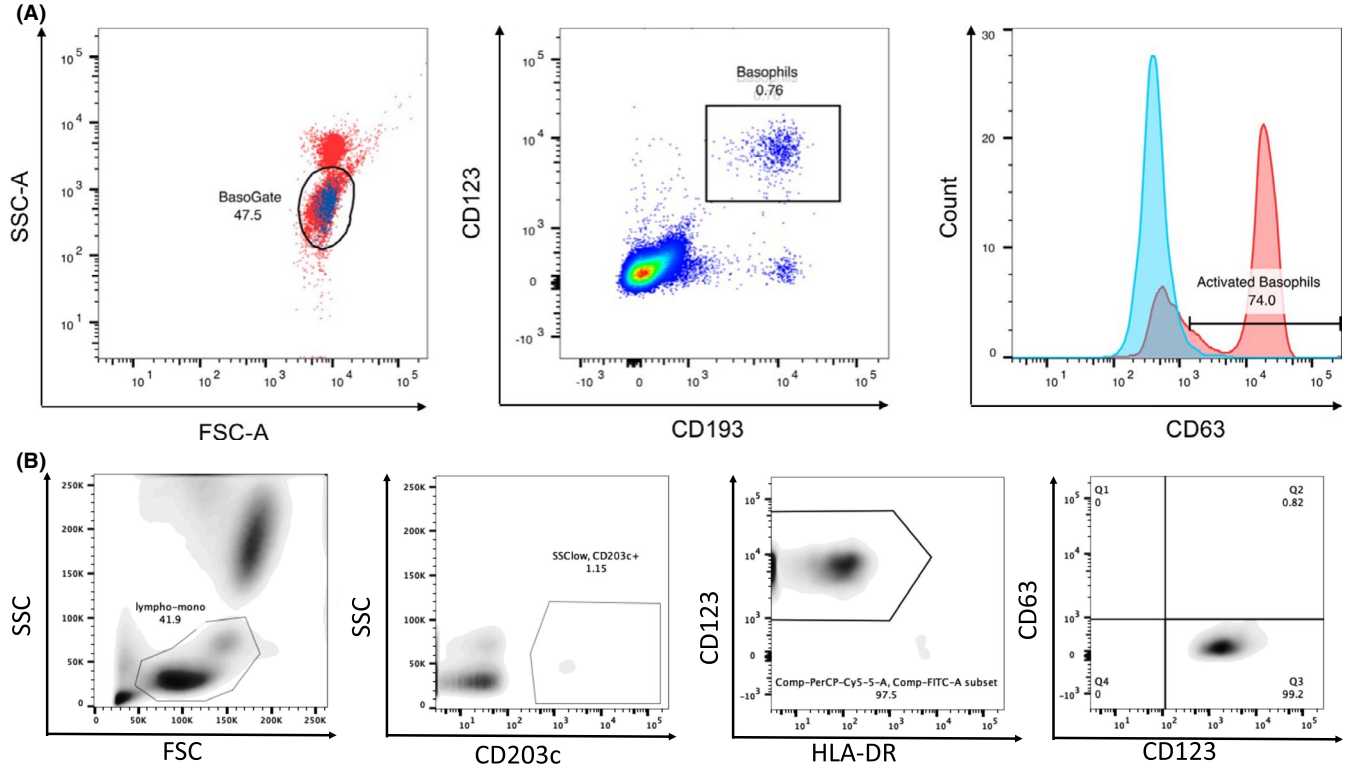

FIGURE 2 Examples of gating strategies for basophils: (a), Basophils were identified as SSClow CD123c^{+} CD193^{+} cells[45,48]: 1. Lymphocyte – monocyte gate on a FSC/SSC plot using a logarithmic scale, 2. Doublet exclusion FSC-H vs. FSC-A, then SSC-H vs. SSC-H, 3. Gate on both markers simultaneously CD123 and CD193, 4. CD63 negative threshold was set to 2.5% and the positive population above that threshold was assessed. (b), Basophils were identified as Lymphocyte/monocyte gate, SSClow CD203c^{+} CD123^{+} HLA-DR-.[2,6,26,37] The CD63 gate is set on the negative control and basophil activation is measured above this gate for the other stimulation conditions, either with allergen or positive controls

