Principles, potential, and limitations of ex vivo basophil activation by flow cytometry in allergology : a narrative review

Principles, potential and limitations of *ex vivo* basophil activation by flow cytometry in allergology: a narrative review.

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COI: The authors have no conflict of interest nor any financial relationship to declare.

Abstract

The major challenge of allergy diagnosis lies in the development of accessible and reliable diagnostics allowing correct prediction of the clinical outcome upon exposure to the offending allergen(s) and cross-reactive structures. Since the late nineties, evidence has accumulated that flow-assisted analysis and quantification of *ex vivo* activated basophils (BAT) might meet this requirement for different IgE-dependent allergies and particular forms of auto-immune urticaria. Other, so called non-diagnostic applications of the BAT involve therapeutic monitoring, follow-up of natural histories and identification of allergenic recognition sites. However, it has also become clear that appropriate use of the BAT necessitates knowledge about degranulation metrics and guidance to guarantee correct execution and interpretation of the results. Here, we review the most relevant applications and limitations of the BAT. Some personal statements and views about its perspectives are made.

Introduction

Degranulation of basophils and mast cells (MCs) can be triggered by various processes (Figure 1). IgE-dependent degranulation involves synthesis of allergen-specific IgE antibodies (sIgE) by plasma cells. These sIgE antibodies bind to their high affinity receptors (FcεRI) present on the surface membrane of MCs and basophils to form sIgE/FcεRI complexes. Encounter of specific allergen that cross-links such sIgE/FcεRI complexes can induce degranulation with release of preformed mediators and *de novo* synthesis of inflammatory mediators. However, IgE-mediated activation is not only achieved by traditional allergens, it can also occur by lectins with a binding specificity that matches the glycosylation of IgE and/or the FcεRI, or by other molecules such as superallergens (protein Fv, HIV gp120) or S. mansoni IPSE/alpha-1 [1, 2]. IgE-independent activation results from occupation of various receptors, e.g. C3aR and C5aR (receptors for the anaphylatoxins C3a and C5a) or the Mas-related G protein-coupled receptor MRGPRX2 on MCs by drugs, as observed in some immediate drug hypersensitivity reactions (IDHRs) [3, 4].

Generally, diagnosis of sIgE-mediated allergies starts with a history taking together with skin tests (STs) and/or quantification of sIgE (including component resolved diagnosis, CRD). However, as none of these tests is absolutely predictive, many have focused on *ex vivo* basophil activation tests (BATs) to close the gaps in their diagnostic instrumentation and to avoid sometimes potentially dangerous provocations. In BATs, functionality of the cells can be explored via quantification of released mediators and phenotyping of intracellular and/or surface changes by flow cytometry (FCM). This review focuses on the main applications and limitations of FCM-based BATs and provides some guidance to guarantee correct execution and interpretation of these tests. It appears that BATs are more than a diagnostic aid but that further standardization and harmonization is required before its entrance in mainstream use. Most of the points to consider relate to the infrastructure and expertise of the laboratory, choice of read-out, allergen preparation, metrics of basophil activation, responder status of the cells and correct positioning of the BAT in the diagnostic algorithm. Needless to stress that state-of-the-art equipment and expertise is required for correct execution of commercial and/or homemade BATs and that inter-laboratory comparison of results should benefit from harmonization and standardization of both instruments and protocols. Users of BATs must realize that data from different laboratories are not always readily interchangeable and that local validation procedures are mandatory.

The basophil activation test via flow cytometry: principles and technical aspects

Basophil identification and activation/degranulation markers

The foundations of modern FCM-based BATs date from 1991, with the first description of the lysosomal associated membrane protein CD63 to be a degranulation marker of basophils [5]. At present, different protocols allowing detection of surface marker alterations, intracellular changes and exteriorization of granular content have been developed [6-8]. As shown in Figures 1 and 2 and Table E1 of repository, traditional FCM-based BATs use whole blood and rely upon cellular identification and quantification of activation and/or degranulation markers on the surface membrane. These changes are detectable and quantifiable on an individual cell level using specific fluorescent-labelled monoclonal antibodies. Actually, in most studies, basophils have been identified by scatter characteristics, reflecting cellular size and granularity combined with staining of surface markers such as IgE/CD203c, CCR3/CD3 or CD123/HLA-DR. Subsequently, after activation, the appearance or up-regulation of specific markers, such as lysosomal CD63 or the lineage-specific ectoenzyme CD203c is measured, with degranulating basophils being defined as $CD203c^{++}CD63^{+}$ cells. Based upon divergent time-kinetics, responses to secretagogues/inhibitory compounds, signalosome and absence/presence of mediator release, a CD63- and CD203c-compartment have been identified. The "CD203c-compartment" is characterized by a rapid and significant upregulation of CD11b, CD13, CD164 and CD203c [9-11]. In the "CD63-compartment", maximum upregulation of CD63 and CD107a is slower and reflects anaphylactic degranulation [11]. For a comprehensive description of these activation marker profiles, their different relationship to piecemeal and anaphylactic degranulation and their signal transduction processes the reader is referred elsewhere in this Journal [12]. Whether the CD63- and/or CD203c-based microfluidic immunoaffinity BAT technique [13], and mass cytometry (CyTOF) [14] can keep promise remains to be established. Other surface markers that can be used to quantify *ex vivo* basophil activation and degranulation are the inhibitory receptors CD300a and CD200R and the activating receptor CD300c [15-17]. Besides, basophilic activation can also be analysed by studying the phosphorylation status of signalling molecules such as p38 mitogen-activated protein kinase (MAPK) or signal transducer and activator of transcription (STAT)5 [18, 19]. Degranulation of basophils can also be explored by measuring the exteriorization of granule matrix and decrease in intracellular histamine content. Briefly, anionic proteoglycans from exteriorized basophil granule matrix are stained by cationic fluorescent avidin probes [6, 20, 21], whereas the quantification of the intracellular histamine relies upon a histaminase affinity technique in which diamino oxidase is coupled to a fluorochrome [22]. Newly synthesized cytokines such as interleukin (IL) 4 and IL-13, or their mRNA, can be trapped and measured intracellularly [23]. Finally, basophil activation can also be measured by imaging of intracellular Ca^{++} [24].

Whole blood versus separated cells and effects of IL-3

Most applied BAT methods use whole blood and study cells in their "natural" environment. However, there are various strategies to enrich and/or purify the cells [25]. If the analyses are performed in whole

blood, one should verify the possibility for *in vivo* stimulation, e.g. by exposure to IL-3. Such exposure is not associated with an up-regulation of CD63, but might be demonstrable by an up-regulated expression of CD69, CD203c, p38MAK and STAT5 [6, 18, 19, 26, 27]. Note that in whole blood settings the outcome of the test can be influenced by blocking antibodies (through CD32) [28] or stoichiometrically via interference with the IgE-allergen interaction [29]. These blocking antibodies can be deleted by washing leukocytes or using purified basophils [25]. Although modern purification protocols are designed to avoid cell loss and activation, e.g. by using low temperatures, Ca2+/Mg2+-free buffers (with added EDTA), and using negative immunomagnetic selection, cell loss and background activation can occur [30, 31]. Alternatively, basophils can be primed intentionally with IL-3 to optimize analytical sensitivity, that is, responsiveness to allergen [6, 32, 33] and autoreactive antibodies [34].

