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In vitro diagnosis of allergic and mast cell-related disorders

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In vitro diagnosis of allergic and mast cell-related disorders

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Dr. Joana Vitte has received speaker and consultancy fees in the past 5 years from Meda Pharma (Mylan), Novartis, Sanofi, Thermo Fisher Scientific. Is president of the Scientific Committee of the French Society for Allergology. Is a member of the Board of the EAACI Working Group "Infection" and chair of the EAACI Task Force "Diagnosis and management of allergic bronchopulmonary aspergillosis".

Table of contents

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Editorial: *In vitro* diagnosis of allergic and mast cell-mediated disorders

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immunoglobulin (Ig) E, *in vitro* diagnosis of allergy, basophil activation test (BAT), mast cell (MC), hereditary alpha-tryptasemia (HαT), molecular allergen, component-resolved diagnosis (CRD), COVID-19 vaccine

Editorial on the Research Topic
In vitro diagnosis of allergic and mast cell-related disorders

Allergic diseases, affecting one in four people, are diagnosed through the demonstration of an adaptive immune response (sensitization) to a trigger (allergen) of a hypersensitivity reaction. In clinical practice, most hypersensitivity reactions referred to allergists are immediate reactions, taking place less than 2 h following exposure to the trigger (1). In most cases, immediate hypersensitivity symptoms develop within minutes after ingestion, inhalation, or injection of the trigger. The occurrence and severity of immediate hypersensitivity reactions are influenced by mast cell conditions, such as hereditary alpha-tryptasemia (HαT), estimated to affect 5 to 8% of general population (2).

Allergen-induced immune responses can be demonstrated using *in vivo* (skin prick test and provocation tests), and *in vitro* tests. Conventional diagnostic strategies require a two-step diagnostic process: first, taking a detailed clinical history aimed at identifying one or a limited number of suspected triggers; and second, proceeding to complementary tests to confirm sensitization to the suspected trigger(s). This decades-old diagnostic paradigm is increasingly shifting towards a precision medicine approach comprising phenotype stratification, personalized therapeutic decision making, risk prediction and even family counselling (3, 4). The allergic patient is increasingly placed at the center of an integrated approach thanks to progress in endotyping the mechanisms at play in the build-up of the allergic host–environment interaction. Key contributors are modern *in vitro* diagnosis concepts and tools, therapeutic breakthroughs and improved knowledge of environmental factors.

Current *in vitro* diagnostic methods are mostly quantitative and increasingly standardized, allowing for reliable comparison and follow-up of allergic patients (5). They are also miniaturized, with hundreds or even thousands of biomarkers assayed in minute volumes of biological fluids. Non- or minimally- invasive tests are being developed, allowing for better pediatric assessment and lower health care resource

utilization. Moreover, new regulatory standards result in improved quality of *in vitro* tests (6).

The Research Topic "*In vitro* diagnosis of allergic and mast cell-mediated disorders" aimed to provide an overview of currently available *in vitro* diagnostic tests for allergic and mast cell-related disorders, and describe their contribution to the prediction, diagnosis and management of patients with a suspected or confirmed allergic or mast cell disorder. Measurement of allergen-specific immunoglobulin (Ig) E, mainly focused on quantitative aspects including the clinical decision associated with low levels of sensitization, and molecular allergen-specific IgE are important tools for phenotype assessment and risk stratification of allergic patients (Balsells-Vives et al., Chantran et al.). Basophil activation test (BAT) provides functional evaluation of a patient's response to IgE-dependent as well as IgE-independent immediate hypersensitivity triggers (reviewed in (Sonder et al.). Using BAT to molecular allergens may improve the diagnostic specificity even further, e.g., discrimination between allergic broncho-pulmonary aspergillosis and *Aspergillus fumigatus* sensitization (Michel et al.). BAT shows promise for the identification of more severe allergic phenotypes at risk of anaphylaxis, such as wheat-dependent exercise-induced anaphylaxis or soy-induced anaphylaxis (Gabler et al., Evrard et al.). However, access to BAT is still limited due to sampling and technical requirements (fresh whole blood samples, flow cytometry facilities, available soluble allergens) and, ideally, need for regulatory approval (Alpan et al.). Serum baseline tryptase determination is, currently, the best screening option for identifying people with HαT, a disease-modifying mast cell condition associated with a higher risk of severe immediate hypersensitivity reactions of various causes, including food allergy (Chantran et al.).

An additional aim of this Research Topic was to provide the reader with the pathophysiological and methodological explanation of *in vitro* diagnostic tools, enabling better understanding of future developments in the field for improved diagnosis and management. Here, Stoffersen et al. investigated the relationship between free serum IgE levels and the performance of a modified BAT using patient's serum and control donor basophils (Stoffersen et al.), while Nicaise-Roland et al. reviewed the causes of hypersensitivity reactions to COVID-19 vaccines and the adequate strategies of *in vitro* diagnosis (Nicaise-Roland et al.).

Taken together, the contributions to the Research Topic "*In vitro* diagnosis of allergic and mast cell-mediated disorders" showcase current hotspots, unmet needs and ongoing research in the field of *in vitro* diagnosis of allergic and mast cell disorders. The manuscripts compiled herein illustrate, through selected examples, how the precision medicine approach using up-to-date *in vitro* diagnostic tools is beneficial to allergic patients, with respect to their culprit allergens as well as to their genetic make-up, paving the way of personalized medical and lifestyle interventions.

Taking a detailed clinical history is the crucial first step to determine the likelihood of an IgE-mediated reaction (Figure 1). The proof of concordant IgE sensitization is the second step allowing to pose the diagnosis of IgE-mediated allergy. Once the diagnosis is confirmed, stratification allows for optimal

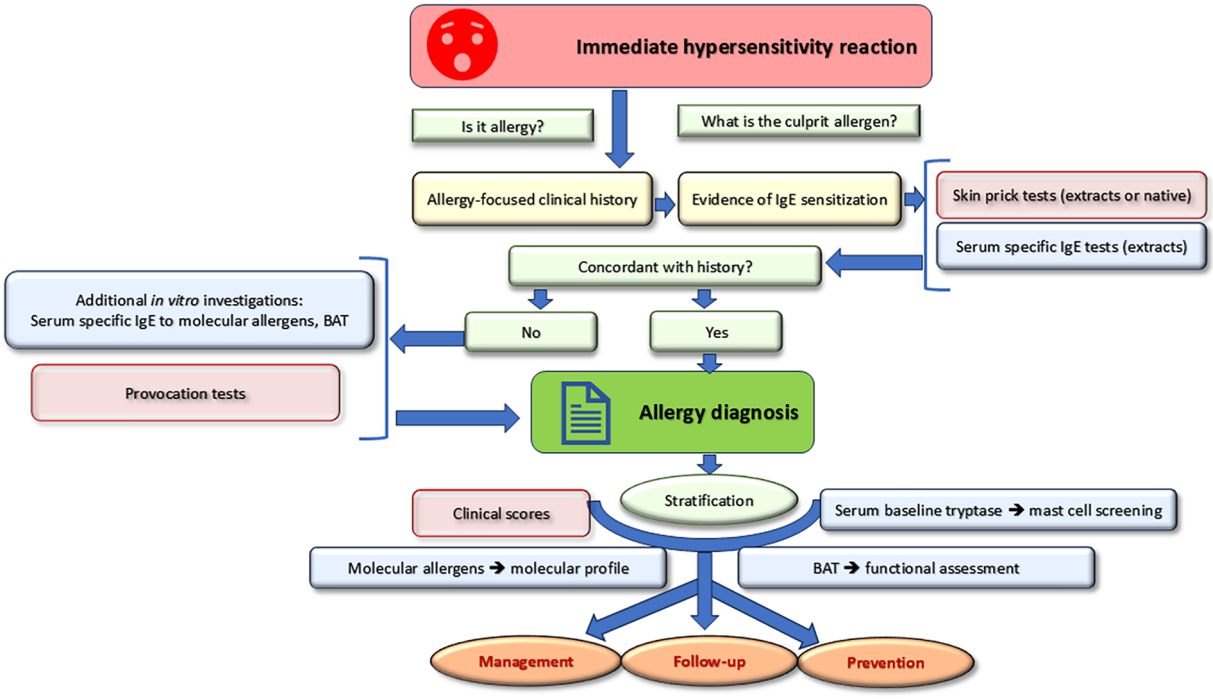

FIGURE 1
General diagnostic strategy for IgE-mediated immediate hypersensitivity reactions.

management. Recent *in vitro* tools such as molecular allergens and basophil activation tests can be used for both diagnostic and stratification purposes. Baseline serum tryptase is a risk marker for the occurrence and severity of systemic hypersensitivity reactions.

Author contributions

JV: Conceptualization, Writing – original draft, Writing – review & editing. AS: Conceptualization, Writing – original draft, Writing – review & editing.

Conflict of interest

JV reports speaker and consultancy fees in the past 5 years from Astra Zeneca, HpVac, L'Oréal, Novartis, Sanofi, Thermo Fisher Scientific, and travel expenses reimbursement from Stallergènes for an international meeting, outside the submitted work. AFS reports grants from Medical Research Council (MR/M008517/1; MC/PC/18052; MR/T032081/1), Food Allergy Research and Education (FARE), the Immune Tolerance Network/National Institute of Allergy and Infectious Diseases (NIAID, NIH), Asthma UK (AUK-BC-2015-01), BBSRC, Rosetrees Trust and the NIHR through the Biomedical Research Centre (BRC) award to Guy's and St Thomas' NHS Foundation Trust, during the conduct of the study; consultancy or speaker fees from Thermo Scientific, Novartis, Allergy Therapeutics, Stallergenes, IgGenix, as well as research support from Buhlmann and Thermo Fisher Scientific through a collaboration agreement with King's College London.

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Basophil Activation to Gluten and Non-Gluten Proteins in Wheat-Dependent Exercise-Induced Anaphylaxis

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Wheat-dependent exercise-induced anaphylaxis (WDEIA) is a cofactor-induced wheat allergy. Gluten proteins, especially ω5-gliadins, are known as major allergens, but partially hydrolyzed wheat proteins (HWPs) also play a role. Our study investigated the link between the molecular composition of gluten or HWP and allergenicity. Saline extracts of gluten (G), gluten with reduced content of ω5-gliadins (G-ω5), slightly treated HWPs (sHWPs), and extensively treated HWPs (eHWPs) were prepared as allergen test solutions and their allergenicity assessed using the skin prick test and basophil activation test (BAT) on twelve patients with WDEIA and ten controls. Complementary sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), high-performance liquid chromatography (HPLC), and mass spectrometry (MS) analyses revealed that non-gluten proteins, mainly α-amylase/trypsin inhibitors (ATIs), were predominant in the allergen test solutions of G, G-ω5, and sHWPs. Only eHWPs contained gliadins and glutenins as major fraction. All allergen test solutions induced significantly higher %CD63$^+$ basophils/anti-FcεRI ratios in patients compared with controls. BAT using sHWPs yielded 100% sensitivity and 83% specificity at optimal cut-off and may be useful as another tool in WDEIA diagnosis. Our findings indicate that non-gluten proteins carrying yet unidentified allergenic epitopes appear to be relevant in WDEIA. Further research is needed to clarify the role of nutritional ATIs in WDEIA and identify specific mechanisms of immune activation.