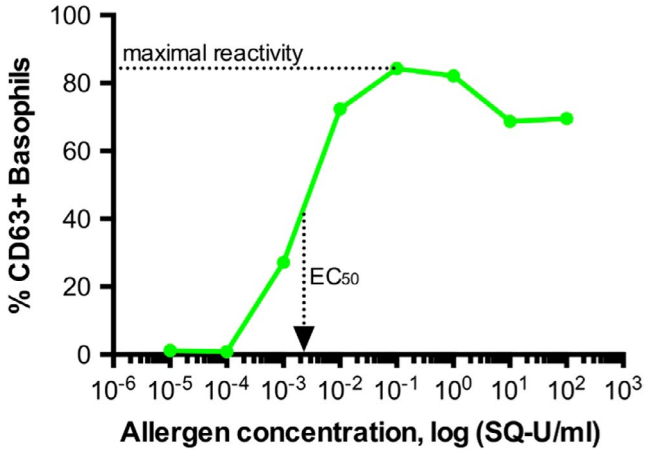

FIGURE 3 A typical dose-response curve of basophil activation with increasing concentrations of allergen of a grass pollen allergic patient from a published study[45,48] is represented. The maximal basophil activation expressed is a measure of basophil reactivity. EC_{50} is the concentration eliciting the half-maximal basophil activation and is a measure of basophil sensitivity

do not get activated in response to a stimulus through IgE/FcεRI, but only to non-IgE-mediated controls are designated 'non-responders'.

Basophils of approximately 10% of the population transiently do not respond to stimulation through FcεRI[25,26] even though they express normal densities of cell surface IgE and upregulate CD63 well to an IgE-independent stimulus. One cause of non-responsiveness is a low level of Syk phosphatase,[27–29] possibly in combination with elevated amounts of CD45.[17] The non-responder state has also been reversed experimentally *in vitro* by culturing basophils in the presence of IL-3.[30] In a large study performed in Singapore,[25] basophil non-responsiveness was associated with lower amounts of basophil Syk, and as the amount of allergen-specific IgE increased, the amount of basophil Syk is transiently decreased by allergen exposure to limit the allergic response. Basophil non-responsiveness was associated with an apparent reduction of the incidence of rhinitis in this study[25] and may be a regulatory mechanism to prevent unwanted reactions against allergens. In a peanut allergy study,[2] the vast majority of subjects with non-responding basophils were not peanut allergic but there was a minority who reacted to peanut on the double-blind placebo-controlled food challenge on the same day that basophils were non-responsive in the laboratory. It is not clear at this time, however, whether it is the clinically relevant allergen that is modulating this basophil response. More studies are needed to explore the immune mechanisms underlying non-responder phenotype and its clinical relevance.

2.4 | Parameters that can influence the results of the basophil activation test

Various factors can affect the results of the BAT, for instance: time between blood collection and the performance of BAT, medication that the patient being tested may be on, material used for basophil stimulation, antibodies used for staining of key markers and flow cytometry analyses.

Blood basophils are best used fresh, ideally on the same day or up to 24 h of blood collection.[32] It is possible to obtain a positive result after 2 days[32,33]; however, a decrease in reactivity is observed over time.[32] Individuals being tested on BAT should stop treatment with oral steroids 3 weeks before the test.[34] Antihistamines and topical treatments with steroids do not influence the result of BAT.[34]

Ideally, standardized extracts, recombinant or purified allergens or parenteral drug preparations should be used for the BAT. If necessary, the patient can bring the relevant allergen with them (Peppy's principle).[5] An allergen the patient brings can be solubilized according to standard methods[5] and should be used at concentrations not toxic to blood cells. Typically, <1% w/v is usually the highest concentration that can be tolerated. Response to more than four sequential log dilutions of allergen should be determined. If a patient's basophils respond to allergen extract, a consecutive, non-sensitized control should be tested for response with the same preparations to confirm specificity of the reaction. Following stimulation, incubation of basophils during the stimulation phase is done at 37°C in either a water bath or an incubator.[35]

Activated basophils are identified by measuring the percentage of CD63 positive cells and the fold change in CD203c MFI compared to negative control. During the gating analysis, it is important to have the same threshold set on a negative control at the same level of reactivity. When diagnosing drug allergy, a threshold of 2.5% $CD63^+$ basophils in the unstimulated condition gives results most concordant with drug provocation testing.[35] The standard positive threshold that is empirically adopted for the positive controls is more than 5% $CD63^+$ basophils. For specific allergens, this empiric cut-off can be used for rare allergens or if there is no study available but ideally the cut-off should be calculated using ROC curve analyses of data collected in rigorous and purposely built diagnostic studies. Methods of automated data analyses have been developed and have the advantage of being more standardized and objective compared to manual gating, which is, however, still considered the gold-standard.[36]