Passive sensitization experiments

Although protocols have been developed to permit BATs up to 24 hours after sampling [14, 35], a major weakness of the test remains the necessity for viable basophils, as sensitivity of the test decreases over time [33, 36]. To circumvent this limitation, several groups have adopted BATs with passive sensitization of stripped donor basophils [37, 38]. This involves stripping of bound sIgE antibodies from their surface FcεRI receptors with the aid of acidic buffers, incubation of these stripped cells with patient's serum (containing sIgE antibodies to the allergen being investigated) and finally challenging the passively sensitized basophils with the allergen at the first stage of the BAT. Donor cells for sensitizing should be from a healthy subject whose basophils are known to be good responders. Both unstripped and stripped donor basophils should be included in the controls. This procedure, apart from being laborious with a number of extra steps, carries the risk of non-specific stimulation or damage to basophils and is difficult to standardize. Results so far indicate rather varying performance. For example, in the study by Moneret-Vautrin et al [39], a food-specific IgE results between of 3.50 and 35 kUA/L was required for effective passive sensitization. In the study by Mueller-Wirth et al [40], passive sensitization was already demonstrable at drug-specific IgE titers of 1.0 KUA/L. Whether, cell lines (e.g. rat basophil leukemia (RBL) or LAD2 MCs) or donor MCs constitute valuable alternatives to circumvent the limitations of BAT remains to be established, but preliminary results seem promising [41-44]. In the comparative study of Larsen et al [37], BAT was shown to be more performant than histamine release testing and passive histamine release testing.

Selection of the optimal "allergen" and "dose" (degranulation metrics)

Other crucial elements to keep in mind are correct selection of source material, preparation and storage of the allergen extract, and metrics of the allergen dose-response curves [12]. For many applications, BATs use crude allergen extracts [45-47]. However, the inherent variability and instability of

natural allergens, complicate selection of the best source material and optimal extraction procedure (for review see: [48]). For example, thermal processing can influence the capacity of peanuts to trigger basophils [49]. This impact seems highly divergent between patients and unpredictable by SDS-PAGE or IgE binding [49]. Or, as shown in Figure E1 of the repository file, in contrast to sesame oil, a whole sesame seed extract might not trigger basophil degranulation in a patient who experienced sesame oil anaphylaxis. Fortunately, it is not all doom and gloom. On several occasions, BATs have shown to benefit diagnosis in difficult patients demonstrating clinically irrelevant sIgE results because of sensitization to cross-reactive carbohydrate determinants (CCDs) [50, 51]. For a summary on allergen concentrations see [52]. Note, these concentrations are only indicative and might not apply to laboratories using different equipment and protocols. It is also important to remember that there can be a significant difference between the stock concentration and final concentration in the aliquot. For drugs and related compounds, it is recommended to express concentrations on a molar base.

Basophil responses are characterized by a broad variability necessitating use of different stimulation concentrations enabling construction of dose-finding curves (Figure 3). These curves encompass different metrics including: basophil sensitivity, EC50 and basophil reactivity that differ according to the stimulation conditions and applied read-out. For an explanation and implications of the metrics of dose-response curves, the reader is referred elsewhere [12, 53-56]. Clearly, clinical validation of BATs cannot be considered appropriate when it failed to carefully establish allergen-specific dose-responses. Ideally, these dose-responses show a sigmoidal shape with plateau or bell-shape. However, because of complexity of most allergens and relative affinity of different epitope-paratope interactions, dose-response curves can show unpredictable complex courses that can vary among allergens and subjects. In diagnostic settings, where only a limited number of allergen concentrations are employed, there is a chance of producing false-negative results. Nevertheless, in our experience, it is not rare to find one or two optimal specific stimulation concentrations, even for drugs that can induce false-negative results because of cytotoxicity. Other explanations for false-negative results are: a non-responders status of the cells (as is observed in about 5-15% of the patients), poor storage conditions of the blood sample (analyses are best performed within 4 hours [33]), use of cytotoxic (concentrations of) allergens, degraded allergens [57], (pharmacologic) interference with surface receptors [58], inhibition by cross-reactive compounds [59], and blunted responses because of a preactivated status of the cell [30]. Therefore, BATs should always include a negative control setting to assess spontaneous expression of the readout, a positive control to verify responsiveness of the cell upon cross-linking of surface sIgE/FcεRI complexes. fMLP, that acts independent from IgE/FcεRI can be of benefit to include such a positive control to verify cell viability. In “non-responders”, who do not react to positive control and allergen, negative results should be considered as uninterpretable. If cells do not respond to positive control but do to allergen, the test can be considered as positive, provided

there is no nonspecific stimulation in at least 3-5 (exposed) control individuals. Non-responsiveness is attributed to disturbances in the signalosome of the FcεRI-pathway, particularly failure to express the downstream tyrosine kinase Syk. Although IL-3 can restore non-releaser status, this approach is of little help in traditional BATs as it takes days for conversion [60]. It should be kept in mind that IL-3 can blunt responses measured via upregulation of expression of CD69 and CD203c and phosphorylation of p38MAPK and STAT5.

Determination of the decision threshold (cut-off limit)

Validation of a diagnostic cannot be considered appropriate when it failed to establish a decision threshold differentiating between positive reactions and responses in control individuals. In such studies one should establish optimal allergen-specific decision thresholds and abandon pre-defined arbitrarily chosen cut-off limits for determination of sensitivity, specificity, predictive values and performance of the test. Normally, the cutoff of in vitro tests is defined by the mean of blank tests + 3.3 SD. However, for BAT this definition is rarely applied. As shown in Figure 3, an alternative method to calculate optimal decision thresholds is analyses of two-graph receiver-operating characteristic (TG-ROC) curves. In TG-ROC curves, the test sensitivity and specificity are plotted against the threshold limit assuming the latter to be an independent variable. For rare conditions it might be difficult to include a minimum of patients to construct TG-ROC curves. In such cases, comparison of the results in the patient(s) with control experiments in minimally 3-5 (exposed) control individuals is proposed. The importance of cut-offs in BAT is stressed by Dreborg, who argues the use of poorly documented decision thresholds [61].

Clinical applications

Over 25 years, BATs have evolved into a useful test in the evaluation of patients with inhalant, food, Hymenoptera venom, *Hevea* latex, immediate drug allergy and some forms of chronic urticaria/angioedema. However, its place within the diagnostic algorithms is highly variable and often still poorly determined. Actually, the position of the BAT is determined by its ease to ascertain a correct execution and interpretation, and the availability of (a) safe, more accessible, better performing alternative(s). For example, as drugs are manufactured according to GMP principles, easily accessible, and only few alternative *in vitro* tests are available, it is likely BATs to deserve a more predominant place in the diagnostic algorithm for IDHRs than for other IgE-mediated allergies. For inhalant, food, Hymenoptera venom and *Hevea* latex allergy, extract preparation and interpretation of results might be less obvious and the merit of the test must be seen in a more global context of other available diagnostics, including CRD.

Inhalant allergy

As shown in Table E2 of the repository file, utility of the BAT has been explored in allergy to house dust mite (HDM), pollen and cat using natural extracts and purified/recombinant components. Although overall performance of BATs in inhalant allergy is good, the technique is rarely beneficial. Diagnosis of inhalant allergies can readily be established by other means such as STs and measurement of sIgE, including CRD. However, in cases of "entopy", that is, local allergic rhinitis, with positive nasal provocation tests and negative SPTs and sIgE, BATs allowed diagnosis in 8 out 16 patients [62]. Correlations between basophil sensitivity and nasal/bronchial provocation tests as well as asthma severity and efficacy of omalizumab treatment have been described [53, 63]. BATs have also been used to monitor allergen-specific immunotherapy (AIT) for, HDM, birch, timothy grass, *Lolium perenne*, mugwort, *Parietaria*, Japanese cedar and cypress [64-68]. In these studies, reduced basophil sensitivity occurs early during AIT and is likely due to interference of blocking IgG antibodies.