Keywords: allergy, amylase/trypsin inhibitor, basophil activation test, gluten, proteomics, wheat

INTRODUCTION

Wheat-dependent exercise-induced anaphylaxis (WDEIA) is a cofactor-induced wheat allergy. It is generally considered to be rare. In Japanese adolescents, the prevalence of food-dependent exercise-induced anaphylaxis predominantly to wheat was 0.017%. However, as these patients tolerate wheat in the absence of association with cofactors, WDEIA may not be recognized in many patients and they are often given the diagnosis of idiopathic anaphylaxis instead (1–3).

Patients with WDEIA may react to intact gluten proteins and/or partially hydrolyzed wheat proteins (HWPs) (4–6). Besides others, Yokooji et al. and Hiragun et al. reported allergic reactions in patients with WDEIA to HWPs in facial soap (6, 7). HWPs are made of gluten subjected to chemical or enzymatic partial hydrolysis to obtain foaming and emulsifying properties for use in foods and cosmetics (6, 8, 9). Depending on the treatment, HWPs differ significantly from one another regarding their functional properties and molecular composition (8, 10–12). Partial hydrolysis may lead to exposure of pre-existent allergenic epitopes otherwise buried within protein aggregates or to the formation of new epitopes, e.g., through deamidation (6, 7). The increase in solubility of HWPs compared with native gluten also affects allergen passage through the skin or the small intestine (6, 7, 13).

About 80% of patients with WDEIA have specific IgE (sIgE) against ω5-gliadins, the major allergens in WDEIA (14), but sensitization to other wheat gluten proteins, such as high- and low-molecular-weight glutenin subunits (HMW-GS and LMW-GS) or α- and γ-gliadins has also been reported (15–20). Water- or salt-soluble non-gluten proteins, such as lipid-transfer proteins (LTPs) associated with baker's asthma, were also suggested to play a role in WDEIA (21–23). Pastorello et al. found sIgE against α-amylase/trypsin inhibitors (ATIs) in WDEIA patients' sera (24), but the role of nutritional non-gluten proteins as causative agents for WDEIA is currently underexplored.

Approaches to diagnose WDEIA include clinical history, skin prick test (SPT), measurement of sIgE against ω5-gliadins, and oral gluten challenge combined with cofactor as golden standard (4). Due to the risk of a serious anaphylactic reaction during the challenge tests, there is a need to establish alternatives. The *in vitro* basophil activation test (BAT) using well-defined allergen test solutions (ATSs) may be suitable, because basophil activation is directly related to the allergenicity of a test substance (25–28). The BAT is already used to diagnose and investigate IgE-mediated allergies, e.g., allergy against antibiotics (29) or bee and wasp venom (30). Schwager et al. evaluated the allergenic potential of natural and recombinant peanut oleosins using the BAT on peanut-allergic and peanut-sensitized patients in comparison with a control group. A complex cocktail of 12 antibodies was used to identify basophils. The activation marker of identified basophils was CD63 (31). The same group improved the BAT workflow for reliable results with a time saving approach to make it suitable for clinical routine. *Inter alia*, they compared the approach of Schwager et al. with a simplified approach using CD63 (activation marker) and CD203c and FcεRIα (identification markers). As they found no significant differences between the results of both strategies, they showed that the necessary simplification to make BAT applicable in clinical routine is possible and reliable. Furthermore, Behrends et al. used different peanut allergens in the BAT, such as oleosins and defensins, Der p 2, Bet v 1, Ara h 8, Ara h 14, and Ara h 15 (31, 32). One important aspect of both studies is the application of single peanut allergens in the BAT. These were either isolated and purified from raw and in-shell roasted peanuts or recombinantly expressed in *Escherichia coli* (31, 32). The robust and optimized BAT setup using these single allergens allowed the differentiation between peanut-allergic and peanut-sensitized individuals (32).

Mehlich et al. tested alpha-gal sensitized patients in comparison with healthy controls for their basophil reactivity to commercial alpha-gal allergens and pork kidney extract. Thereby, CCR3 was assessed as an identification marker and CD63 as an activation marker for basophils. Similar to the peanut-BAT, they were able to differentiate between patients with alpha-gal syndrome and asymptomatic alpha-gal sensitization within the sensitized patient group using BAT (33).

Chinuki et al. used the BAT to examine the allergenicity of a HWP product in 10 WDEIA patients. The HWP had been produced by acid hydrolysis, but further details on its molecular composition were not provided (5, 34).

We already demonstrated that BAT using CCR3 as identification marker and CD63 as activation marker for basophils allowed the discrimination of patients with WDEIA from controls. ATSs made from peptic hydrolysates of ω5-gliadins, HMW-GS and total gluten showed the best sensitivity and specificity at optimal cut-off (20). Although these three peptic hydrolysates work very well in BAT, one drawback of using those ATSs is that they cannot be easily prepared in routine clinical practice, because the procedure involves elaborate gluten fractionation and digestion (20).

Therefore, we aimed to provide aqueous ATS from gluten samples with different molecular properties that can be easily made for use in BAT. We included four ATSs to cover a wide range of variability in molecular composition. These ATSs were prepared as saline extracts from one representative sample of wheat gluten (G) and of slightly hydrolyzed wheat proteins (sHWPs) and extensively hydrolyzed wheat proteins (eHWPs) selected from our previous work (10). The fourth sample was produced from flour of wheat variety Pamier, a wheat/rye translocation line with an 89% lower content of ω5-gliadins (G-ω5; 2.40 mg/g), the main allergen in WDEIA, in comparison with representative gluten (G; 22.3 mg/g) (35). If G-ω5 truly induced lower allergenic responses, products made of this variety might be nutritionally beneficial for patients with WDEIA. We combined allergenicity assessment using SPT and BAT with the characterization of allergenic proteins in the ATS to identify which proteins are present in those saline ATSs.

METHODS

Materials

Gluten and HWPs were from Hermann Kröner GmbH (Ibbenbüren, Germany), Tate & Lyle PLC (London, UK), and Manildra Group (Gladesville, Australia). G in the present study corresponds to G1, G-ω5 to G4, sHWP to HWP7, and eHWP to HWP3 (7). All reagents and chemicals were from Sigma Aldrich (Darmstadt, Germany), Merck (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), Honeywell (Offenbach, Germany), J. T. Baker (Arnhem, The Netherlands), and Fresenius Kabi Deutschland GmbH (Bad Homburg, Germany). Water was purified with an Arium 611VF water purification system (Sartorius, Goettingen, Germany). Pepsin (from porcine mucosa,

10 FIP U/mg), trypsin (from bovine pancreas, TPCK treated, 10.000 BAEE U/mg protein), α-chymotrypsin (from bovine pancreas, TLCK-treated, ≥40 U/mg protein), and thermolysin (from *Geobacillus stearothermophilus*, 30–175 U/mg protein) were purchased from Sigma Aldrich (Darmstadt, Germany) and Merck (Darmstadt, Germany).

Allergen Test Solutions

To prepare saline ATS for BAT, the sample (eHWP: 25 mg, sHWP: 100 mg, G: 100 mg, and G-ω5: 100 mg) was weighed into a 2 ml tube followed by addition of glass beads for better homogenization and 1 ml 0.9% isotonic NaCl solution. The suspension was homogenized by vortex mixing for 1 min, stirring for 20 min at room temperature, and ultrasonic treatment for 3 min. After centrifugation (2,300 × *g*, 15 min, 20°C), the supernatant was filtered (0.45 μm, regenerated cellulose, GE Healthcare, Chicago, IL, USA) and the protein/peptide concentrations measured at 205 nm by a micro volume UV/VIS spectrophotometer NanoDrop One (Thermo Fisher Scientific, Carlsbad, CA, USA). The ATS from eHWP was diluted 1:5 (v/v) with 0.9% isotonic NaCl solution to adjust protein/peptide concentrations of all ATS for BAT experiments.

Several supernatants of G, G-ω5, and sHWP were prepared, pooled, and lyophilized for ultra-performance liquid chromatography (UPLC)-TripleTOF-MS analysis. The lyophilized powder was carefully homogenized with mortar and pestle and weighed into 2 ml tubes (6 mg). eHWP was used directly (4 mg), because it was completely soluble in isotonic NaCl solution.

Study Population

Twelve patients with a clinical history of WDEIA based on positive oral food challenge (5 women, 7 men, 26–60 years, median age: 48 years) and 10 individuals without a history of any wheat-related disorder were included in the study as healthy controls (9 women, 1 male, 25–76 years, median age: 44 years). Five of the control subjects were atopic. Further details on the study population are reported in Gabler et al. (20). The study protocol was approved by the ethics committee of the Technical University of Munich and all participants gave written informed consent before being included in the study.

Skin Prick Test

Skin prick test was carried out on the forearm with gluten (G, G-ω5) and hydrolyzed wheat proteins (eHWPs and sHWPs). Histamine dihypochloride solution (10%) from ALK-Abello (Hørsholm, Denmark) served as a positive control and isotonic NaCl solution from Fresenius Kabi Deutschland GmbH (Bad Homburg, Germany) as a negative control. The SPT was defined as positive, when the wheal diameter caused by the tested substance was ≥3 mm larger than the diameter of the negative control (4).

Basophil Activation Test

Flow CAST (Bühlmann Laboratories AG, Schönenbuch, Switzerland) was used for quantitative determination of *in vitro* basophil activation, as described previously (20). Anti-FcεRI-mAb and *N*-formyl-methionyl-leucyl-phenylalanine were used as positive controls. Flow cytometry was performed using a FACSCalibur system (Becton-Dickinson Immunocytometry System, Heidelberg, Germany) with a 488 nm, 15 mW and a 635 nm, 10 mW argon laser. Basophils were gated as low side scatter CCR3/side scatter^{low}. CCR3 was used as identification marker for basophils and CD63 as basophil activation marker, labeled with anti-CCR3-phycoerythrin mAb and anti-CD63-fluorescein-isothiocyanate, respectively. BD CellQuest (Becton-Dickinson Immunocytometry System) was used to analyze the data. At least 450 basophils were counted per measurement (13, 28). The following BAT parameters were studied: basophil activation (%CD63$^+$ basophils) expressed as percentage of basophil granulocytes expressing CD63 divided by the total number of counted basophil granulocytes per single measurement and %CD63$^+$ basophils/anti-FcεRI ratio as quotient of the basophil activation (%CD63$^+$ basophils) triggered by ATS and by the anti-FcεRI mAb as positive control (33).

Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis was carried out according to Lagrain et al. (36). In brief, lyophilized ATS (G, G-ω5, sHWP) and eHWP (used directly, because of complete solubility in isotonic NaCl) were incubated with reducing extraction buffer for 12 h at room temperature, heated to 60°C for 10 min and centrifuged (5,000 × *g*, 20°C, 5 min). A homogeneous NuPAGE 10% polyacrylamide Bis-Tris gel (10 mm × 1 mm wells) (Invitrogen, Carlsbad, CA, USA) was used with a MOPS running buffer. The PageRuler Unstained Protein Ladder served as a molecular mass (M_r) standard (Thermo Fisher Scientific). The running time was 30 min at 200 V and 115 mA. Protein bands on the gel were fixed with 12% trichloroacetic acid (w/w) (30 min), stained with Coomassie blue (30 min) and destained in two steps. The gels were scanned using the Gel Doc EZ Imager (Bio-Rad Laboratories, Munich, Germany) and the Image Lab software (Bio-Rad Laboratories) (10, 37).