2.5 | Reactivity and sensitivity may be distinct measures of basophil response

Basophil reactivity refers to the proportion of basophils that express CD63 compared to the negative control and can be expressed as $\%CD63^+$ basophils at a given allergen concentration (Figure 3) or as the ratio of $\%CD63^+$ to allergen and the IgE-mediated positive control (anti-IgE or anti-FcεRI). It serves to document the presence of biologically relevant sensitization to allergen through IgE. Two recent studies of peanut allergy found a relationship between reactivity and symptom severity[26,37]; however, in a study of wasp venom allergy, basophil reactivity to wasp allergen extract could not predict patients symptom severity.[38] Basophil sensitivity[39] has been shown to be useful in the diagnosis of allergic asthma,[40] rhinitis,[41]

TABLE 1 Sensitivity and specificity of the basophil activation test to diagnose different allergic conditions

Allergic disease	Examples	Allergen stimulation	Optimal cut-off	Sensitivity	Specificity
Food allergy	Peanut allergy[2]	Peanut extract 0.1-10,000 ng/ml	8.11% $CD63^+$ basophils	98%	96%
	Egg allergy[113]	Ovalbumin 0.1–100 μg/ml	5% $CD63^+$ basophils	77%	100%
Drug allergy	Beta-lactams[114]	Various	5% $CD63^+$ basophils	55%	80%
	Neuro-muscular blocking agents[115]	Rocuronium	4% $CD63^+$ basophils	80%	96%
Insect venom allergy	Wasp venom[116]	Wasp venom, 0.0001–1 μg/ml	10% $CD63^+$ basophils	85%	83%
	Bee venom[116]	Bee venom, 0.0001–1 μg/ml	10% $CD63^+$ basophils	91%	93%
Respiratory allergy	Grass pollen[40]	Grass pollen extract, 100–0.0001 SQU/ml	2.5% $CD63^+$ basophils	ND	ND
	Aspergillus[117]	A fumigatus extract (10 μl) or rAsp f 1	ND	ND	ND

food allergy,[2,37,42,43] allergen immunotherapy[44–48] and anti-IgE therapy.[49–51]

Basophil sensitivity refers to the allergen concentration eliciting half-maximal basophil activation and can be expressed as EC_{50} or CD-sens which is the inverse of EC_{50} multiplied by 100 and can be calculated based on the slope of the dose-response curve[26,39] (Figure 3). EC_{50} decreases whereas[45,48] CD-sens increases with the severity of allergic reactions.[39] Determination of sensitivity of basophils to allergen by flow cytometry was preceded by studies determining basophil sensitivity to allergen by measuring the release of histamine, PGD_2 or Cys-Leukotrienes.[5] Activation of blood basophils should be assessed at each of 5–12 log dilutions of allergen. The degree of reactivity at each allergen concentration is plotted against allergen concentration, and both maximal reactivity and half-maximal reactivity are determined by fitting a non-linear curve to the dose-response. Basophil sensitivity correlates with the patient´s sensitivity to allergen at the clinical level, both in respiratory[40,52] and in food allergies[2,26,42,43,53] and changes in sensitivity reflect the clinical improvement in allergic rhinitis.[44,45,47,48,54,55] Basophil reactivity and basophil sensitivity appeared to be distinct parameters of activation[56,57]; however, systematic analyses of signalling molecules in the pathway leading from IgE crosslinking to degranulation show that they are interdependent and both are regulated by Syk.[58,59]

3 | WHAT CAN BAT TELL US ABOUT ALLERGIC REACTIONS?

Acute immediate allergic reactions and anaphylaxis result from the effect of mediators released by basophils and mast cells following exposure to the allergen. Blood basophils are more readily available in peripheral blood than tissue mast cells and thus constitute an accessible relevant sample to study immediate allergic reactions and anaphylaxis. There is clear evidence that basophils contribute to the allergic reactions from studies measuring basophil activation ex-vivo during food and nasal allergen challenges and from studies of fatal or near-fatal anaphylaxis in which mast cell tryptase was not measurable over time, despite the concomitant persistence of allergic symptoms.

The BAT has been shown to reflect the allergic status of patients sensitized to food, inhalant and insect venom allergens in different studies, with the basophils of allergic subjects typically showing a dose-dependent increase in the $\%CD63^+$ basophils or in the mean fluorescence intensity of CD203c.[60,61] Such studies have led to a growing force into applying BAT to the diagnosis of IgE-mediated allergic disease, given its very high specificity with retained high sensitivity compared to IgE sensitization tests, namely in food allergy (Table 1). In a peanut allergy study,[2] which was recently validated further,[62] the specificity of BAT to peanut ranged between 96 and 100%. Such high specificity strongly supports its use to confirm the diagnosis of food allergy and dispense patients from risky and stressful exposure to the allergen during challenges.[63] In patients with allergic asthma, CD-sens was correlated with the allergen dose used in bronchial challenge causing a 20% drop in forced expiratory volume in 1 s (PD_{20}). This correlation was mostly due to patients with low AHR and was not seen in patients reacting with high AHR, which further suggests that this correlation is allergen-specific and that BAT reflects the allergic component in the bronchial responsiveness.[40] In venom allergy, BAT can add clinical value to IgE testing and can be particularly useful in cases of undetectable IgE sensitization or double sensitization to both wasp and bee venoms.[64] In case of dual sensitization, if the concentration to which the basophils react between bee and wasp venom allergens is more than 10-fold different, the primary sensitizer allergen is likely to be the one to which the basophils respond at a lower concentration.[64] Autoimmune chronic spontaneous urticaria can also be diagnosed with BAT.[65]