Food allergy

The performance of the BAT in food allergy has already extensively been studied and reviewed. From these reports, the BAT has emerged as a potential diagnostic for primary and secondary food allergies including the tick-borne alpha-gal syndrome [69-71]. However, it is premature to claim BATs to be "food challenges in a test tube" [70]. The utility of BATs is allergen-dependent and requires validation for different allergens and phenotypes (e.g. oral allergy syndrome *vs*. anaphylaxis). However, such a validation might be challenging for reasons already addressed above but also because of distinct age and geographic-related sensitization patterns [72, 73]. Finally, it should be kept in mind that the test performance can depend on the control group. For example, in exploring its performance in the context of cross-reactivity syndromes, a comparison between patients and healthy control individuals is likely to result in an overestimation of its specificity [51, 73, 74]. In other words, a comparison between true patients and healthy control individuals might not be representative for the general clinic population in which one might need to differentiate between clinically relevant and irrelevant sensitization, rather than to dichotomize between patients and controls. Table E3 of the repository file summarizes the sensitivity and specificity from BATs in food allergy. Predictive values are summarized in table E9. BATs can be useful to discriminate between clinically relevant and irrelevant sIgE results or when no alternative *in vitro* tests are available [55, 74-76] or for reducing the need for challenges in difficult cases who experienced severe anaphylaxis [70, 77]. Alternatively, as already mentioned higher, one should keep in mind the possibility of clinically irrelevant BAT results induced by dietary lectins [1, 2].

With respect to BATs using components, it is noteworthy a single component rarely to cover the entire sensitization profile [78, 79], and that outcomes have been reported to be age and/or population dependent [73]. BATs have also been used to monitor AIT and allergen-specific oral immunotherapy (OIT) for peanut [56, 80-82], sesame [83], cow's milk [84-86] and egg [87]. In line with the findings for aeroallergens, reduced basophil allergen sensitivity during AIT and OIT food is likely due to interference of blocking

sIgG antibodies [80-83, 86, 87]. Acosta *et al* [88], recently provided an immunological explanation why birch-associated apple allergy cannot be effectively treated by administration of the sensitizing pollen allergen (SLIT with recombinant (r) Bet v 1, the major allergen from birch (*Betula verrucosa*)). They found that treatment with rBet v 1 promotes specific IgG antibodies that cross-react with rMal d 1 from apple (*Malus domesticus*) but lack sufficient affinity to inhibit BAT with apple allergens and to induce cross-protection. In contrast, treatment with the apple allergen induced a food allergen-specific de novo antibody response characterized by IgG1 antibodies with IgE-blocking bioactivity and specific for epitopes exclusive of the apple allergen. Some have claimed BATs also to predict clinical severity and prognosis of food allergy [54, 89-91] and food challenge responses [55, 91-93], thresholds of reactivity [54, 94], to help determination when food can safely be (re)introduced [95], and to distinguish degrees of tolerance [96]. However, not all studies seem promising [72, 97]. Therefore, and because of the possibility of reporting bias, additional comprehensive studies are required for confirmation. Ideally, these studies should compare the BAT with STs and/or CRD, as these might provide similar information but in an easier manner. Recently passive BAT was used to demonstrate that mammalian glycolipid can activate allergic effector cells via surface bound sIgE in alpha-gal allergy [38] and to identify unique epitopes of certain peanut components [98]. BAT has also been used to monitor the effect of omalizumab in food allergy [99].

Hymenoptera venom allergy (HVA)

The utility of the BAT in wasp and honeybee venom allergy has been largely explored (Table E4 of repository). BAT can benefit diagnosis of HVA, especially in difficult cases that yield equivocal or negative sIgE and/or ST results. About 4-6% of patients with HVA demonstrate negative sIgE and ST results. In some of these cases, BATs can be useful to identify the culprit insect and guide treatment [100, 101]. Besides, BATs can also help to take the sting out of difficult cases presenting with double positive sIgE results resulting from sensitization to α-1,3-fucose containing CCDs present on various Hymenoptera venom proteins [100, 102, 103]. However, utility of the BAT in HVA needs to be reviewed in the global the context of other diagnostics. With the venue of CRD using non-glycosylated recombinant proteins, the BAT likely lost ground but the technique remains useful in patients with negative skin test and sIgE investigations. BAT appears not predictive for severity of sting reactions [104, 105]. With respect to venom immunotherapy (VIT) it has been shown treatment to decrease basophil sensitivity but not reactivity [104, 106-110]. Effects of VIT on basophils include early basopenia and a decrease in intracellular histamine content during maintenance treatment [109]. Some studies suggest basophil sensitivity to be predictive for side effects during the build-up phase of VIT [107, 111]. However, this is not the experience of others [112]. In patients with mastocytosis the BAT adds little, if at all, to the diagnosis in cases with negative sIgE and ST results [113, 114].

Hevea latex allergy

As shown in Table E5 of the supplementary file, the first descriptions of BAT in the diagnosis of allergy to latex from *Hevea brasiliensis* dates back from almost 2 decades ago [51]. The technique predominantly proved to be helpful to discriminate between clinically relevant and irrelevant sIgE results, the latter mainly resulting from sensitisation to CCD and profilin [115]. However, as in HVA, with the venue of CRD using non-glycosylated components, utility of the test lost ground. Alternatively, the BAT can help when other tests are unavailable, e.g. sensitization to Hev b 12, the non-specific LTP from *Hevea* [116].

Cannabis sativa

Allergy to *Cannabis sativa* (Can s) has become a significant health problem and can be associated with complex cross-reactivity syndromes involving many vegetables, fruits and latex [117, 118]. Diagnosis of cannabis allergy can be challenging, mainly because of the moderate specificity of sIgE, STs or BATs using natural extracts [119]. In such difficult cases, BAT with Can s 3, the non-specific lipid protein of C. sativa can benefit correct diagnosis; especially as traditional sIgE testing is still not available [119]. The utility of BAT with Can s 4, the oxygen-evolving enhancer protein 2 (OEEP2), in Can s 3 negative patients, remains to be established [120]. Note that Can s 3 covers approximately two-thirds of the Cannabis IgE reactivity profile [118, 119].

Drug hypersensitivity

Although drug provocation tests (DPT) are considered the gold standard, DPT might be difficult because of ethical and practical considerations. By consequence, confirmation of IDHRs generally relies on STs and quantification of sIgE. Unfortunately, diagnosis of IDHRs is not always straightforward, mainly because of uncertainties associated with STs and unavailability of drug-sIgE assays. As shown in Tables E6-E8 from the repository file, many have explored BATs as a confirmatory diagnostic in IDHRs. It has emerged the performance of BATs in IDHRs to vary considerably according to the investigated drug (class), decision threshold [61, 121], time elapsed between index reaction and testing [122]. Alternatively, application of *ex vivo* basophil experiments in IDHRs might extend beyond diagnosis [123]. Different groups have claimed utility of the BAT in diagnosing IDHRs to neuromuscular blocking agents, β-lactam antibiotics, carboplatin, opiates, iodinated contrast media, biologicals [124-126]. However, for some of these drug(s) (classes) evidence is limited [127] or controversial [57, 121, 128, 129]. In our experience, BAT adds little to the diagnosis of (amino)penicillin hypersensitivity (unpublished data). In addition, the technique could deepen our insights in immune IgE/FcεRI and non-immune mechanisms of IDHRs [59, 127, 129, 130], unveil cross-reactivity between structurally related substances [127, 131, 132], benefit identification of antibody recognition sites [130] and monitor effects of rapid desensitisation strategies [133, 134]. For some drugs such as opiates, BAT is likely to constitute the only diagnostic [127, 135]. Figure E2 of the repository file shows an example of a BAT to opiates in a patient who experienced anaphylaxis from pholcodine but tolerated a codeine and morphine DPT.

It is evident that the BAT only adds to diagnosis in IDHRs that involve basophil degranulation. BATs cannot document IDHR resulting from enzymatic inhibition of cyclo-oxygenase as happens in non-selective hypersensitivity to non-steroidal anti-inflammatory drugs (NSAIDs) and angioedema to angiotensin converting enzyme inhibitors. Therefore, we do not advocate use of the BAT in this context. Alternatively, for selective NSAID reactors, BAT might benefit diagnosis and help to depict presence or absence of parent drug/metabolite reactive IgE antibodies [136].