Gel Permeation HPLC

Two different gel permeation (GP)-HPLC systems, previously reported by Gabler at al. and Scherf et al. were used to analyze the M_r distribution of proteins and peptides in the ATS compared with protein markers of known M_r (10, 38). Measurements were performed on a Jasco HPLC Extrema (Jasco, Gross-Umstadt, Germany). A BioSep-SEC-s3000 column (300 mm × 4.6 mm, 29 nm, 5 μm, Phenomenex, Aschaffenburg, Germany) was used for protein separation with an isocratic gradient (50:50, 0.1% trifluoroacetic acid (TFA) in ultrapure water/0.1% TFA in acetonitrile) with a flow rate of 0.3 ml/min at 20°C. Chromatography was carried out on a BioBasic SEC-60 column (150 mm × 7.8 mm, 6 nm, 5 μm, Thermo Fisher Scientific) with an isocratic gradient (70:30, 0.1% TFA in ultrapure water/0.1% TFA in acetonitrile) at a flow rate of 1.0 ml/min for small proteins/peptides. The injection volume was 3–5 μl.

Reversed-Phase HPLC

The protein/peptide concentration of the ATS was analyzed according to Gabler et al. using reversed phase (RP-)HPLC on a Jasco XLC instrument (Jasco) using a C_{18} column at 60°C (Acclaim 300, C_{18}, 2.1 mm × 150 mm, 300 nm, 3 µm, Thermo Fisher Scientific). The elution solvents were 0.1% TFA in ultrapure water (A) and 0.1% TFA in acetonitrile (B) at a flow rate of 0.2 ml/min. Gradient elution was performed: 0 min 0% B, 0.1–0.5 min 24% B, 0.6–15 min 56% B, 15.1–19.1 min 90% B, 19.2–35.0 min 0% B. The injection volume was 20 µl. Prolamin Working Group (PWG)-gliadin was used for external calibration (10, 39).

Ultra-Performance Liquid Chromatography (UPLC)-TripleTOF-MS

Reduction and Alkylation

Lyophilized ATS from G, G-ω5, and sHWP as well as eHWP were dissolved in 320 µl of TRIS-HCl buffer (0.5 mol/L, pH 8.5) and 320 µl 1-propanol. For reduction, 50 µl of Tris-(2-carboxyethyl)-phosphine (TCEP) solution (22 mg/ml TCEP in TRIS-HCl buffer) were added and the samples shaken for 30 min at 60°C under nitrogen. After cooling, 100 µl of chloroacetamide (CAA)-solution was added for alkylation (34 mg/ml CAA in TRIS-HCl buffer). The samples were shaken for 45 min at 37°C in the dark. The solutions were evaporated to dryness (37, 40).

Enzymatic Digestion

Different protein digestions were carried out: pepsin + trypsin (PT), pepsin + chymotrypsin (PC), pepsin + trypsin + chymotrypsin (PTC), trypsin + chymotrypsin (TC), and thermolysin (TLY). Digestion was performed by adding pepsin [750 µl, 0.2 mg/ml in 0.15 mol/L HCl, pH 2, enzyme/substrate (E:S) ratio of 1:20 (w/w)] to the alkylated residues and shaking for 60 min at 37°C. After the peptic digest, the pH was adjusted to 6.5 with PBS (50 mmol/L). Then, trypsin and/or chymotrypsin [E:S of 1:20 for T or C, E:S of 1:40 for TC (w/w)] were added and the samples were hydrolyzed for 120 min at 37°C. For TC digestion, TC was added to the alkylated residues [1 ml, 0.12 mg/ml T/C in 0.1 mol/L TRIS-HCl-buffer, E:S of 1:50 (w/w)] followed by incubation for 16 h at 37°C. The digestions were stopped by heating for 10 min at 95°C (37, 40). TLY digestion [E:S of 1:20 (w/w)] was carried out in TRIS-HCl $CaCl_2$ buffer (0.2 mol/L TRIS, 0.5 mmol/L $CaCl_2 \cdot 2H_2O$, pH 6.5) at 37°C for 16 h. The reaction was stopped with formic acid (FA) (41–43).

Solid Phase Extraction

Enzymatic digests were purified by solid phase extraction (SPE) using 100 mg Discovery DSC-18 cartridges (Supelco, Bellefonte, PA, USA). After activation with methanol, equilibration with 80/20 (v/v) acetonitrile 0.1% FA in water and washing with 2/98 (v/v) acetonitrile/0.1% FA the cartridges were loaded with sample and washed again. Elution was carried out using 40/60 (v/v) acetonitrile/0.1% FA in the first step and 80/20 (v/v) acetonitrile/0.1% FA in the second. Both eluates were united and evaporated to dryness. The residues were dissolved in 500 µl 0.1% FA and filtered immediately before UPLC-TripleTOF-MS analysis (40, 44).

UPLC-TripleTOF-MS

The UPLC-TripleTOF-MS analysis was performed using an UPLC system ExionLC coupled to a TripleTOF 6600 MS (SCIEX, Darmstadt, Germany). A bioZen peptide PS-C18 column (100 mm × 2.1 mm, 10 nm, 1.6 µm) (Phenomenex) was used. Peptides (injection volume 10 µl) were separated using linear gradient elution (0–65 min 5% B to 100% B, 65–69 min 100% B, 69–70 min 100% B to 5% B, 70–75 min 5% B; solvent A: 0.1% FA in water, solvent B: 0.1% FA in acetonitrile) with a flow rate of 0.35 ml/min at 40°C. The MS was operated in positive electrospray ionization mode and the following settings: ion spray voltage 5,500 eV, source temperature 550°C, heating gas 0.45 MPa, nebulizing gas 0.38 MPa, curtain gas 0.24 MPa.

The MS was operated in information-dependent acquisition (IDA) mode. The mass-to-charge range for MS1 was 350–1,800, using an accumulation time of 250 ms, collision energy of 10 V, and a declustering potential of 80 V. The IDA criteria for the precursor ion included intensity of >100 counts/s and the resolution was set to 0.5 Da. MS2 spectra of the 20 most abundant compounds were recorded in a mass-to-charge range of 350–1,800, using an accumulation time of 40 ms, collision energy of 35 V, declustering potential of 80 V, and a collision energy spread of 5 V. Instrument control and data acquisition were performed with Analyst TF software (v 1.7.1., SCIEX).

Analysis of UPLC-TripleTOF-MS Data

The raw data were analyzed against the proteome of *Triticum aestivum* (UniprotKB, download 08/2019) using the proteomics software MaxQuant (version 1.6.3.4) (45). The search parameters including specific and unspecific digestion are reported in **Supplementary Table 1**. All other parameters were kept as default settings. The intensity based absolute quantitation (iBAQ) algorithm implemented in MaxQuant was used to estimate wheat protein abundances in the ATS. A total sum normalization of protein iBAQ intensities between sample measurements was performed to correct for different total protein injection amounts (37, 40).

Statistical Analysis

A statistical analysis was performed with Origin 2020 (OriginLab Cooperation, Northampton, MA, USA) and SigmaPlot 14 (Systat Software GmbH, Erkrath, Germany). One-way ANOVA with Dunn's *post-hoc* test ($p < 0.05$) was used to identify significant differences between the ATS analyzed by HPLC, SPT, and BAT. Receiver operating characteristic (ROC) analyses were carried out to estimate how well BAT parameters, such as area under the ROC curve (AUC) distinguished between patients and controls. The optimized discrimination threshold (cut-off) for the %$CD63^+$ basophils/anti-FcεRI ratio was determined based on the ROC curve for best selectivity and specificity.

RESULTS

Allergenicity of Gluten and HWP for Patients With WDEIA

Skin Prick Test

As expected, all patients with WDEIA showed sensitizations to the positive control (wheal and erythema mean diameter (W/E): 5.8 and 13.4 mm), but none to the negative control (0 mm). A positive reaction was triggered in all patients with WDEIA for G (W/E 6.1 and 15.4 mm), in 11 of 12 patients for sHWP (W/E 5.8 and 13.7 mm), in 10 of 12 patients for G-ω5 (W/E 3.8 and 7.0 mm), and in 9 of 12 patients for eHWP (W/E 6.2 and 11.1 mm) (**Figure 1** and **Supplementary Table 2**). Large interindividual differences were observed that resulted in wide ranges of minimal and maximal diameter for each substance, ranging from 0.5 to 16.5 mm for W and from 2.0 to 31.0 mm for E overall. There were no significant differences ($p > 0.05$) in mean wheal diameter between the four substances, even if G, sHWP, and eHWP triggered wheals that were comparable in size with those of the positive control and about 60% larger compared with G-ω5. The mean erythema diameter caused by G, sHWP, and eHWP was also similar to that of the positive control. The erythema following SPT with G was significantly higher ($p < 0.05$) than that with G-ω5, but all other pairwise comparisons were not significantly different from one another.

Basophil Activation

All ATS for gluten and HWP induced basophil activation in the blood of patients with WDEIA, except for p5, p7, and p8 (**Figure 2** and **Supplementary Figures 1–8**). As already observed in the SPT, the responses were highly individual, e.g., with blood from patients p1 and p6 showing the highest basophil activation for eHWP, p9 for sHWP and eHWP, and p11 for G and G-ω5. Contrary to expectations, G-ω5 did not lead to lower basophil activation in comparison with G in general. The basophil activations (%CD63$^+$ basophils) of patients were in a range between 0.2 and 63.0% (median: 9.4%) for G, 0.6–82.6% (median: 11.6%) for G-ω5, 0.4–72.7% (median: 8.2%) for eHWP, and 2.2–80.0% (median: 23.1%) for sHWP. Significant differences in %CD63$^+$ basophils between patients and controls were found for sHWP ($p < 0.05$), but not for G, G-ω5, and eHWP. In contrast, patients showed significantly higher %CD63$^+$ basophils/anti-FcεRI ratios compared with controls with all ATS ($p < 0.05$) (**Figure 3**). Consequently, the %CD63$^+$ basophils/anti-FcεRI ratio was used as characteristic parameter for further investigations.

There were no significant differences ($p > 0.05$) in patient %CD63$^+$ basophils/anti-FcεRI ratios between the four different ATS (median G = 0.113, G-ω5 = 0.178, eHWP = 0.130, and sHWP = 0.408), *inter alia*, due to high interindividual variability. The %CD63$^+$ basophils/anti-FcεRI ratios were low for all ATS in controls (median: G: 0.018, G-ω5: 0.016, eHWP: 0.069, and sHWP: 0.019). The ROC curves generated for all ATS from the %CD63$^+$basophils/anti-FcεRI ratio of patients and controls revealed that BAT with sHWP gave the highest AUC (0.925) with excellent sensitivity (100%) and specificity (83%) to discriminate between patients with WDEIA and controls (**Supplementary Figure 9** and **Supplementary Table 3**).