Basophil activation test can also be useful to describe more detailed aspects of allergic patients' phenotype. For instance, patients with different phenotypes of milk and egg allergy have shown different profiles of CD63 upregulation following allergen stimulation

with children tolerating baked milk/egg while reacting to fresh milk/whole egg showing an intermediate degree of basophil activation between children who were allergic to all forms of milk and children who had outgrown their milk/egg allergy.[66,67] A greater proportion of activated basophils has been associated with increasing severity of allergic reactions and basophil sensitivity with the threshold dose at which patients reacted during challenges to peanut.[37,62,68–71] This is another example of how BAT can be used to define more subtle characteristics of the allergic response beyond the dichotomic classification of allergic vs. non-allergic.

4 | CHANGES IN BAT WITH IMMUNOMODULATORY TREATMENTS

Apart from identifying patients' allergic status at a given time point, BAT may be a useful tool to monitor natural changes in allergic status over time or with immunomodulatory treatments.

As basophils express FceRI and bear IgE, they are an effector cell of interest to explore the long-term effects of immunotherapy[72]; the suppressive effects of blocking antibodies induced during treatment. A change in basophil sensitivity during the first 3 weeks of allergen immunotherapy correlated strongly with the clinical effect of treatment during the first year[45] as well as after 3 years of treatment[48] and could be developed into a diagnostic biomarker for allergen immunotherapy. BAT may also be valuable in replacing sting challenges to guide when to stop immunotherapy to hymenoptera venom.[73] The decreased basophil activation that accompanies AIT can be due to intrinsic (eg, cellular anergy) or extrinsic (eg, blocking antibodies) changes to the basophils. Passive sensitization approaches in which pre and post-treatment plasma are used to sensitize primary basophils or to pre-incubate with allergen prior to adding sensitized cells are ways to assess the function and suppressive effects of post-treatment plasma containing blocking antibodies.[73–75] Another experimental setup that can be used to explore the effects of blocking antibodies is the washed BAT, in which plasma surrounding basophils is removed, and its comparison with whole blood BAT.[45] Typically, post-treatment plasma contains allergen-specific antibodies of different isotypes to IgE, namely IgG and IgA, that compete with IgE for allergen binding reducing the amount of allergen that is able to cross-link IgE antibodies on the surface of mast cells and basophils and therefore reducing the chance of inducing an allergic reaction or its severity.[76] Evidence that blocking antibodies can induce inhibitory cell signalling through ITIM-coupled receptors is lacking in natural tolerance or desensitization through IT.[77]

Various studies have documented a decrease in basophil reactivity and sensitivity following allergen-specific immunotherapy to food, respiratory and insect venom allergens.[45,48,78–80] In food allergy, a decrease in basophil reactivity during treatment has been observed to the culprit allergen and a bystander allergen as well as IgE-mediated (but not non-IgE-mediated) positive controls suggesting changes intrinsic to the basophil during the course of oral immunotherapy. These changes, which are typical of basophil anergy, accompany clinical desensitization to the allergen, as measured by the increase in threshold of reactivity while on treatment.[81] The decrease in basophil reactivity can be stronger in oral compared to sublingual immunotherapy to foods, mirroring the difference in efficacy of oral immunotherapy (OIT) compared with sublingual immunotherapy (SLIT) in terms of the dose of allergen tolerated during treatment.[82] As the reduction in basophil reactivity can be transient, which is similar to the clinical effect of oral immunotherapy in some patients following discontinuation of treatment,[82] it may be a good test to monitor relapse of the allergy.