For diagnostic purposes, the fact that positive basophil responses cannot discriminate between sIgE/FcεRI cross-linking and alternative mechanisms is not a drawback. However, evidence of usefulness of BAT in IDHRs from IgE/FcεRI-independent pathways is limited and should be interpreted carefully. The reporting, basophils to constitutively express the MRGPRX2 receptor [30] contradicts earlier observations [8, 137] and is difficult to align with the observation various MRGPRX2 agonists not to trigger basophil degranulation in control individuals [121, 128, 129]. Figure E3 of the supplementary file shows plots of MRGPRX2 by MCs and basophils [8]. Figure 4 shows an algorithm indicating the place of BAT in the diagnostic work-up of IDHR. Particular attention is paid to its place in discriminating between sIgE/FcεRI- and MRGPRX2-mediated reactions.

Urticaria and angioedema

Acute and chronic histaminergic urticaria's and angioedema's rest upon MC and basophil degranulation via diverse innate and adaptive immune responses, including auto-immune processes [138, 139]. At present, in chronic spontaneous urticaria, two groups of MC degranulating signals have been identified, that is IgE autoantibodies to auto-allergens and IgG autoantibodies that target FcεRI or IgE/FcεRI complexes present on the MC surface. The presence of such anti-IgE or anti-FcεRI anti-bodies can be assessed functionally using patients' sera in an autologous serum skin test (ASST) and/or to passively sensitize donor basophils in the autoimmune BAT (Figure E4 of the repository file) [140-142].

Conclusion and future directions

The BAT provides the physician and clinical laboratory with a promising diagnostic approach, especially in difficult cases where traditional tests are unavailable or yield uncertain results. However, before entrance in mainstream application, additional larger scale studies are required to confirm and critically verify some observations. Ideally, such studies should assess how the BAT relates to other diagnostics. Further automation of data analyses and bioinformatic tools should advance standardization and quality assurance and thus accelerate transition to the clinics [143]. Although the non-diagnostic applications of BAT are still in their infancy, with increasing employment, it is expected the technique to become an attractive and valuable asset to study various domains of basophil activation/degranulation biology. Evidence is emerging that the BAT might help to deepen our understandings in mechanistic endotypes of IDHRs, benefit identification of antibody recognition sites,

expand our understandings of desensitisation and tolerance induction strategies, predict natural disease courses and prognosis.

Acknowledgements

Didier Ebo is a senior clinical researcher of the Research Foundation Flanders (FWO: 1800614N). Vito Sabato is a senior clinical researcher of the Research Foundation Flanders (FWO: 1804518N). The authors would like to thank Prof. Dr. Jean-Pierre Timmermans and Dr. Isabel Pintelon of the Antwerp Centre of Advanced Imaging (ACAM), Department of Veterinary Sciences, University of Antwerp, Antwerp (Belgium) for the use of the Leica SP8/LSM confocal microscope (GOH4216N).

Legends of figures main text

Figure 1: **basophil identification and activation/degranulation markers**

Basophils express various molecules that can be used in isolation or in combination to identify the cells and measure their activation/degranulation status by flow cytometry in a technique designated as basophil activation test (BAT). Basophil activation/degranulation can occur via IgE/FcεI-dependent and IgE/FcεRI-independent pathways. Individual cell activation/degranulation can be measured flow cytometrically on 4 levels. First, via appearance or up-regulation of surface markers (such as CD203c, CD63, CD300a, MRGPRX2 and avidin binding of membrane-associated exteriorized anionic proteoglycans released from granules). Second, via phosphorylation of signalling molecules (such as p38MAPK and STAT5). Third, via changes in mediator content (such as decrease of histamine or increase of trapped cytokines or their mRNA). Fourth, via increased intracellular calcium staining. Intracellular molecules are denoted in pink. For more details on the identification and activation/degranulation markers see text and Table E1 of repository. For technical details see [6, 8, 144].

Figure 2: **Confocal microscopy image.**

Confocal microscopy image of anti-IgE-stimulated basophils stained with anti-CD203c-APC, anti-surface IgE-AF405, anti-lysosomal associated protein CD63-PE, avidin-AF488 (binding membrane-associated exteriorized anionic proteoglycans released from granules) and composite view.

Figure 3: **Allergen dose-response curve and TG-ROC analyses.**

Dose-response (DR) curve for *ex vivo* basophil degranulation by recombinant Can s 3 (rCan s 3), the non-specific lipid transfer protein from *Cannabis sati*va, in 9 patients sensitized to Can s 3 (closed symbols). In contrast, in 7 control individuals cell are unresponsive to Can s 3 (open symbols). Results are obtained from individuals responsive to positive control stimulation and expressed as % $CD63^{+ve}$ basophils, the dashed rectangle denotes 1 µg/mL to be the most discriminative stimulation concentration (left). Two-graph receiver operating characteristics curve combining sensitivity (squares) and specificity (circles) for the BAT rCan s 3 (1 µg/mL) (right). For a comparative validation of BAT rCan s 3 in cannabis allergy not restricted to Can s 3-sensitized patients see [119].

Figure 4: **Resolving MRGPRX2- and IgE-binding properties of drugs: a guidance.**

(a) Referring physicians should provide complete information and tryptase values. (b) Allergological work-up of immediate drug hypersensitivity generally starts with skin tests (STs). (c) Negative STs do not preclude sensitization. (d) Diagnosis of drug allergy rarely can rely upon a positive sIgE result in isolation. (e) ST negative cases have been reported in IgE-mediated drug allergy. An IgE-dependent reaction might be confirmed in a passive mast cell activation test (*indirect MAT). (f) In patients with

evocative history and positive ST but incongruent negative combined sIgE, BAT and indirect MAT results may indicate an MRGPRX2-dependent reaction. (g) In cases with negative investigations drug challenges might be indicated. Note other mechanisms of IgE/FcεRI-independent MC activation by drugs can occur.

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Figures and legends of repository text

Figure E1: **BAT sesame vs. sesame oil.**

Individual plots for sesame and sesame oil in a patient who experienced urticaria after eating a salad dressed with sesame oil and anaphylaxis with hypotension during an open sesame challenge because of repetitive negative sIgE and skin test responses to sesame. Note the response to sesame oil (up-regulation of CD63 up to 35% and of CD203c up to 83%). For sesame no activation of the cells was demonstrable. Sesame extract was obtained using the extraction procedure described in [1] and starting from crushed seeds.