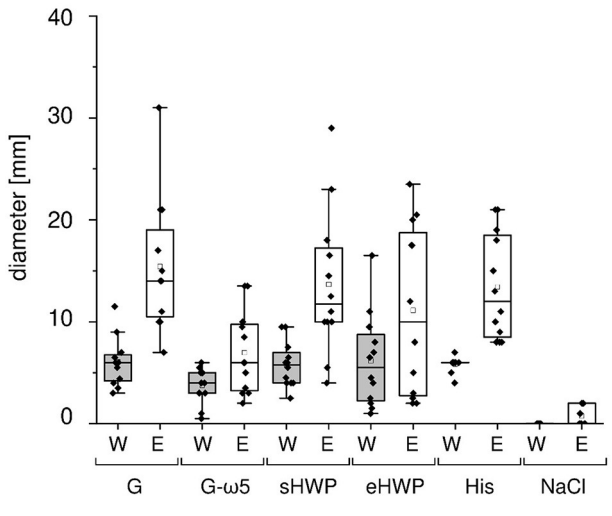

FIGURE 1 | Skin prick test results of patients with wheat-dependent exercise-induced anaphylaxis. Allergen test solutions from gluten G and G-ω5 and from slightly and extensively hydrolyzed wheat proteins sHWP and eHWP were used, as well as histamine dihypochloride (10%) solution (His) as positive control and isotonic sodium chloride solution as negative control (NaCl). The diameter of the wheals (W) and erythema (E) were documented in mm. A double determination was performed for each patient ($n = 2$), except patient 4 ($n = 1$). The data for gluten G were added for comparison and were already reported in Gabler et al. (20).

Identification of Allergenic Proteins in the Test Solutions

Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis

In SDS-PAGE, all protein bands from the ATS had M_r below or equal to 60 kDa (**Figure 4**). The lack of larger and hydrophobic proteins, such as HMW-GS was expected, because the ATSs were aqueous extracts of G, G-ω5, and sHWP or were completely soluble in water as in case of eHWP. The band pattern of G and G-ω5 was similar with bands at 60, 57, 47, and 37 kDa and three additional ones at 52, 40, and 27–24 kDa for G-ω5. Bands with M_r about 60 kDa typically belong to ω-gliadins and the additional band at 52 kDa in G-ω5 is likely to be from ω-secalins. The other bands in the range from 37 to 47 kDa can be assigned to gliadins and LMW-GS (46). While eHWP showed a weak and blurred band at 20–27 kDa and its main band at 10–16 kDa, sHWP had only one band at 10–16 kDa. This indicates that proteins were degraded through hydrolysis in sHWP and eHWP. The most intense protein band in all ATSs was at M_r 10–16 kDa and this range corresponds to non-gluten proteins of the water-/salt-soluble albumin/globulin fraction, such as grain softness proteins, puroindolines, purothionins (Tri a 37), non-specific lipid-transfer protein (Tri a 14), and ATIs (Tri a 15, Tri a 28, Tri a 29, Tri a 30, and Tri a 40), many of them already known as allergens (47).

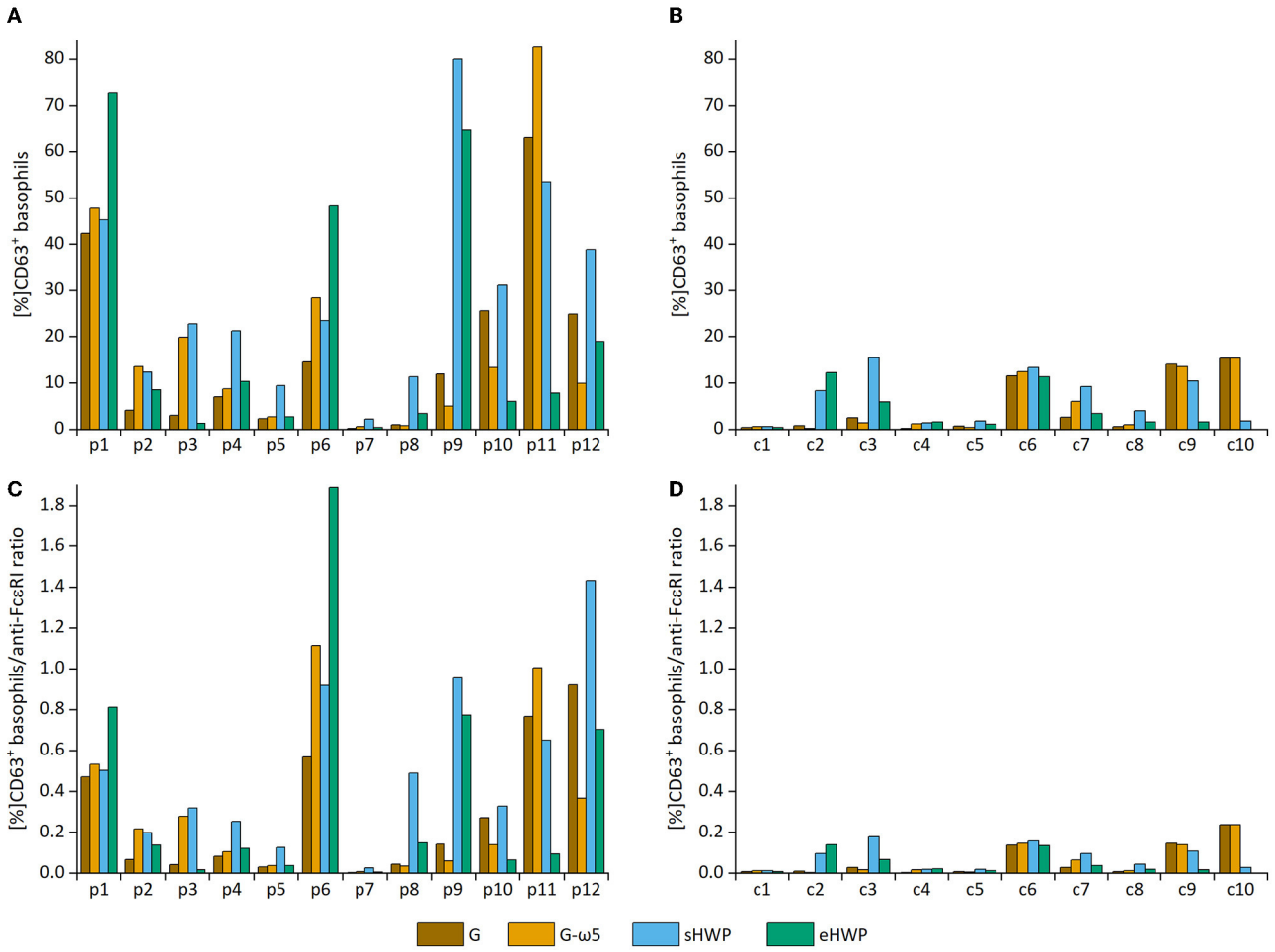

FIGURE 2 | Basophil activation in individual patients and controls. Allergen test solutions from gluten G and G-ω5 and from slightly hydrolyzed wheat proteins (sHWPs) and extensively hydrolyzed wheat proteins (eHWP) were used. **(A)** %CD63$^+$ basophils from patients p1–p12, **(B)** %CD63$^+$ basophils from controls c1–c10, **(C)** %CD63$^+$ basophils/anti-FcεRI ratio from patients p1–p12, **(D)** %CD63$^+$ basophils/anti-FcεRI ratio from controls c1–c10.

High-Performance Liquid Chromatography

Gel permeation- and RP-HPLC analyses were carried out to obtain further information complementary to SDS-PAGE on the M_r distribution of the proteins in the ATS and their hydrophobicity profile. Both GP-HPLC systems showed that there were high percentages of proteins with M_r of about 14 kDa present in the ATS (**Figure 5**). More than 68% of all proteins in the four ATS had a M_r about or below 14 kDa, according to system I suitable for a M_r range from < 14 to 200 kDa (G: 81.9%, G-ω5: 68.3%, sHWP: 84.4%, and eHWP: 92.2%) (**Supplementary Figure 10**). System II suitable for a M_r range from < 2 to ≥ 14 kDa confirmed that over 75% of proteins in the ATS had a M_r about 14 kDa (G: 85.6%, G-ω5: 91.5%, sHWP: 83.3%, and eHWP: 75.7%) (**Supplementary Figure 11**). These results corresponded well to the protein band pattern on the SDS-PAGE gel.

The RP-HPLC chromatograms of the G and G-ω5 ATS showed the typical hydrophobicity profile of the albumin/globulin fraction. In contrast, the peaks in the chromatograms of sHWP and eHWP could not be clearly assigned to any reference chromatogram of intact wheat proteins, again indicating protein degradation (**Supplementary Figure 12**). The protein concentrations of the ATS used for the BAT experiments determined by RP-HPLC were 2.10 mg/ml (G), 2.05 mg/ml (G-ω5), 3.96 mg/ml (sHWP), and 3.00 mg/ml (eHWP). Higher protein concentrations were not achievable with this preparation procedure for G, G-ω5, and sHWP, because of limited solubility. The concentration range of the four ATS, in which the allergenic basophil activation was triggered, was not directly comparable between the ATS. The concentrations were not set in a specific range, but resulted from preliminary tests, which were primarily intended to exclude non-specific activations in the control group while triggering specific activations in patients.

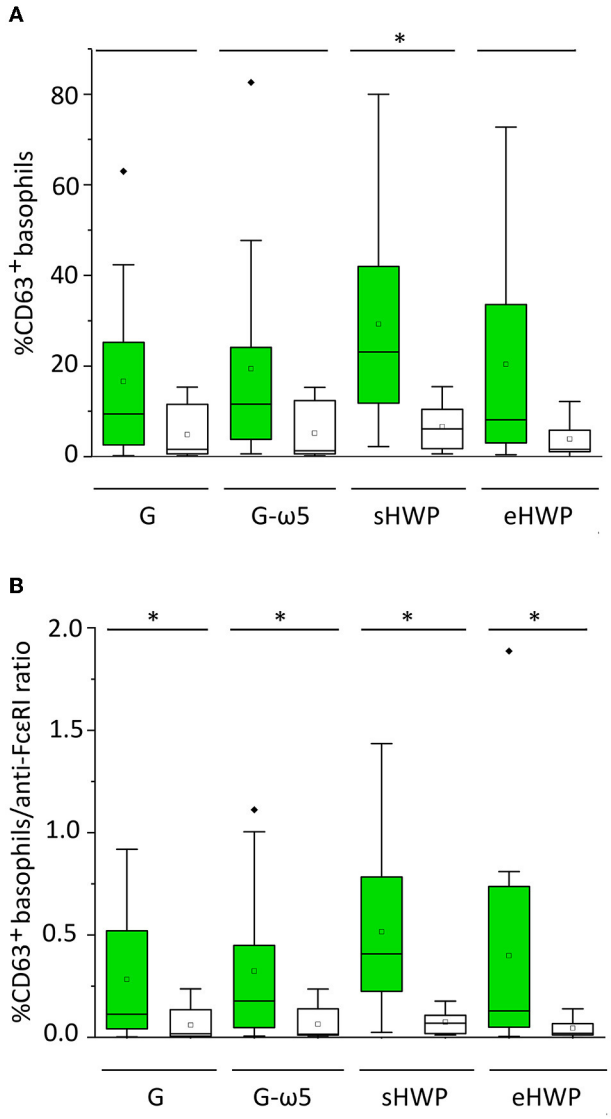

FIGURE 3 | Basophil activation in patients (green) and controls (white). Allergen test solutions from gluten G and G-ω5 and from sHWPs and eHWPs were used. **(A)** %CD63$^+$ basophils, **(B)** %CD63$^+$ basophils/anti-FcεRI ratio. Significant differences between patients and controls are indicated by asterisks (one-way ANOVA, Dunn's *post-hoc* test, $p < 0.05$). There were no significant differences between patients' reactivity to different ATS. Diamonds indicate individual outliers outside the first or third quartiles. Squares represent the mean and lines the median. Whiskers mark the 1.5 interquartile range. The box corresponds to the range in which the middle 50% of the data are located.