Basophil activation test has also shown to be useful in monitoring the response to treatment with omalizumab.[50,83–85] In a peanut study, the BAT was used to make decisions about the need to adjust the dose of omalizumab.[86] Given that the anti-IgE antibody captures IgE in circulation and reduces the IgE that is bound to receptors on the surface of circulating basophils and tissue mast cells, it leads to a progressive reduction in surface expression of FceRI on effector cells and in response to the allergen in vitro in the BAT.[50,51,87] However, because the reduction in receptor density on the surface of these effector cells enhances their intrinsic sensitivity,[88] omalizumab can paradoxically increase basophil reactivity to the allergen. As a result, the patients that are most likely to better respond to omalizumab are the ones with higher allergen-specific activity, that is, the ones whose proportion of IgE that is allergen-specific is higher.[89,90] BAT can potentially be useful in assessing the response to other biologicals in terms of their effect on the risk of acute reactions to a given allergen. The BAT has also been useful in confirming the diagnosis of autoimmune urticaria, in identifying subtypes of chronic urticaria and in assessing response to omalizumab in this context.[65,91,92]

5 | THE USE OF THE BASOPHIL ACTIVATION TEST IN CLINICAL TRIALS

Basophil activation test has a huge potential in clinical trials, both as a biomarker for inclusion and as a biomarker of clinical response to treatment, and also in the exploration of possible underlying mechanisms at the effector cell level. However, there are practical aspects that need to be considered in order for the results to be informative, reproducible and comparable between study sites. Table 2 presents some of the practical issues and suggestions to circumvent them and reach an optimal use of BAT in the context of clinical trials.

In addition to being a surrogate of clinical outcomes of therapies, a key application of BAT in future clinical trials is to confirm eligibility of patients for allergy treatment. This is particularly important in the context of food allergy. At the moment, eligibility for food immunotherapy requires the performance of allergen challenge in patients that have been previously diagnosed with food allergy. Having to undergo an oral food challenge for a patient known to be allergic can be quite stressful and additional challenges are often required in study protocols to assess clinical response to IT.[93,94] This approach is unlikely to be well accepted by patients and families in clinical practice, as patients being considered for treatment have already been diagnosed with food

allergy and may be fearful of exposure to the allergen, even in the context of an oral food challenge. Giving a blood sample for a BAT may be more acceptable. Depending on the thresholds of reactivity required, challenges done as part of study protocols can exclude allergic patients with high threshold of reactivity that would otherwise benefit from such treatment. Similar considerations can be made for biologicals, which are often reserved for patients with severe allergic conditions, that may be at additional risk of undesirable outcomes during allergen challenges.

6 | THE USE OF BASOPHIL ACTIVATION TEST IN CLINICAL PRACTICE

The BAT can have different applications in the day-to-day clinical setting – Figure 4 and Table 3 summarize some of the possible indications of BAT, which can be categorized into three main groups: 1) confirmation of an allergy, 2) eligibility for a specific therapy and 3) monitoring of the response to therapy or natural resolution of an allergy. The confirmation of allergy is important for several reasons. Firstly, it improves the safety profile of the diagnostic work-up, as it may defer the need for an oral food challenge, preventing potential anaphylactic reactions. Secondly, it allows confirming the indication for immune modifying therapies that may require prolonged exposure to medications before the clinical response is seen. Examples for this is the use of omalizumab in allergic asthma and initiation of oral food immunotherapy, both of which require many months on therapy to assess response. Thirdly, BAT may be useful to measure the response to treatment and act as a surrogate of in vivo allergen exposure, like in a food challenge. Even in cases where basophils show no response to allergen and the positive control, anti-IgE (known as non-releaser or anergic basophils), data is emerging that is suggestive of this finding is more likely to indicate low clinical reactivity to allergen.[25] Furthermore, BAT also has value in autoimmune chronic spontaneous urticaria[65] and rare allergic disorders, such as allergic bronchopulmonary aspergillosis, as an additional criterion for diagnosis, particularly in patients who do not fulfil the minimal diagnostic criteria.[95,96]

The use of BAT in clinical practice requires: analytical validation of the methodology, clinical validation of the test against patients' phenotype and continued quality assurance.[36,60,97,98]

6.1 | Analytical validation of the basophil activation test

Analytical validation determines the accuracy of the testing procedure from the draw of the blood sample to the reporting of the results. There are several important components of the analytical validation of a basophil activation test[99]:

TABLE 2 Practical issues and considerations for optimal use of BAT in clinical trials