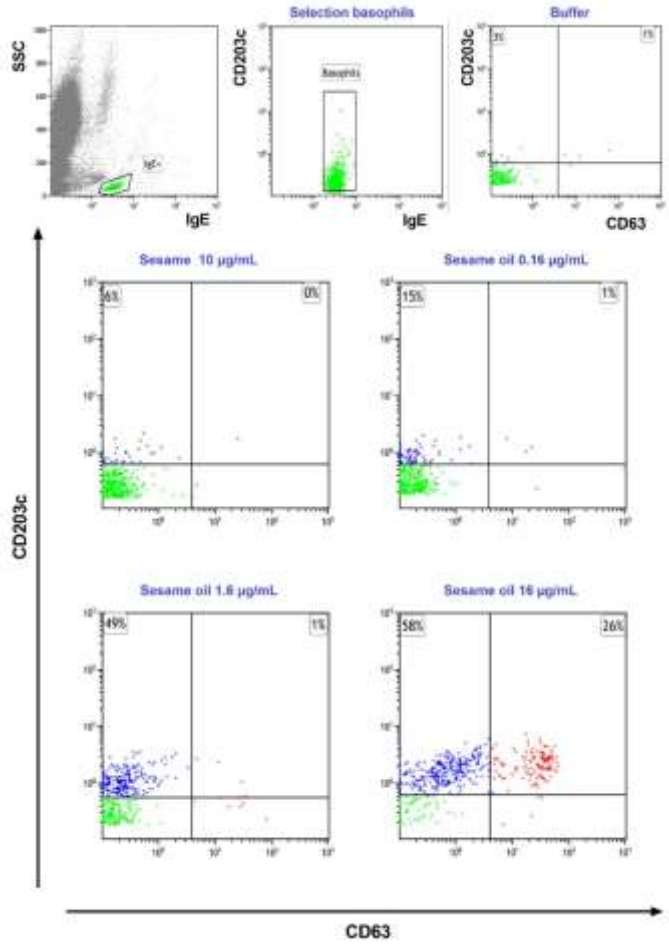

Figure E2: **BAT pholcodine and cross-reactive structures**

Representative plots of basophil activation test (BAT) in a patient who experienced an immediate hypersensitivity reaction to the opiate antitussive pholcodine and who was uneventfully challenged with the structurally closely related compounds codeine and morphine. In the context of the pholcodine-hypothesis the patient was also tested for the aminosteroid-derived curarizing neuromuscular blocking agent rocuronium. Skin tests and BAT rocuronium were negative. See [2-4]. Basophils are selected as $IgE^{+ve}/CD203c^{+ve}$ cells. Pholcodine induces a clear dose-dependent upregulation of the lysosomal degranulation marker CD63 (up to 52% of the cells). In contrast, upon stimulation of cells with codeine and morphine, expression of CD63 remains merely unchanged.

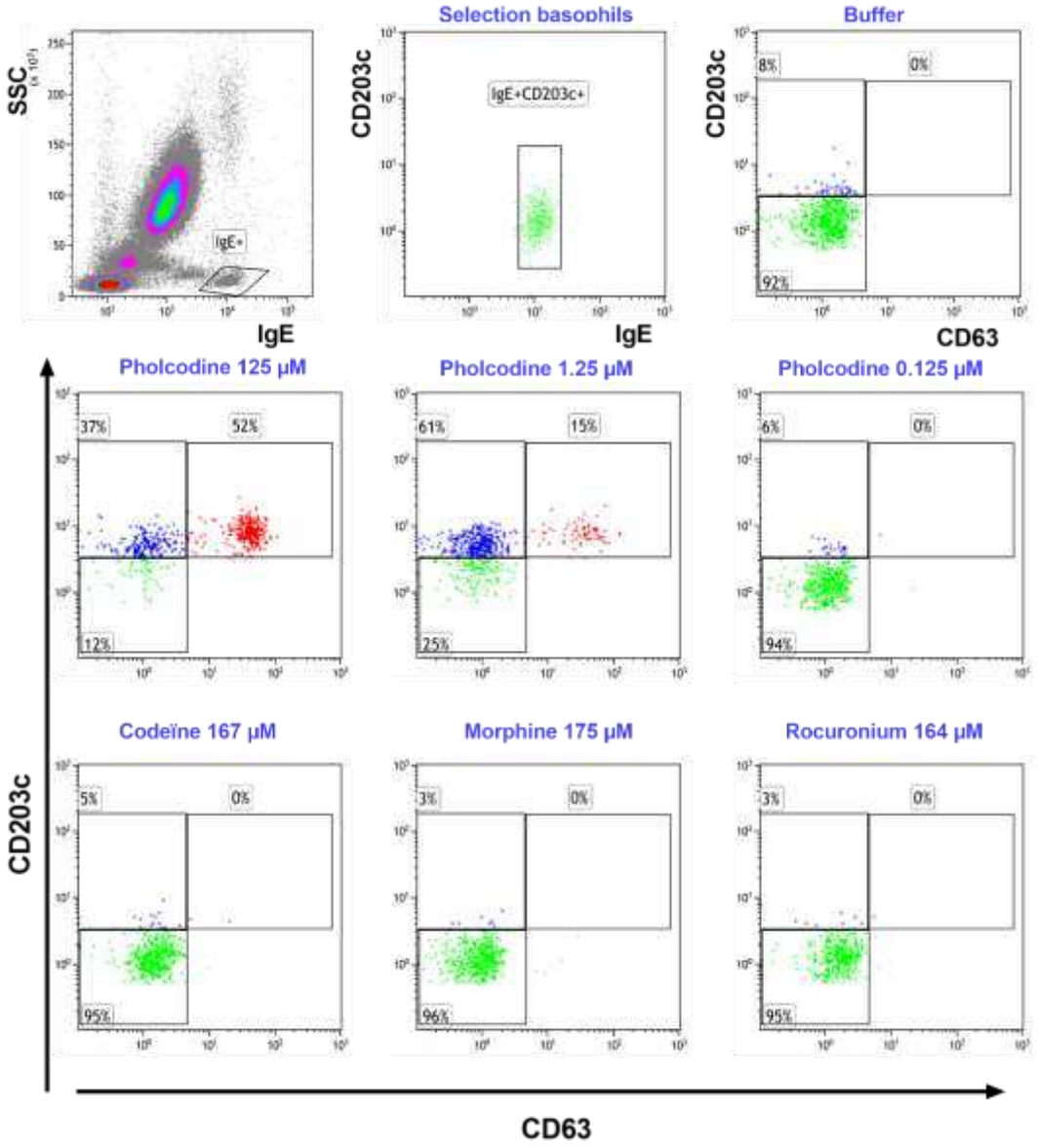

Figure E3: **Expression of MRGPRX2 by mast cells and basophils**

Intracellular and surface expression of the Mas-related G protein coupled receptor MRGPRX2 by CD117^{+ve}/CD203c^{+ve} mast cells (MCs) and IgE^{+ve}/CD203c^{+ve} basophils (A). Unlike MCs, resting basophils barely express MRGPRX2 on their surface (B). However, MRGPRX2 is quickly up-regulated by basophils in response to positive control stimulation with anti-IgE (C). For details on MRGPRX2 staining in basophils see [5].

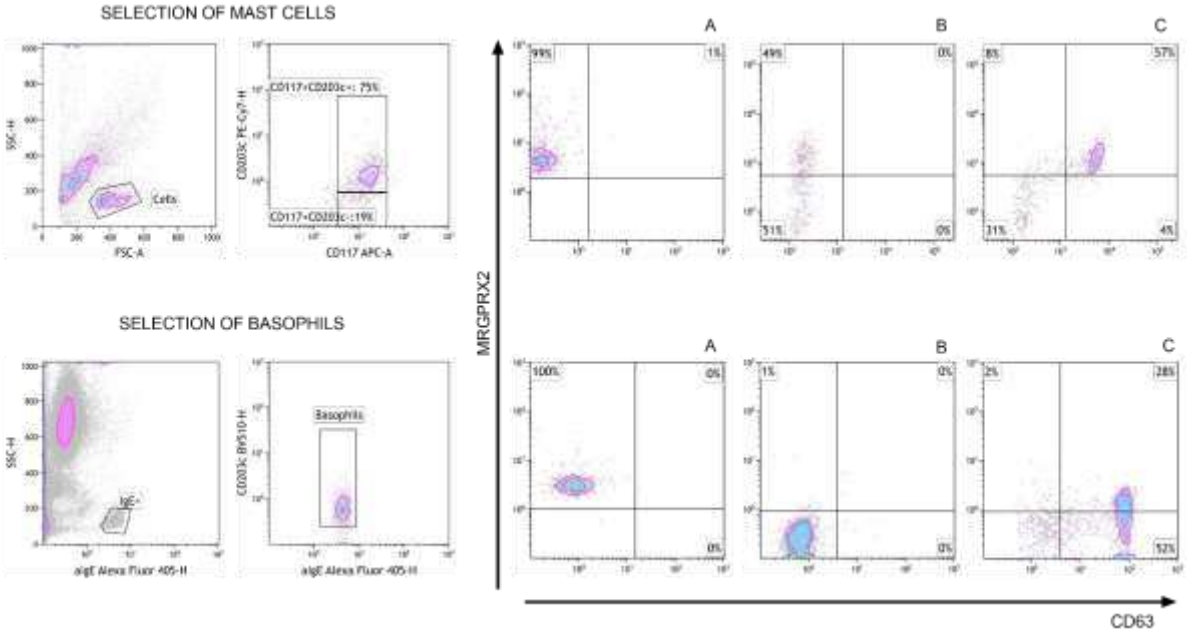

Figure E4: Representative plots of auto-immune basophil activation test (AIBAT) for suspected auto-immune chronic spontaneous urticaria. Donor basophils are identified as $CD123^{+ve}/HLADR^{-ve}$ cells. After incubation with control serum there is no upregulation of CD63 (AIBAT: negative). After incubation of cells with serum of a patient, 94% of the basophils up-regulate expression of CD63.