Proteomics-Based Untargeted Liquid-Chromatography Mass Spectrometry of the ATS

While SDS-PAGE and HPLC already provided valuable information on the identities of the proteins in the ATS, untargeted UPLC-TripleTOF-MS of different enzymatic digests of the ATS was performed to identify the specific proteins in the ATS and their proportions. Different enzyme combinations were used to maximize protein identifications and avoid bias, because gluten proteins, and especially ω5-gliadins, are known to be resistant to cleavage with P, T, or C (40). Of the PT, TC, PTC, PC, and TLY digestions used (**Figure 6** and **Supplementary Figure 13**), PT turned out to be the most suitable, because percentages of identified proteins in the ATS were the highest in comparison with other digestions. Consequently, the peptides and corresponding proteins identified in the ATS after PT digestion are reported in **Supplementary Tables 4–11**, using both specific and unspecific digestion mode for data evaluation.

The identified proteins in the ATS made from gluten samples G and G-ω5 contained 96.6 and 99.3% of ATIs, such as ATI-types CM1, CM2, CM3, CM16, 0.28, and 0.53 in G and CM2, CM3, CM16, and 0.19 in G-ω5. ATIs are soluble in aqueous salt solutions whereas gluten, by definition, remains mostly insoluble. Therefore, it appears reasonable that ATIs were enriched during ATS preparation with isotonic NaCl solution. Small proportions of LMW-GS and α-gliadins were present in G, as well as α- and ω-gliadins in G-ω5.

The solubility of sHWP in aqueous solutions was comparable with that of gluten samples (10) as was the composition of the ATS. It consisted of 88.8% of ATIs, such as ATI-types CM1, CM2, CM3, CM16, 0.19, and 0.28, as well as a slightly higher proportion of 3.7% of gluten proteins (LMW-GS, gliadins) compared with G and G-ω5. In contrast, eHWP contained 70.2% of gluten proteins, with 58.2% gliadins (α-, β-, γ-gliadins) and 12.0% glutenins (LMW-GS). This difference can be explained by the fact that eHWP was strongly hydrolyzed and completely soluble in aqueous solutions. ATIs (CM1 and CM3) only represented 16.0% of proteins in eHWP and the remaining 13.8% were other proteins, such as enzymes and uncharacterized proteins. The investigations using TC, PTC, PC, and TLY digestions showed some variation in protein composition compared with the PT digestion, but the overall picture of identified protein groups in the ATS was similar (**Supplementary Figure 13**).

The sequences of the identified proteins in the ATS were analyzed for known WDEIA epitopes (15, 48, 49). Only the epitope QQPGQ was identified two times in an ω-gliadin (Uniprot accession: C0KEH9) present in G-ω5. All other identified proteins in the ATS contained none of the known WDEIA epitopes.

DISCUSSION

We expected to see differences in allergenicity to patients with WDEIA between G and G-ω5, because G-ω5 was gluten from a wheat/rye-translocation line (35) that contains a significantly lower amount of ω5-gliadins. SPT results showed that wheal and erythema diameters caused by G-ω5 were the lowest compared with other substances, but the differences were not significant except for the comparison of erythema diameter between G and G-ω5. However, the BAT %CD63$^+$ basophils/anti-FcεRI ratio was similar for G and G-ω5 with almost identical median values and ranges, as were all parameters derived from the ROC curves. It was reasonable to assume that gluten with a significantly lower

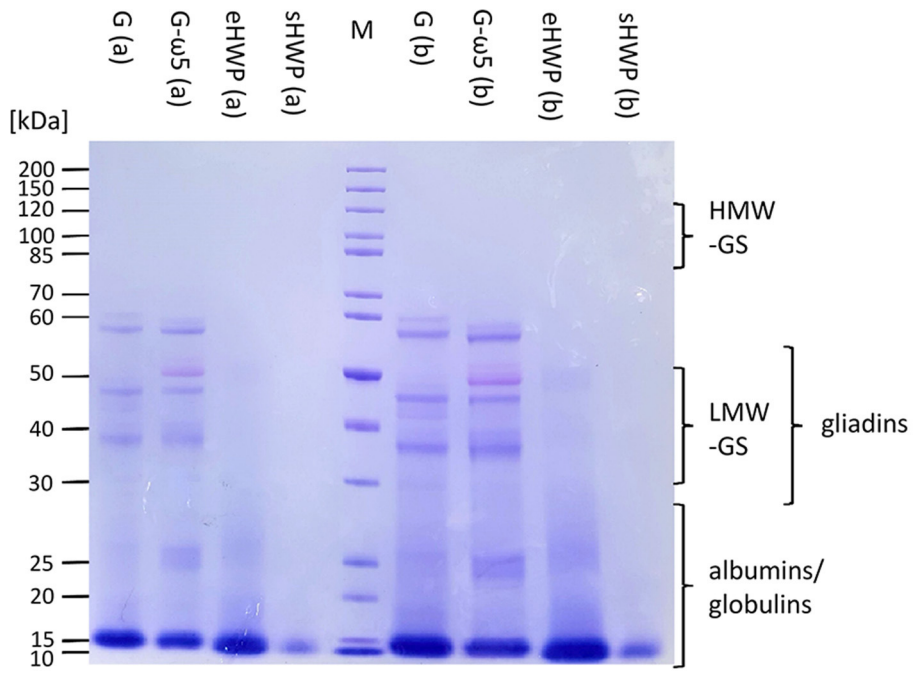

FIGURE 4 | Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of allergen test solutions. Gluten G and G-ω5 as well as sHWPs and eHWPs were analyzed. Protein marker (M) 3.5 μg, samples 5.3 μg (a), and 15.0 μg (b). HMW-GS, high-molecular-weight glutenin subunits, LMW-GS, low-molecular-weight glutenin subunits. The albumin/globulin fraction may consist of, e.g., grain softness proteins, puroindolines, purothionins (Tri a 37), non-specific lipid-transfer protein (Tri a 14), and amylase/trypsin-inhibitors (Tri a 15, Tri a 28, Tri a 29, Tri a 30, and Tri a 40) (40).

ω5-gliadin content would trigger lower basophil activations in patients than representative gluten, since ω5-gliadins are considered to be the main allergen of WDEIA (14). In several cases, it appeared as if even the opposite was the case, because stronger basophil activities occurred for G-ω5 in comparison to G in p1, p2, p3, p4, p6, and p11. These results indicate that other allergenic proteins need to be relevant and present in the ATS.

Altenbach et al. used transgenic wheat with reduced content of ω5-gliadins and assessed its allergenicity in sera of eleven patients with WDEIA using a two-dimensional immunoblot analysis. Seven out of eleven patients showed reduced levels of immunoglobulin E (IgE) reactivity to ω5-gliadins using transgenic wheat, but the same sera showed IgE reactivities to other gluten proteins at the same time. Additionally, sera from three patients generally had the highest IgE reactivity not to ω5-gliadins, but to HMW-GS, α-gliadins, and non-gluten proteins. They concluded that this transgenic wheat line was not beneficial for the nutrition of patients with WDEIA because of the complexity of the immune response in the participating patients with WDEIA. Without knowing to which wheat protein groups, a patient with WDEIA is sensitized, it is too risky overall to consume transgenic wheat. Even if the ω5-gliadin content is reduced therein, other wheat proteins were shown to trigger IgE reactivity in patients with WDEIA (50). Our findings support their conclusion and still leave a wheat-free diet and/or avoidance of cofactors as the only safe option for patients with WDEIA. Further, the identified proteins in ATS from G and G-ω5 both contained over 96% of ATIs (non-gluten proteins) and only very low proportions of gliadins, so that a potential difference in ω5-gliadin content was most likely negligible.

We expected to find LTPs in the aqueous ATS, as they are known to be soluble and to cause basophil activity in patients. Pastorello et al. described three cases of exercise-food challenge confirmed patients with WDEIA. They identified a 9 kDa LTP as the allergenic protein in these patients by immunoblotting. Simultaneously, these patients showed no reactivity to the gliadin and glutenin fractions (23). The protein band of the albumin/globulin fraction (10–15 kDa) of G-ω5 and sHWP in the SDS-PAGE gel of the lyophilized ATS suggested that LTPs may be present. However, no LTPs were identified with the proteomics UPLC-TripleTOF-MS approach (PT-digestion), but high percentages of ATIs. In our previous study, the same WDEIA patient cohort was tested for sIgE against LTP. All patients showed negative results (< 0.1 KU/L; LTP/Tri a 14) (20).

Based on the heterogeneous molecular properties of sHWP and eHWP, we expected differences among the parameters investigated, but we did not find any significant differences in SPT or $CD63^+$ basophils/anti-FcεRI ratio. The only parameters that differed were those derived from the ROC curves indicating that sHWP yielded higher sensitivity/specificity (100%/83%) compared with eHWP (75%/70%) to discriminate between patients and controls. Due to a lack of studies so far, it remains unclear how degree and type of protein hydrolysis affect the allergenicity of gluten in WDEIA. Hydrolysis to a certain

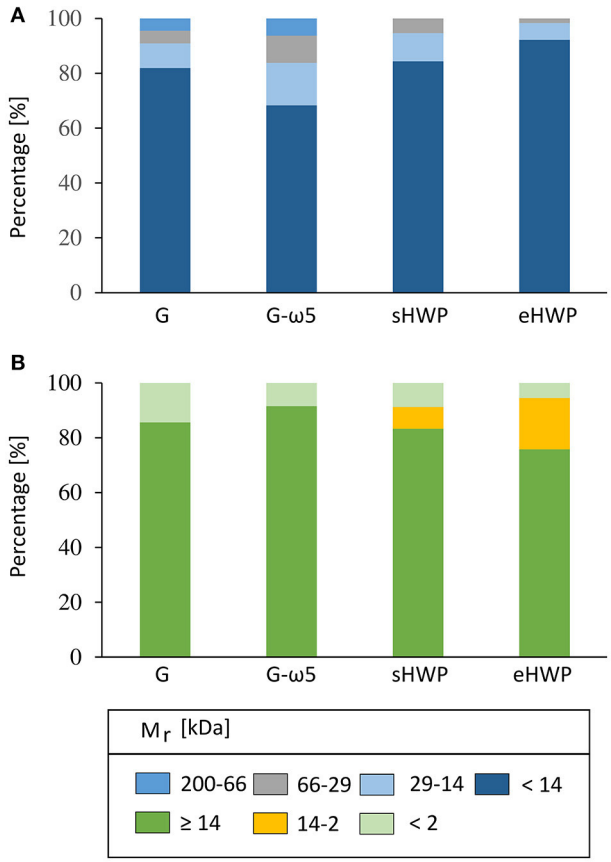

FIGURE 5 | Gel-permeation high-performance liquid chromatography (GP-HPLC). Relative molecular mass distribution (M_r) [%] of the allergen test solutions from gluten G and G-ω5 and from sHWPs and eHWPs. Areas within each fraction were set by marker substances. Two different systems were used. **(A)** 1: 200–66 kDa; 2: 66–29 kDa; 3: 29–14 kDa; 4: <14 kDa. GP-HPLC; **(B)** 1: ≥14 kDa; 2: 14–2 kDa; 3: <2 kDa.