Practical issues	Ruggestions	Implications for clinical trials
Basophil reactivity is reduced over time.[33]	Perform BAT within a few hours (up to 24 h) of blood collection.	• Good transportation system between sites to ensure timely delivery of samples. • Test samples of all study sites within the same time frame.
Basophil reactivity can be affected by vibration and changes in temperature.[33]	Ensure method of transportation that ensure stability of temperature transfer of samples.	• Prefer transport system with temperature control for samples.
Immunosupressors, including oral corticosteroids, can reduce basophil response.[34]	Avoid performing BAT in patients who are on immunosupressors.	• Need to continue treatment with immunosupressors should be an exclusion criteria of studies using BAT.
Exposure to allergen, chronic inflammation and infection can induce basophil degranulation and homing to the tissues.[118]	Avoid performing BAT after allergen exposure or during infection or active chronic inflammatory condition.	• Blood for BAT needs to be collected prior to allergen exposure (namely challenge but not SPT). • Active infections and inflammatory conditions should be an exclusion criteria of studies using BAT.
Basophil activation can vary with the anticoagulant used.[33]	BAT can be performed in blood collected into heparin or EDTA.	Blood for BAT should be collected using the same material and methodology during studies and between sites.
Measurement of basophil activation can be influenced by the markers used to identify the basophils, by the BAT protocol and by flow cytometry.[6]	BAT should be performed with a validated method and standardized conditions.	The same reagents and protocol should be used throughout a clinical trial and flow cytometers should be standardized.
Quantification of basophil activation can vary with the method adopted for data analyses.[6,36]	Criteria should be defined for each step of flow cytometry data analyses. Automated data analyses can be considered.	The exact same methodology of analyses of flow cytometry data needs to be used between centres and throughout the clinical trial.

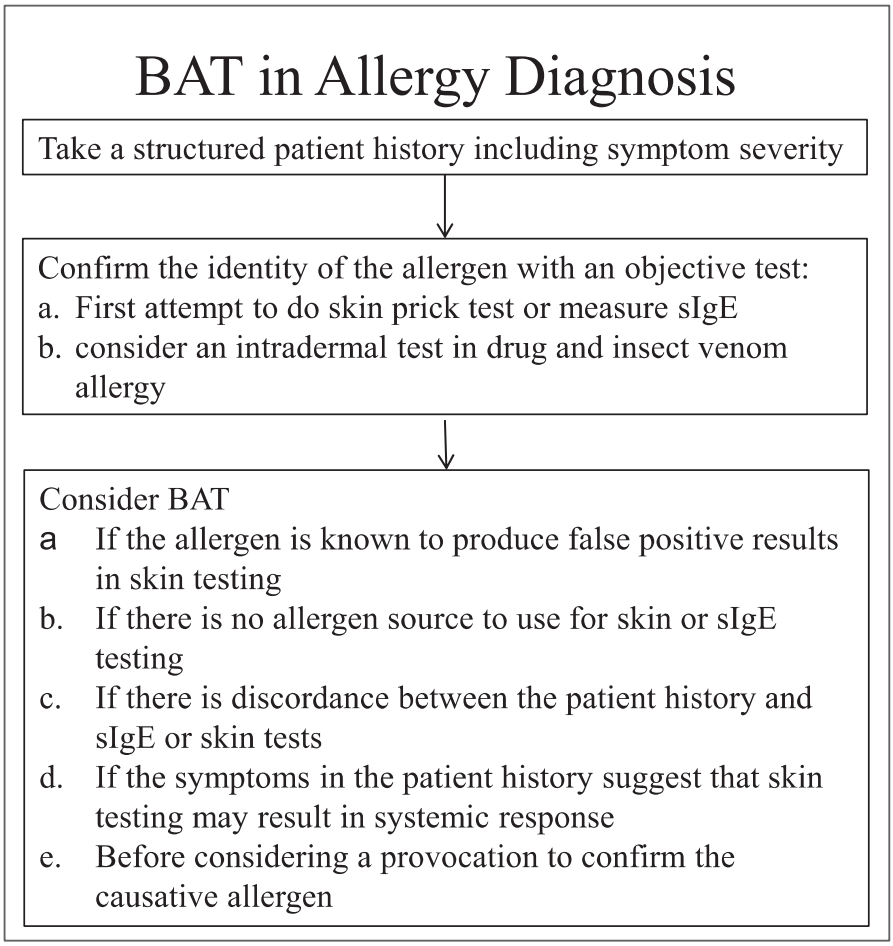

FIGURE 4 Indications to consider performing the basophil activation test for diagnostic purposes (with permission from the EAACI Position Paper on Clinical Utility of the Basophil Activation Test, BAT[5])

1. *Inter and intra-run precision*: Inter-run precision analyses samples at different time points, whereas intra-run precision assays for repeats of samples at the same time point on the same day. The precision analysis for the BAT shows good correlation.[97]
2. *Analytical interferences*: A given allergen does not stimulate/induce basophils of non-allergic patients and basophil activation in a given patient are specific to the allergen being tested and the concentration of the allergen. A given concentration of allergen does not induce the same basophil response in all patients, hence the importance of clinical correlations for each allergen at a number of concentrations.
3. *Stability of samples*: The question of stability of the samples before reaching the laboratory has mostly been resolved.[33] When transported in heparin tubes, samples can stay stable up to 24 h even when shipped in ambient conditions. EDTA is an alternative calcium chelating anticoagulant that stabilizes basophils before testing but requires addition of calcium prior to stimulation. Allergens should be prepared freshly, even if previously stored frozen or lyophilized.
4. *Proficiency Testing*: For a sustained high-quality use of BAT in the clinical setting, constant quality control is necessary. In 2017 the EU approved the in vitro diagnostic medical devices regulation (IVDR), that has to be implemented by 2024.[100] Since BAT is not a widely available assay and regulatory bodies have not yet established proficiency testing, laboratories have created individualized quality control measures to assure that the validated assays continue to perform accurately. RfB (www.rfb.bio) and INSTAND (www.instand-ev.de) are planning to offer external quality assurance systems. Standardization of BAT procedures, allergen preparations and sharing databases in which annotated raw data can be deposited are important as they allow comparison of results in different centres and would ensure consistency.