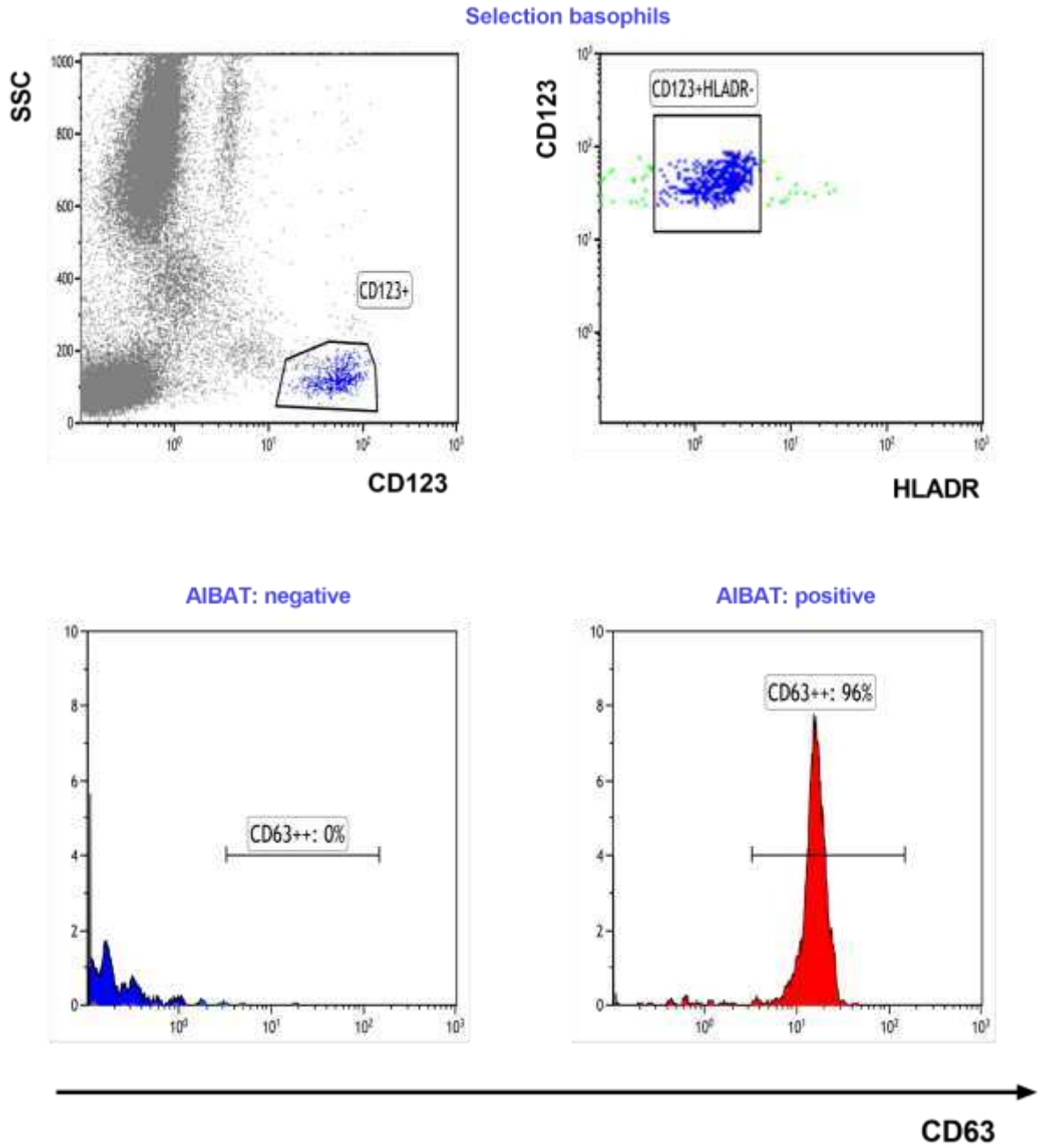

Table E1: Basophil identification, activation and degranulation makers (actualized from [6])				
	Marker	Other cell expression		References[(3)]
Identification markers[(1)]	CD193 (CCR3)	MCs, Th2 lymphocytes	CD3 required for differentiation	7-20
	CD203c (ENPP3)	MCs, $CD34^{+ve}$ progenitors	widely used identification marker	13, 15, 20-28
	CD123 (IL-3 receptor)	plasmacytoid DC	HLADR is frequently used to discriminate between $HLADR^{+ve}$ DCs	8, 12, 24, 25, 28-36
	IgE	monocytes, DCs	expression can be highly variable	13, 28
	CD294 (CRTH2)	MCs, eosinophils, Th2 lymphocytes	Differentiation from eosinophils via SSC or T lymphocytes by CD3	13, 21, 22, 24, 26, 37
Activation markers	CD63[(2)] (LAMP3)	MCs, macrophages, platelets	Widely used degranulation marker	5, 7-16, 20-22, 25, 26, 28-43
	CD203c[(2)] (ENPP3)	See above		5, 14, 20-26, 28, 34, 35, 37-42, 44-46
	CD107a (LAMP1)/ CD107b (LAMP2)	MCs, T lymphocytes, NK	CD63 compartment	21-23, 38
	CD11b	Macrophages, PMN, NK	CD203c compartment	47, 48
	CD13	myeloid cells	CD203c compartment	23, 38-41
	CD16	NK, neutrophils, monocytes, macrophages		20
	CD164	$CD34^{+}$ progenitors	CD203c compartment	11, 23, 38, 41
	CD300a/CD200R	MCs, NK	Inhibitory receptors	49-51
	Phoshorylated p38 MAPK	Various cell types	phosphorylated members of the signalosome	52-54
	Phosphorylated STAT5	Various cell types	phosphorylated members of the signalosome	55
	Histamine	MCs	staining via DAO	21, 28, 56-59
	Exteriorized granule matrix	MCs	staining via avidin	60-64
	Trapping cytokines (IL-4, IL-13) or mRNA	Th 2 lymphocytes		65, 66

MCs: mast cells; DCs: dendritic cells; NK: natural killer cells;
CCR3: eotaxin receptor; ENNP3: ectonucleotide pyrophosphatase/phospshodiesterase family member 3; CD123: interleukin receptor α chain, CRTH2: prostaglandin D2 receptor; MRGPRX2: Mas-related G protein-coupled receptor X2; LAMP: lysosomal associated membrane protein; CD11b: part of complement receptor 3 (CR3), CD13: surface aminopeptidase-N; CD16: Fc receptor (FcγRIII); CD164: sialomucin core protein 24; CD300a: inhibitory receptor p 60 (IRp60); CD200R: OX2 receptor; MAPK: mitogen-activated protein kinase; STAT: signal transducer and activator of transcription.
Intracellular histamine content can be quantified flow cytometrically by using histaminase diamine oxidase (DAO) that is conjugated to a fluorochrome.
Exteriorized proteoglycans can stained by fluorescent avidin probes.
[(1)] Identification markers are generally used in combinations (see text).
[(2)] CD203c compartment: rapid and significant upregulation of CD13, CD164 and CD203c; CD63 compartment: slower upregulation of CD107a and CD63 [38].
[(3)] Not exhaustive.