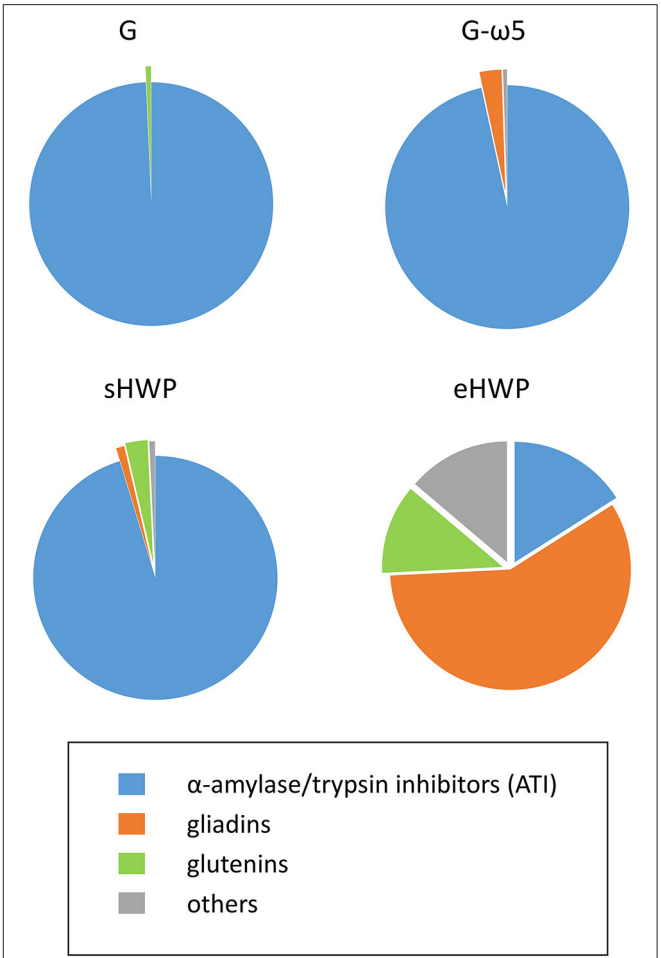

FIGURE 6 | Percentages of protein groups in allergen test solutions. Groups include α-amylase/trypsin inhibitors (ATI, blue), gliadins (orange), glutenins (green), and others (gray) in allergen test solutions from gluten G and G-ω5 and from sHWPs and eHWPs. Percentages were determined using the intensity based absolute quantitation (iBAQ) algorithm with ultra-performance liquid chromatography (UPLC)-TripleTOF-MS data (protein score > 40) in specific digestion mode (pepsin + trypsin). Details on specific proteins are available in **Supplementary Tables 4–11**.

degree may increase the allergenicity by exposing epitopes or generating new ones (6, 7, 51). Beyond that degree, continued hydrolysis is expected to decrease allergenicity, because epitopes are degraded.

Neither SPT nor BAT revealed clear differences between gluten samples (G, G-ω5) and HWP (sHWP, eHWP) in terms of allergenicity. SDS-PAGE and GP-HPLC revealed that all ATS contained high percentages of proteins with M_r 10–16 kDa (SDS-PAGE) and about 14 kDa (GP-HPLC). UPLC-TripleTOF-MS analysis showed that high percentages of ATIs were present and their M_r correspond exactly to this mass range. These findings raise the question, whether ATIs are implicated not only in baker's asthma, but also in WDEIA. Until now, the main focus was on gluten proteins, such as ω5-gliadins and HMW-GS as major WDEIA allergens (19, 48, 52), though there are reports that ATIs may also play a role in WDEIA (24). IgE immunoblotting with patients' sera showed reactions to ATIs present in wheat protein fractions and ATI-types CM1, CM3, CM16, and 0.19 were identified in the allergenic fraction (24), similar to our results.

The sequences of all identified proteins in the ATS were analyzed for known WDEIA epitopes (15, 48, 49). Only one known epitope (QQPGQ) was identified, indicating that other epitopes appear to be relevant in WDEIA which are currently unknown. Western blotting using patient samples can be used in further studies to support their identification.

Sandiford et al. and Battais et al. reported possible cross-reactive epitopes between gliadins and ATIs (53, 54). Pastorello et al. used wheat flour within their study, which naturally contains ATIs, whereas we used gluten. By definition, gluten is poorly soluble in water and salt solutions, but residues of the soluble albumin/globulin fraction still remain in the gluten polymer, even after extensive washing to remove starch and other flour constituents (38, 55).

Besides ATIs, small percentages of gliadins and/or LMW-GS were present in the ATS from G, G-ω5, and sHWP. Overall, these three ATS showed a high degree of similarity regarding protein composition and solubility, again confirming that sHWP was only slightly hydrolyzed.

The basophil activation triggered by eHWP was according to expectations, because over 70% of proteins were gliadins and glutenins, but rendered soluble due to more extensive hydrolysis compared with sHWP. As gluten proteins are already known as relevant allergens in WDEIA, basophil activation in patients was anticipated (4, 48). Chinuki et al. reported a HWP product in soap, which was produced by acid hydrolysis and triggered allergic reactions in patients with WDEIA (5, 19). Apparently the degree of hydrolysis was enough to solubilize all wheat proteins in water, but not enough to significantly destroy allergenic epitopes in eHWP.

The %CD63$^+$ basophils/anti-FcεRI ratio was identified as an appropriate BAT parameter to differentiate reactivity to ATS between patients with WDEIA and control subjects. The discriminability estimated from the AUC of each ROC curve varied between gluten samples and HWP, with the best results for sHWP (AUC ROC: 0.925). Specificity (83%) and sensitivity (100%) of %CD63$^+$ basophils/anti-FcεRI ratio using sHWP at optimal cut-off were very good. Another advantage is that ATS preparation was easy and fast and did not require complex extractions or enzymatic digestions, as in the case of gluten isolates (20, 34, 55). We found no correlations between BAT results (CD63$^+$ basophils, %CD63$^+$ basophils/anti-FcεRI ratio), diameter of wheals and erythema in SPT, sIgE, or disease severity. This is understandable regarding the levels of sIgE, because only clinical routine IgE determination for WDEIA was available. As mainly ATIs were identified in the ATS (G, G-ω5, and sHWP), the allergic reactions to these are found here and those did not appear to be related to sIgE against wheat flour, gluten, gliadin, ω5-gliadin, or LTP. Further insights into possible correlations between BAT and sIgE levels could be gained by measurements of sIgE against ATI types. One possible reason for the lack of correlation between BAT results and disease severity may be that basophil granulocytes are only one part of the whole allergic reaction that has many other influencing factors (e.g., mast cells) (56).

Three of the twelve patients (p5, p7, and p8) showed low basophil activations to G, G-ω5, sHWP, and eHWP in general. P8 showed low basophil activations to the ATS assessed here, but showed a high reactivity to ω5-gliadins in our previous study (20). Patients p5 and p7 had low basophil reactions in the present and in our previous study (20). The IgE positive control showed a basophil activation in both cases, but the basophil granulocytes did not react to the allergens tested in either case. The exact reasons remain unclear at this point in time, but warrant further investigations.

In our previous study, we investigated the basophil activity to isolated ω5-gliadins in the context of WDEIA with the same patients and controls as in the present study. The BAT parameter %CD63$^+$ basophils was identified as the best parameter in this case to differentiate between patients and controls. Thereby, the ATS from isolated ω5-gliadins showed a test sensitivity of 100%, a specificity of 90%, and an AUC ROC of 0.975 at a concentration of 4 mg/ml (20). In comparison with G, G-ω5, and eHWP, the results for ω5-gliadins were better, but comparable with sHWP (sensitivity 100%, specificity 83%, and AUC ROC 0.925). As we identified high amounts of ATI in the ATS of sHWP, this underlines the result of the present study, that non-gluten proteins carrying yet unidentified allergenic epitopes appear to be relevant in WDEIA.

One acknowledged limitation of our study is the comparatively small number of patients with WDEIA and controls. The main reason is that the prevalence of WDEIA is very low overall and the participants were only recruited from the surrounding area of one specialized center. Our main intent was to identify the causative proteins in the ATS first, before we continue studies with more patients with WDEIA from several centers.

In conclusion, we found differences in allergenicity of gluten and HWP samples with varying molecular composition in individual patients with WDEIA using SPT and BAT. The %CD63$^+$ basophils/anti-FcεRI ratio was the most promising parameter to distinguish patients from controls. The procedure to prepare ATS from sHWP is easy and can be performed even in routine clinical practice to help establish BAT as another option to complement the WDEIA diagnosis. Since the ATS made of G, G-ω5, and sHWP predominantly contained ATIs and only small concentrations of gluten proteins, more research is needed to clarify the role of non-gluten proteins in WDEIA and identify specific mechanisms of immune activation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee, Technical University of Munich. The patients/participants provided their written informed consent to participate in this study.

AUTHORS CONTRIBUTIONS

AG, JG, M-CN, BE, TB, KS, and KB: conceptualization. AG, JG, and M-CN: formal analysis, investigation, and methodology. AG, JG, and BE: data curation. BE, TB, KS, and KB: funding acquisition, resources, and supervision. AG: visualization and writing—original draft. JG, M-CN, BE, TB, KS, and KB: writing—review and editing. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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BRIEF RESEARCH REPORT
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Low Levels Matter: Clinical Relevance of Low Pru p 3 sIgE in Patients With Peach Allergy

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Many clinical lab settings still use 0.35 KU_A/L as the cut-off for serum specific-IgE (sIgE) immunoassays, while the detection limit is 0.1 KU_A/L. The clinical relevance of -low-level sIgE (0.1–0.35 KU_A/L) remains controversial. Pru p 3 sIgE is considered to be the main routine tool for assessing lipid transfer protein (LTP) sensitization. We aimed to evaluate the clinical relevance of Pru p 3 sIgE low levels in a population diagnosed with LTP allergy. Adults diagnosed with LTP allergy and Pru p 3 sIgE $\geq$ 0.1 KUA/L between 2012 and 2019 were included. Clinical data were reviewed. nPru p 3 basophil activation test (BAT) was performed and basophil reactivity (BR) and sensitivity (BS) correlated with the peach allergy symptoms. Pru p 3 sIgE from 496 subjects was recorded, 114 (23.0%) between 0.1 and 0.34 KU_A/L (grLOW), the rest $\geq$ 0.35 KU_A/L (grB). A total of 44.7% in grLOW and 59.9% in grB were allergic. Urticaria was more frequent in grLOW. In grLOW, Pru p 3 sIgE was higher in patients with local compared with systemic symptoms. In grB, Pru p 3 sIgE was higher in allergic patients. Pru p 3/Total IgE ratios were higher in allergic vs. tolerant in both groups. In BAT, BR was similar in both groups. In grLOW, it was higher on allergic compared with tolerant ($p = 0.0286$), and on those having systemic vs. local symptoms ($p = 0.0286$). BS showed no significant difference between groups. Patients with low levels represent a non-negligible fraction and around 45% are peach allergic. BAT showed functional sIgE in them. Pru p 3 sensitizations should be carefully evaluated even when sIgE levels are low.