It is important to note that regulations and reimbursement/coverage by healthcare systems vary for flow cytometry-based assays in different parts of the world. In the United States, BAT is used as a diagnostic test as a part of clinical decision making in allergy practices that have access to a flow cytometry laboratory.[101–103] At the time of this review there are such set-ups in private clinical practice as well as academic institutions. In Europe, the BAT is mostly used in research but has been adopted as a clinical test in some countries, such as Sweden, Spain, Germany, Denmark and Italy. Basophil testing has gained acceptance throughout the world, including South Africa, Eastern Europe and South America. Many allergy clinics use in house procedures (also referred to as "Laboratory Developed Tests") detecting CD63, others use kits that are commercially available. Efforts are underway to facilitate the standardization and quality assurance of the BAT across clinical laboratories.[5,104]

TABLE 3 Indications for the basophil activation test in the clinical setting

Indications	References
Confirmation of diagnosis	
Food allergy	Santos & Shreffler 2017[60]
Drug allergy	Aranda 2011[21]; Ebo 2006[119]
Venom allergy	Eberlein 2012[64]
Occupational allergy	Hansen 2014[120]
Allergic rhinitis	Nopp 2013[41]
Local allergic rhinitis	Campo 2015[121]
Allergic asthma	Dahlen 2011[40]
Allergic bronco-pulmonary aspergillosis	Gernez 2016[117]
Eligibility for treatment	
Allergen-specific immunotherapy	Schmid 2014[45]
Anti-IgE	Johansson 2009[90]
Other immunomodulatory treatments	
Monitoring	
Natural resolution of food allergy	Wanich 2009[66]; Berin 2008[67]
Response to allergen-specific immunotherapy	Schmid 2014/2020[45,48]
Response to anti-IgE	Nopp 2007[50]

6.2 | Clinical validation of the basophil activation test

An essential aspect of clinical validation of BAT is to determine its sensitivity and specificity for clinical correlates of interest. The sensitivity and specificity of BAT for food allergies are high, despite showing significant differences between foods.[60,105] The sensitivity of BAT for drug allergies is lower, but still BAT can be extremely useful in the case of life-threatening drug allergies in which patients cannot be re-challenged or in the case of drugs for which no other tests are available or their results are equivocal, before considering provocation tests. A summary of the specificity and sensitivity is shown in Table 1 and has been previously reviewed.[5,61]

Food allergy is the area of Allergology in which there is the largest evidence about the diagnostic performance and cut-offs for tests, such as specific IgE and skin prick testing and in which some of the largest studies on the clinical utility of the BAT were done.[105,106] Although the SPT and specific IgE are very sensitive and positive cut-offs have been determined to improve their specificity, the majority of food sensitized patients fall into an immunologically grey area, that is, have results for SPT and specific IgE that are detectable but are below the 95% PPV cut-off. For most foods, this immunologically grey zone is wide and in such cases, BAT provides significant value in differentiating true allergy from sensitization.[2,60,63,105] Even for foods for which there are informative allergen components, for instance Ara h 2 in the case of peanut, BAT can clarify equivocal cases and reduce the number of patients requiring OFC[2].

OFC is often also required to confirm eligibility for treatments for food allergy, such as OIT. For clinics that do not routinely perform OFC before starting OIT, BAT may be used as an alternative to identify allergic patients. BAT may also provide prognostic information about which patients would benefit the most from this treatment.[75] In a peanut OIT study, participants entering the study with low basophil responsiveness were more likely to achieve treatment success.[107] In another study, using grass pollen SCIT, basophil sensitivity improved within 3 weeks of the start of the allergen immunotherapy (AIT) and correlated with clinical outcomes after 3 and 4 years based on in vivo allergen challenge.[48]