Santos el al, demonstrated that expression of CD123 can decrease upon activation of cells [25]. However, the authors and others do not share this experience [36, 67].
Corvan et al, provide evidence for IgE-independent activation of CD203c^{+} KU 812 basophil-like cells by the nematode *Trichostrongylus colubriformis* (a parasite of herbivorous mammals) [23].

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Table E2: BAT in inhalant allergy (data for CD63-BAT, except if denoted in *italics*)					
Allergen	Reference test	Sensitivity	Specificity	Number	Reference
House dust mite (HDM) Dactylis glomerata	H + IgE and/or ST	56-78 73-100	91-100 100	20	[68]
Cypress pollen	H + ST + PT	91	100	75	[69]
HDM and Lolium perenne	H + ST + PT	93	98	166	[70]
nFel d 1	H + IgE and/or ST	100 *95*	95	39	[71]
nPhl p 5 (45.1 µg/mL)	ST	*100*	*100*	40	[72]
H: history, ST: skin test, sIgE: specific IgE, PT: provocation test. *Italics*: CD203c-BAT. Predictive values are summarized in table E9.					

Table E3: BAT in food allergy (data for CD63-BAT, except if denoted in *italics*)					
Allergen	Reference test	Sensitivity	Specificity	Number	Reference
Various foods	H, ST, OFC	58	97	64	[73]
Birch-celery Birch-carrot Birch-hazelnut	H (OAS)	85 80 90	80 95 90	49	[74]
Birch-apple	H (OAS)	100 88[$]	100 75[$]	61	[75]
Birch-rMal d 1 (apple) Birch-rApi g 1 (celery) Birch-rDau c 1 (carrot)	H (OAS)	75[£] 75[£] 65[£]	68[£] 77[£] 100[£]	55	[76]
Macrobrachium Rosenbergii	H	100	100	45	[77]
Peanut	H + sIgE and/or ST	87 *90*	94 *97*	75	[78]
Egg	H + sIgE and/or ST	89 *63*	100 *97*	68	[78]
Wheat PBS fraction Wheat ethanol fraction Wheat alkaline fraction Native Ω-5 gliadine Recombinant Ω-5 gliadine	OFC or severe proven H	*86* *83* *84* *85* *82*	*58* *69* *67* *77* *63*		[45]
Cow's milk Casein Egg's white (HEA+ vs HEA-) Egg's white (REA+ vs REA-) Ovomucoid (HEA+ vs HEA-) Ovomucoid (REA+ vs REA-)	OFC or recent convincing H	*89* *67* *74* *77* *80* *83*	*83* *71* *62* *63* *73* *83*	Total of 71 (milk and egg)	[46]
Peanut Ara h 9	H and sIgE and ST	92 88	95 100	45	[79]
Sesame	OFC or recent convincing H	86 *84*	85 *80*	82	[80]
Peanut	DBPCFC	92	77	34	[81]
Peanut	OFC or severe proven H	98 95	96 96	104	[82]
Cow's milk	DBPCFC	100	100		[83]
Pru p 3 (Antwerp) Pru p 3 (Barcelona) Mal d 3 (Antwerp) Mal d 3 (Barcelona)	H and sIgE	NAV NAV NAV 63	NAV NAV NAV 67	219	[84]
Alpha-gal syndrome	No sensitivity and specificity data available. However, BAT discriminates between asymptomatic sensitization and patients with a genuine alpha-gal syndrome.				[16]

H: history, ST: skin test, sIgE: specific IgE, OFC: oral food challenge, DPPCFC: double blind placebo controlled food challenge. HEA: heated egg allergic, REA: raw egg allergic. *Italics*: CD203c-BAT.

[£] Lower sensitivity in [76] as compared to [74] is likely to reflect the Bet v 1 homologues (Api g 1 and Dau c 1) not to cover the entire sensitisation profile. The same holds true for casein that does not cover the cow's milk reactivity profile but ovomucoid vs. egg's white [46].

[$] according to a separate analysis between birch pollen allergic patients with and without apple-related oral allergy syndrome (OAS).

The study by Tokuda et al [45] shows allergen-specific decision thresholds.

The study by Decuyper et al [84] emphasizes the importance of geographically distinct sensitization profiles and component-related differences. NAV: no added value for diagnosis.
For additional information on BAT in food allergy see: [85].
Predictive values are summarized in table E9.

Table E4: BAT in hymenoptera venom allergy (data for CD63-BAT, except if denoted in *italics*)					
Allergen	Reference test	Sensitivity	Specificity	Number	Reference
Wasp and honeybee	H	100	100	55	[86]
Wasp and honeybee	H	*91*	*85*	35	[87]
Wasp (Ves v 5) Honeybee (Api m 1)	H	*79* *69*	*100* *100*	34	[88]
Wasp and honeybee	H and ST	77	90	44	[89]
Wasp Honeybee	H	85 91	87 (overall)	34 23	[90]
Wasp	H	92	80	70	[91]
Wasp Honeybee	H + ST + sIgE	97 100	100 100	38 12	[92]
Wasp and honeybee	H + ST + sIgE	89 *97*	100 *89*	68	[93]
Wasp	H	84	100	94	[94]
Wasp Honeybee	H	87 90	97 95	178	[95]
Wasp and honeybee	H	90		204	[96]
Wasp and honeybee (children)	H	67-75		15	[97]

H: history, ST: skin test, sIgE: specific IgE. *Italics*: CD203c-BAT

Wasp venom allergen 5 (Ves v 5) and honeybee venom phospholipase A2 (Api m 1) do not cover the entire IgE reactivity profile of wasp and honeybee venom, respectively [88]. Overall sensitivity of BAT using Ves v 1, Ves v 2, Ves v 5, Api m 1, Api m 2 and Api m 4 was 90%.

From the study by Eberlein-König et al [92], it appears BAT to be highly utile. However, it is poorly discriminative, as 75% of honeybee venom allergic patients had a positive BAT wasp, and, vice versa, in patients with wasp venom allergy 55% had a positive BAT for honeybee venom.

Note, as indicated in the manuscript, in patients with mastocytosis the BAT adds little to the diagnosis in difficult cases with negative sIgE and ST results [98, 99].

Predictive values are summarized in table E9.

Table E5: BAT in *Hevea* latex allergy (data for CD63-BAT, except if denoted in *italics*)					
Allergen	Reference test	Sensitivity	Specificity	Number	Reference
Latex	H + sIgE + ST	93	92	102*	[100]
Latex	H + ST	93	100	73	[101]
Latex	H + ST	50 *75*	100 *100*	28	[102]
Latex	H + ST + GPT	95	100	43	[103]
Latex	H + sIgE ± ST	80	97	79	[104]
Latex rHev b 5, rHev b 6	H + sIgE + ST	95	100	33	[105]
H: history, ST: skin test, sIgE: specific IgE, PT: glove provocation test. *Italics*: CD203c-BAT * Includes atopic control individuals. *Hevea brasiliensis* (Hev b) is the rubber tree. Predictive values are summarized in table E9.					

Table E6: BAT in immediate β-lactam hypersensitivity (data for CD63-BAT, except if denoted in *italics*). Actualized from [106].

Stimulus	Reference test	Activation marker	Sensitivity	Specificity	Number of patients and controls	Ref.
β-lactams	H	CD63	50	93	88	[107]
β-lactams	H + DPT[1]	CD63	39	93	53	[108]
β-lactams	H ± ST ± sIgE ± DPT	CD63	49	91	110	[109]
Amoxicillin	H ± ST	CD203c CD63	52 22	100 79	41	[110]
β-lactams	H	CD63	50	89-97	262	[111]
β-lactams	H ± ST ± sIgE	CD63-CCR3 CD63-IgE	55 53	100	39	[10]
Amoxicillin	H	CD63	29	/	14 patients, no controls	[112]
Amoxicillin	H ± ST ± DPT	CD63	50	/	61 patients, number of controls not mentioned	[113]
Amoxicillin	H ± ST	CD63	50	/	30 patients	[114]
Amoxicillin Clavulanic acid	H ± ST ± DPT	CD63	47 62	93 89	57 58	[115]
Cefazolin	H + ST	CD63 *CD203c*	33 *67*	94 *94*	16 patients, 17 controls	[116]

H: history, ST: skin test, DPT: drug provocation test, N: number of patients and control individuals. *Italics*: CD203c-BAT.
Note that several studies were not restricted to a single compound but investigated BAT in a drug class . For comment on [115] see [117].