Keywords: serum specific-IgE, low levels, lipid transfer protein, clinical relevance, BAT

INTRODUCTION

Allergen-specific IgE (sIgE) levels cannot be used as individual predictors of clinical reactivity or severity, although high-sIgE concentrations correlate with increased risk of reactions (1). The importance of establishing sIgE cut-offs to provide clinical relevance in the assessment of food allergy has been extensively reported (2–4). The cut-off for the most common immunoassays used to quantify serum sIgE (e.g., ImmunoCAP® ThermoFisher Scientific, Uppsala), has traditionally been 0.35 KU_A/L; and it is still used in many clinical lab settings, despite the reports showing

that the cut-off may differ depending upon the factors, such as the allergenic source and patient age (3). Indeed, the technical detection limit for the *in vitro* singleplex fluorescence enzyme-immunoassay ImmunoCAP® (ThermoFisher Scientific) is 0.10 KU_A/L. Little evidence has been reported on the clinical relevance of sIgE levels between 0.1 and 0.35 KU_A/L and it is a matter of discussion in the field.

Lipid transfer proteins (LTPs) are widely cross-reacting panallergens related to complex clinical profiles regarding severity and food triggers (5, 6). LTPs are the most important cause of plant food allergy in adults and children in the Mediterranean, but indeed emerging in other areas (6). Pru p 3, the peach LTP, is considered to be the prototype protein, and routinely used as the main marker to assess LTPs sensitization (7). High Pru p 3 sIgE has been related with systemic reactions and a higher prevalence of hazelnut, peanut, and walnut allergy (4, 8). Pastorello et al. established Pru p 3 2.69 KU_A/L to discriminate patients at risk of reactions (4), but other authors have found overlapped values between allergic and tolerant (9). Nevertheless, Pru p 3 allergic patients have also been reported with sIgE levels < 0.35 KU_A/L (10). We aimed to evaluate the clinical relevance of low levels of Pru p 3 sIgE by ImmunoCAP®.

MATERIALS AND METHODS

Study Population

Adult patients evaluated in the Allergy Section of Hospital Clinic (Barcelona, Spain) between 2012 and 2019 with an LTPs food allergy and Pru p 3 sIgE $\geq$ 0.1 KU_A/L were selected. Serum samples obtained following routine practice were analyzed in the Immunology Department of the same hospital. Pru p 3 sIgE (by ImmunoCAP®, Thermo Fisher Scientific) is measured per protocol in all LTPs allergic patients regardless of the presence of symptoms with peach. Sensitization to other plant food allergens was analyzed by microarray ImmunoCAP® ISAC (Thermo Fisher Scientific.) Patients sensitized to other panallergens (PR-10; TLP; Profilin) were excluded. The study was approved by the local ethic committee (HCB/2020/0373).

Clinical Characterization

Demographical and epidemiological data were retrospectively recorded from clinical history. Peach allergy symptoms were classified as: local (gastrointestinal symptoms–GI-, Oral Allergy Syndrome–OAS-, and contact urticaria–CU-) and systemic (generalized urticaria and/or angioedema–U/AE-, anaphylaxis-AN-). Peach tolerance (–TOL-) and peach avoidance (-AV-; due to medical advice, fear, or dislike) were also recorded and also the involvement of cofactors, including exercise, alcohol, non-steroidal anti-inflammatory drugs (NSAIDs), and/or menstruation.

Basophil Activation Test

Pru p 3 basophil activation test (BAT) was performed in some patients to assess sIgE functionality. Briefly, after the patient informed consent, 10 ml of heparinized peripheral blood was obtained and immediately taken to the laboratory for BAT using the Flow2CAST™ kit (Bühlmann Laboratories AG, Switzerland) and following the manufacturer's procedures. Purified Pru p 3 (1 mg/ml, Bial Aristegui, Bilbao, Spain) was tested at 25, 12.5, 5, and 2.5 ng/ml final concentrations. Basophils were identified by flow cytometry (FACS-Canto II, BD Biosciences, Germany). A minimum of 500 basophils was gated and those CD63+ were defined as activated ($\geq$15% was considered a positive test). Basophil reactivity (BR, i.e., number of basophils responding to a stimulus) was calculated as the CD63+ expression post-stimulus minus basal CD63+ expression, represented as % CD63+. Basophil sensitivity (BS) is calculated as CD-sens, i.e., inversion of EC50 (concentration inducing 50% of maximum response) $\times$ 100 (11).

Statistical Analysis

Pru p 3 sIgE centralization and dispersion measurements were calculated considering a quantitative and asymmetric distribution. Free distribution was considered in our analysis so non-parametric tests were used to verify heterogenicity between our variables. Quantitative data were compared using the Mann Whitney *U*-test or the Kruskal–Wallis test. Qualitative data were compared using the chi-squared test and Fisher's exact test for a small sample size. *P* values lower than 0.05 were considered statistically significant. The GraphPad Prism 8.0.2 software (Inc., CA, USA) was used for the statistical analysis.

RESULTS

Groups Characterization

A total of 496 subjects with Pru p 3 sIgE $\geq$ 0.1 KU_A/L were recorded between 2012 and 2019. A total of 284 (57.3%) subjects were women, median [Interquartile range, IQR] age of 42 (17–92) years. Of 496 subjects, 114 (23.0%) had Pru p 3 sIgE between 0.1

TABLE 1 | Clinical picture.

	grLOW *n* = 114	grB *n* = 328	*P* value
Peach allergic	44.7%	59.9%	*ns*
Peach tolerant	20.2%	25.9%	*ns*
Peach avoidance	35.1%	14.1%	****
Peach-related symptoms			
Local	50.4%	55.1%	*ns*
CU	21.9%	25.1%	*ns*
OAS	23.7%	24.6%	*ns*
GI	4.4%	5.5%	*ns*
Systemic	22.8%	25.4%	*ns*
U/AE	21.2%	17.5%	*
AN	1.9%	8.1%	*ns*

*Clinical relevance frequencies among studied patients. grLOW, Pru p 3 sIgE from 0.1 to 0.34 KU_A/L; grB, Pru p 3 sIgE >0.35 KU_A/L; CU, contact urticaria; OAS, oral allergy syndrome; GI, gastrointestinal symptoms; U/AE, generalized urticaria or angioedema; AN, anaphylaxis. Chi-squared test and Fisher's exact test for small simple size were used to test p (*0.01 to 0.05, ****< 0.0001 and ns, non-significant). Patients avoiding peach were not included on the symptom statistical analysis because tolerance or allergy could not be guaranteed.*

TABLE 2 | Pru p 3 sIgE values distribution.

classification	Peach sIgE median [IQR] KU_A/L	Pru p 3 sIgE median [IQR] KU_A/L	Pru p 3/Peach sIgE median [IQR]	Pru p 3/Total sIgE median [IQR]	Pru p 3 sIgE on CCD+ median [IQR] KU_A/L
grLOW [0.1–0.35]	0.20 [0.14–0.28]	0.19 [0.07–0.26]	1.16 [0.92–1.46]	0.00 [0.00–0.01]	0.29 [0.22–0.31] *ns*
grB [≥0.35]	3.73 [1.35–10.28]	3.37 [1.16–9.67]	1.19 [1.04–1.38]	0.03 [0.01–0.07]	16.30 [4.58–20.85] *p**
	*p****	*p****	*ns*	*p****	

*IgE values distribution among groups: grLOW (Pru p 3 sIgE from 0.1 to 0.34 KU_A/L) and grB (Pru p 3 sIgE >0.35 KU_A/L). Pru p 3, peach and Pru p 3/Peach ratio sIgE median and IQR (interquartile range) results are included as well as CCD+ Pru p 3 sIgE median [IQR]. Differences between grLOW/grB and between CCD+/CCD- Pru p 3 sIgE in grLOW/grB were statistically evaluated with the Mann–Whitney–test (*0.01 to 0.05, ***0.0001 to 0.001, ns, non-significant).*

and 0.34 KU_A/L (grLOW = group low levels) and 382 (77.0%) ≥ 0.35 KU_A/L (grB = group high levels).

44.7% of patients of grLOW and 59.9% in grB were allergic ($p > 0.05$), with similar peach-related symptoms and a higher presence of local symptoms. However, U/AE was more frequent in grLOW ($p = 0.020$). Peach avoidance was statistically superior in grLOW ($p < 0.0001$) (**Table 1**).

Pru p 3 sIgE Levels and Symptoms

Peach sIgE values were higher in grB, as well as Pru p 3/total IgE ratio ($p < 0.05$), whereas no differences were observed in Pru p 3/Peach sIgE (ratio) between groups (**Table 2**). In grLOW (**Figure 1A**), Pru p 3 sIgE was higher in patients with local compared to systemic symptoms ($p = 0.0385$). In grB (**Figure 1B**), Pru p 3 sIgE was higher in allergic compared to tolerant ($p = 0.0009$). The medians from the ratios Pru p 3/peach sIgE were superior to 1 for either grLOW or grB. Moreover, when classifying patients according to their clinical symptoms, no statistically significant differences were found. Pru p 3/Total IgE ratios were lower than 1% in grLOW, unlike grB. In both groups, these ratios were statistically higher ($p < 0.0001$) in allergic compared to tolerant (**Supplementary Table 1**).

Co-sensitization

Co-sensitization to other LTPs was analyzed in 70 patients of grLOW and 318 of grB (**Supplementary Figure 1**; **Supplementary Table 2**). In grLOW, co-sensitization was statistically less frequent (64.3 vs. 95.9%). Mal d 3, Ara h 9, and Jug r 3 were the most frequent ones, and Tri a 14 the rarest in both groups.

Cross-reactive carbohydrate determinants (CCD) reactive sIgE may cause false-positive results in Pru p 3 measurements by binding the test cellulose matrix (12). CCD sensitization data were available for 80 (70.2%) patients of grLOW and 226 (59.2%) of grB. In grLOW, of the 7 CCD+ (8.7%), 5 avoided eating peach, 1 tolerated and 1 referred local symptoms. In grB, of the 19 CCD+ (8.4%), four avoided the ingestion of peach, three tolerated, six had local, and six systemic symptoms (two anaphylaxis). Tolerant and allergic frequencies were not statistically different between CCD+ and negative (**Table 2**). In grB, were found significant differences on sIgE to Pru p 3 from CCD+ compared with CCD–, being higher on CCD+.