The utility of the BAT is influenced by patient selection, allergens used and criteria for cut-off values.[63] There are also practical issues to consider when incorporating BAT as part of routine diagnostic work-up. For instance, although BAT to peanut showed overall best diagnostic accuracy compared to all other tests available,[2] it is faster and more cost-effective to perform skin prick test or specific IgE and therefore these tests can be used as first line. BAT has been proposed as a second-line test in patients with equivocal outcome following clinical history and IgE sensitization tests,[60] before referring patients for OFC. This proposed approach reduced the number of OFC by 67% in a previous study of peanut allergy.[2] To circumvent the need for fresh blood and the 10%–15% non-responders for whom BAT in uninterpretable, the mast cell activation test (MAT) may be used to complement the BAT.[108] The MAT uses a mast cell line grown in the laboratory to which plasma from the patients is added to mimic the patients' own mast cells. The mast cells are then stimulated with allergens or controls and analysed by flow cytometry for the expression of activation markers such as CD63 on their surface. The MAT has shown to be very specific to diagnose peanut allergy and to identify patients at high risk of severe reactions.[108] It has also been shown to be useful to test the function of IgE following allergen IT.[109] Figure 5 represents an integrated approach using various allergy tests to support the diagnosis of food allergy.

6.3 | Quality assurance of the basophil activation test

For a sustained high-quality use of BAT in the clinical setting, constant quality control, as laid out in ISO 15189:2012, ISO15189:2013 and ISO 9001:2016, is necessary and increasingly required by national legislation. For the test to be reimbursed by health care systems and insurance companies, rigorous quality assurance process needs to be in place in certified laboratories.

Representatives of European laboratories developing basophil testing have discussed opportunities of basophil testing since 2006[110,111] and have met regularly in the EUROBAT meeting series to strengthen the development of basophil tests. These meetings continue every second year under the auspices of the Interest Group Allergy Diagnosis and Systems Medicine within the European Academy for Allergy and Clinical Immunology (EAACI). To meet the increasing demand for certification described in ISO

FIGURE 5 Proposed sequence of tests to support the diagnosis of food allergy, before referring patient for an oral food challenge. CRD, component-resolved diagnosis; BAT, basophil activation test; MAT, mast cell activation test

TABLE 4 Clinical applications of the basophil activation test

Key clinical messages
• A basophil activation test above the positive cut-off supports the diagnosis of IgE-mediated allergy.
• Basophil reactivity and basophil sensitivity decrease over the course of allergen-specific immunotherapy and EC50 (or CD-sens) seem to be particularly important in reflecting the change in clinical allergen threshold.
• Omalizumab decreases basophil response to allergen as a consequence of the decrease in IgE receptor density and circulating IgE.

9001/15189 for biomedical laboratories, EAACI launched a task force with the aim of standardizing basophil testing and establishing external quality assurance under the control of EAACI. Engaging EAACI as the European organization representing professionals working with allergy in quality assurance of a cutting-edge diagnostic test would uniquely enhance the quality of the test. Standardizing the method of analysis dramatically improved coherence of the results (CV <10% for detection of $CD63^+$ basophils) in ten European laboratories.[35] As this is in stark contrast to the heterogeneity of results obtained in external quality assurance of IgE testing,[112] it is important to maintain the momentum of this process and bring it to IgE testing as well.

In the United States, at the present time, there are 9 laboratories in 6 states that provide BAT for the common food allergens with an inter-laboratory quality assurance system in place and harmonized protocols. Similar to its European counterpart, AmeriBAT was created between these laboratories that offer clinical grade BAT to establish a network of quality assurance and control (QA/QC). In this quarterly process, a blood sample from Donor A is processed the day it is collected (Day 0) in Lab 1 and then mailed to lab 2 where it is processed the following day (Day 1) and a blood sample from Donor B is processed in lab 2 on Day 0 and mailed to Lab 1 for processing on Day 1. The temperature during shipping is measured with a temperature strip to ensure that the sample is within 2-37C range. The %CV between the results for the two locations should be below 25% but results as high as 35% can be accepted as basophils can be considered rare events in whole blood.

7 | CONCLUSION

The BAT can be seen as a surrogate of immediate allergic reactions in vitro and thus support the diagnosis of allergic diseases and its monitoring during immunomodulatory treatments (Table 4). A robust laboratory method which can provide consistent and reliable results that have been clinically validated can be extremely valuable both for clinical practice and for clinical trials into existing and novel treatments for allergic disease. Standardization and continuous quality assurance as well as training of health care professionals on the interpretation of BAT results are important for further implementation of BAT in clinical practice and allergy research.

CONFLICT OF INTEREST

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ORCID

Alexandra F. Santos https://orcid.org/0000-0002-7805-1436
Oral Alpan https://orcid.org/0000-0001-7467-8541
Hans-Jürgen Hoffmann https://orcid.org/0000-0002-6743-7931

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