Predictive values are summarized in table E9. See also [118].

Table E7: BAT in immediate neuromuscular blocking agent (NMBA) hypersensitivity (data for CD63-BAT, except if denoted in *italics*). Actualized from [106].

Stimulus	Reference Test	Activation marker	Sensitivity	Specificity	N	Ref.
NMBAs	H + ST	CD63 CD45	64 43	81 96	26	[119]
NMBAs	H ± ST	CD63	54	100	56	[7]
NMBAs	H + ST	CD63 *CD203c*	79 *36*	100 *100*	31	[120]
NMBAs	H ± ST	CD63	36-86[1]	93	92	[121]
Rocuronium	H + ST	CD63	92[2]	100	22	[30]
NMBAs	H ± ST ± sIgE	CD63	60	100	92	[122]
Rocuronium	H + ST	CD63	80	96	104	[31]
NMBAs	H+ST	CD63	68	100	56	[123]
Atracurium	H + ST	CD63	71[3]	100	75	[124]
Rocuronium	H + ST	*DAO*	100	100	13	[59]
NMBAs[4,5]	H ± ST ± sIgE	CD63 *CD203c*	48 *58*	87 *89*	120	[125]

H: history, ST: skin test, N: number of patients and control individuals. *Italics*: CD203c-BAT

[1] Increasing sensitivity when only the reactions that occurred during the 3 years were taken into account,

[2] taking into account the non-responders sensitivity is 76%,

[3] taking into account the non-responders sensitivity is 63%.

[4] sensitivity and specificity for %CD63 and %CD203c. Overall sensitivity and specificity is 77% and 76%, respectively.

[5] BAT also positive in some patients with negative skin tests (note that CD300a was used in some cases). BAT was also found to supplement skin tests in Van Gasse et al [126].

DAO: conjugated diamine oxidase is used to measure intracellular histamine content (proof-of-concept in IDHR) [59].

Predictive values are summarized in table E9. See also [118].

Table E8: BAT fluoroquinolone (FQ) hypersensitivity (data for CD63-BAT, except if denoted in *italics*). Actualized from [106].

Stimulus	Reference Test	Activation marker	Sensitivity	Specificity	N	Ref.
FQs	H + DPT	CD63	0	_	4	[127]
FQs	H + ST + DPT	CD63	0	100	18	[128]
FQs	H	*CD203c*	*100*	*100*	5	[129]
FQs	H + DPT	CD63	71	_	75	[130]
FQs	H + DPT	*CD203c*	NA	*100*	34	[131]
Moxifloxacin	H + DPT	CD63	9.1	77.8	11	[14]
		CD203c	*36.4*	*94.4*	11	
Ciprofloxacin		CD63	83.8	88.9	6	
		CD203c	*0*	*94.4*	6	
Moxifloxacin	H	CD63	13.3	100	24	[132]
		CD203c	*46.7*	*100*	24	

H: history, ST: skin test, DPT: drug provocation test, N: number of patients and control individuals
Italics: CD203c-BAT
Predictive values are summarized in table E9. See also [118].

Table E9: positive and negative predictive values of BAT (not exhaustive, for drug also see [118])					
Allergen	Reference test	PPV (%)	NPV (%)	Numbers	References
Inhalant allergens					
HDM and Lolium perenne	H + ST + PT	99	89.7	166	[70]
Food allergens					
Various wheat fractions	OFC or severe proven H	77.1-86.8	66.7-72	58	[45]
Ovomucoid Egg's white Cow's milk Casein	OFC or recent convincing H	100 94.7 85.7 75	31.3 21.9 86.4 59.1	Total of 71 (milk and egg)	[46]
Peanut	OFC or severe proven H	95	98	104	[82]
Drugs					
β-lactams	H	93.5	49.1	88	[107]
β-lactams	H ± ST ± sIgE ± DPT	50.8	92.4	110	[109]
Amoxicillin	H ± ST	85.2	52.5	41	[110]
β-lactams	H	90.5	43.8	262	[111]
β-lactams	H ± ST ± sIgE	81.5	52.6	39	[10]
FQs	H + DPT	90.1	66.7	75	[130]
FQs	H + DPT	100	78.9	34	[131]
Moxifloxacin Ciprofloxacin	H + DPT	81.8	66.7	35	[14]
NMBAs	H + ST	86.9	78.1	26	[119]
NMBAs	H + ST	100	48.6	56	[7]
NMBAs	H + ST	100	76.9	31	[120]
Rocuronium	H + ST	100	87.3	22	[30]
NMBAs	H ± ST ± DPT	84.9	58.3	92	[121]
Rocuronium	H + ST + IgE	97	75	104	[31]
NMBAs	H + ST	100	82.9	56	[123]
Atracurium	H + ST	100	70	75	[124]
In the study by Sanz et al [70], the likelihood ratio for a positive value was 58.3, for a negative result 0.07 In the study by Tokuda et al [45], the positive predictive value (PPV) and negative predictive value (NPV) for sIgE wheat by ImmunoCAP (Thermo Fisher Scientific) was 74.5% and 65.2%, respectively. In the study by Leysen et al [31], the PPV for ST and sIgE rocuronium was 98% and 83%; the NPV for ST and sIgE rocuronium was 96% and 72%.					

Table E10: Basophil activation tests: requirements and points to consider to advance further entrance in mainstream use
Technical
Standardization between systems and instruments (required for accreditation) - E.g. Euroflow standard operating procedure.
Standardization of sample preparations (extraction procedure, storage, preservatives) - E.g. aqueous vs. alcoholic extraction procedures, lyophilization, spiking with purified or recombinants - For recombinants: cave presence of maltose binding protein (MBP) See also: Larsen et al. Methods Mol Biol. 2019;2020:63-76. doi: 10.1007/978-1-4939-9591-2_5.
Sample conditions: chelators or heparin, temperature, preservation, whole blood vs. purified cells - E.g. when EDTA is used as anticoagulant correct Ca restoring is mandatory to avoid false negative outcomes
Stimulation conditions (dose-findings (with reporting of end concentrations in aliquot), incubation time, incubation temperature (prewarming of reagents), use of primers (e.g. IL-3), stopping by chelators - For drugs: take into account pharmacokinetics, cytotoxicity (viability should be monitored), protein binding, pharmacologic inhibition (e.g. via nicotine receptors), use of metabolites, photodegradation, interaction with fluorochromes, stimulation concentrations are best expressed in µmol/L
Calculation of allergen-specific decision thresholds (absolute % of positive cells, MFI, stimulation indices). Arbitrarily chosen cut-off should be abandoned. In BAT traditional cut-off, defined as result of blank (stimulation with buffer) + 3.3 SD, is rarely adopted.
Non-responders should be reported and included in performance analyses Basophils can show inherent biological variability from one day to another - Repetition of analyses could be worthwhile - Whether passive BAT using stripped donor basophils, humanized rat basophilic leukemic cell lines (e.g. RBL-2H3), tumoral MC lines (e.g. LAD2) or mast cell activation tests (MAT) using cultured donor MCs remains to be established
Automation and introduction of bioinformatics should improve efficiency, transparency and standardization.
Clinical
Identification and enrollment of sufficient numbers of well-documented cases and exposed control individuals. - Consensus about mechanistic nomenclature - Consensus scores about clinical presentation and severity - Application of gold standard or reference test (e.g. challenges)
Establishment of sensitization profile. - E.g. not allergens are equally potent
Identification of confounders - E.g. underlying cross-reactivity

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