Basophil Activation Test Results

nPru p 3 BAT was performed on 12 patients per group as previously reported (10). All in grB were BAT+, being 3 (25%) tolerant and 9 (75%) allergic (5 local/4 systemic reactions). In grLOW (**Table 3**), 7 (58.3%) were BAT+: 6 (85.7%) allergic (2 local/4 systemic reactions) and 1 (14.3%) avoided peach. In BAT-: 2 (40%) were tolerant and 3 (60%) allergic (2 local/1 systemic reactions). The median [IQR] for Pru p 3 sIgE for grLOW was 0.26 [0.10–0.28] KU_A/L. The ratio Pru p 3/peach sIgE median was 0.99 [0.79–1.09]. In addition, from these BAT- patients were 0.21 [0.18–0.23] (Pru p 3 sIgE) and 0.98 [0.97–0.99] (Pru p 3/peach sIgE ratio). BAT reactivity (BR, %CD63+ basophils) was not statistically different between groups (BR median: 17.8% grLOW/ 27.3% grB), neither when only allergic patients of each group were compared. In grLOW, BR was significantly higher on allergic individuals vs. tolerant ones ($p = 0.0286$), and on those having systemic symptoms vs. local ($p = 0.0286$). No statistically significant differences in basophil sensitivity were found between groups, although being higher in grLOW (CD-sens median: 819.0 grLOW/ 75.4 grB).

CONCLUSION

In summary, the ratio Pru p 3/Peach was similar in both groups and superior to 1, which would confirm a sensitization due to Pru p 3 on our population (13). About 45% of our patients of grLOW are allergic, highlighting the importance of considering Pru p 3 sIgE > 0.1 as potentially clinically relevant, despite 0.35 has traditionally been used as the cut-off, BAT reactivity (similar in both groups) demonstrated the presence of functional sIgE in patients with low levels.

Besides the theory reported by Kleine-Tebbe and Jakob (14) exposing that a 0.01 or greater ratio of specific IgE to total IgE, translated as a fraction of 1% of bound total IgE, is enough for basophil half-maximal activation, we observe basophil activation with a lower percentage. Thus, reliable quantitative detection of sIgE and the ratios analysis of specific and total IgE on these patients is relevant for an accurate diagnosis (13, 15).

A definite answer for the reason why Pru p 3 sIgE levels are higher on patients with local symptoms compared with those with systemic is not clear. Little is known about the real correlation between LTP sIgE levels and symptoms severity,

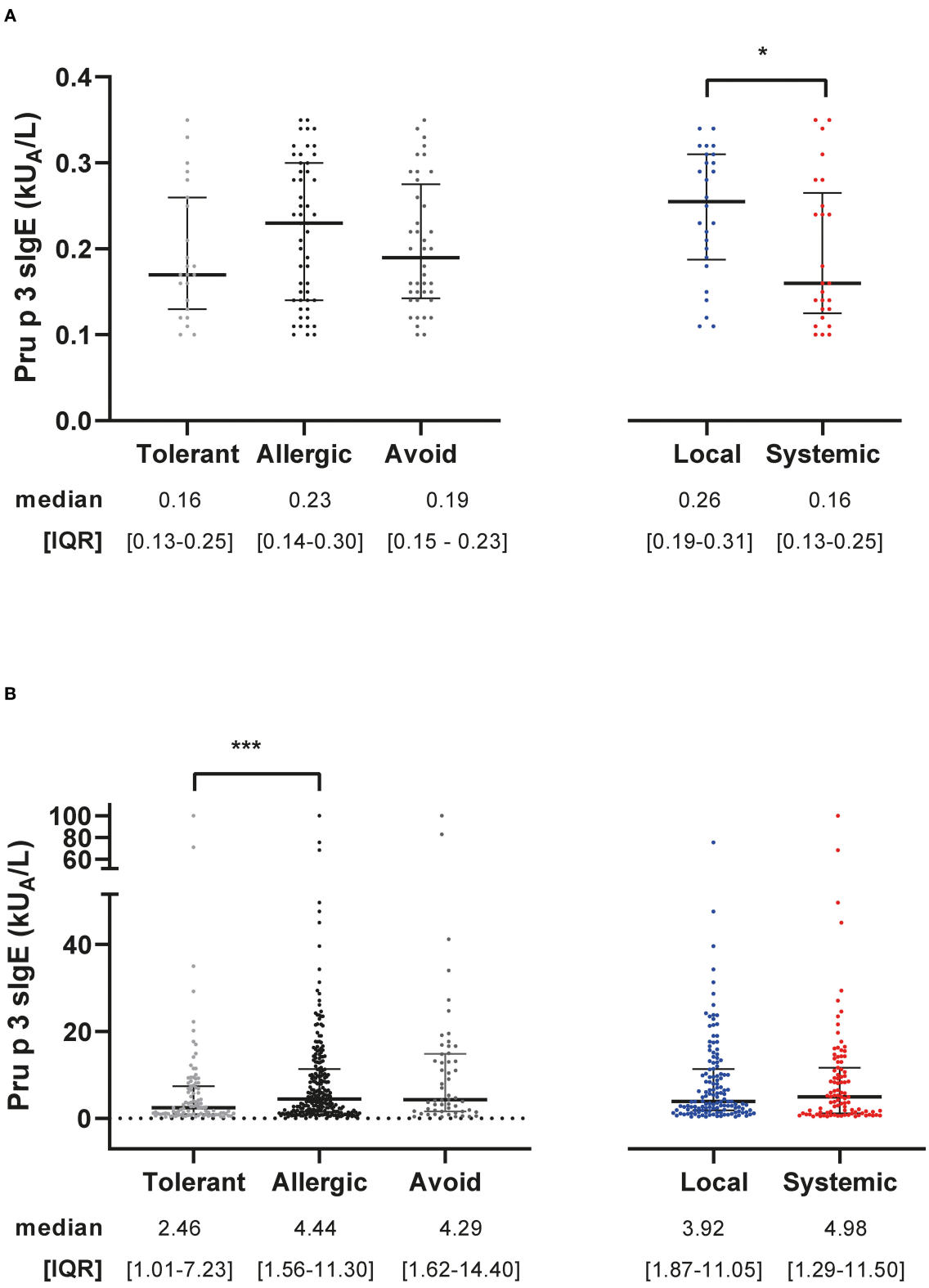

FIGURE 1 | Levels of Pru p 3 sIgE per group. Pru p 3 sIgE distribution, median, and IQR (interquartile range) values from grLOW **(A)** and grB **(B)** according to symptom classification (tolerance vs. allergy, local vs. systemic). The Mann–Whitney test was used to test *p* (*0.01 to 0.05, ***0.0001 to 0.001).

TABLE 3 | Characteristics and BAT results of the allergic patients from grLOW.

	BAT	EC50	% CD63+				Symptoms	Pru p 3 sIgE (KU_A/L)	Ratio Pru p 3/peach sIgE
			2.5	5	12.5	25			
			ng/mL Pru p 3						
P1	-	3.29	0.60	0.40	0.20	1.40	TOL	0.26	NA
P2	-	3.71	0.60	0.50	0.00	0.20	TOL	0.20	20
P3	-	32.48	0.20	0.00	0.00	0.00	CU, OAS	0.25	0.96
P4	-	-	0.00	0.00	0.00	0.00	OAS	0.30	0.73
P5	+	0.04	16.60	26.80	31.90	37.20	OAS	0.34	0.97
P6	+	0.13	16.70	2.40	14.80	0.20	GID	0.22	NA
P7	-	-	0.00	0.00	0.00	0.00	AN	0.16	1.00
P8	+	0.02	41.70	25.70	16.90	84.30	U/AE	0.12	0.52
P9	+	0.00	57.80	66.20	62.40	55.90	U/AE (exercise)	0.12	1.09
P10	+	0.09	9.20	17.40	19.60	25.20	U/AE	0.26	1.18
P11	+	0.00	54.10	48.40	59.40	60.40	SHOCK	0.29	1.07
P12	+	0.22	12.80	15.70	12.10	0.70	AVOID	0.28	0.43

Characteristics and BAT results of the grLOW patients (n = 12) tested under a Pru p 3 stimulation. %CD63+, % of activated basophils; EC50, the concentration inducing 50% of maximum response. Tolerance (TOL), local (CU, contact urticaria; OAS, oral allergy syndrome; GI, gastrointestinal symptoms) and systemic symptoms (U/AE, generalized urticaria or angioedema; AN, anaphylaxis). In parentheses the presence of cofactors is detailed. Pru p 3 and Pru p 3/peach sIgE are included. A ratio ≥1 indicates a greater proportion of sIgE Pru p 3 compared to peach sIgE. NA, not available.

and conflicting results have been published (9, 16, 17). It has been reported that high Pru p 3 sIgE concentrations correlate with an increased risk of reactions (18). Ciprandi et al. (19) described Pru p 3 sIgE levels variation as an age-dependent event. They reported an increase from infancy to young adulthood (highest from 21 to 30 years) that posteriorly decreased. Also, values have been inversely related with an early onset peach allergy (16).

Moreover, it has been described that mono-sensitization to LTP correlates with a more severe clinical reactivity (20) which could be explained by the fact that IgE receptors are mostly occupied by LTP sIgE, which would induce a more efficient cross-linking of the FcεRI and effector cell activation, but not actually related to sIgE levels.

In the previous studies from our group and collaborators (21–23), a trend to lower levels of sIgE has been observed in those groups with severe symptoms compared with those with mild symptoms. From our point of view, we think that this might be explained by the differential affinity of sIgE to the antigen and differential efficiency on the cross-linking in effector cells in which the ratio of sIgE to total IgE of 0.01 is enough for half-maximal activation of the effector cells.

CCD sensitization was similarly distributed in both the groups, ruling out that low levels detected were merely artifacts of CCD interaction not deserving clinical consideration.

Finally, a lower co-sensitization to other LTPs was found on grLOW although sensitization profiles (peanut, walnut, and apple) were similar in both the groups. This study has some limitations, besides being retrospective. Mainly, oral food challenges could not be done to confirm food diagnosis due to logistic limitations; and the fact that avoidance may have caused sIgE concentrations to decrease in patients with a history of a severe reaction.

In conclusion, our data show that, regardless of patients with low Pru p 3 sIgE may represent a minority in our daily practice, this sensitization can be clinically relevant, with up to 20% of systemic reactions. Therefore, Pru p 3 sensitizations should be carefully evaluated even when sIgE levels are low.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital Clínic de Barcelona Ethical Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SB-V has contributed to the acquisition, analysis, and interpretation of data, as well as drafting the manuscript for publication. RC-S and MR-Z have contributed to the acquisition of clinical data. CS, JR, and MT contributed to the performance of laboratory tests. JB, RM, and MP have contributed to the design and interpretation of the data and critically revised. All authors have participated

sufficiently in the work, approved the final version, agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/falgy.2022.868267/full#supplementary-material

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ORIGINAL RESEARCH
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The Allergen-Specific IgE Concentration Is Important for Optimal Histamine Release From Passively Sensitized Basophils

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Background: The basophil histamine release (HR) assay can be used for allergy diagnosis in addition to the conventional measurement of allergen-specific IgE (sIgE). Passive sensitization of basophils increases the versatility and allows testing the biological relevance of allergen-induced IgE cross-linking in any serum unbiased by the cellular component. However, not all the patient sera perform equally well and we hypothesized that the absolute level and fraction of sIgE affect the performance. Choosing birch pollen allergy as a model, we investigated the concentration of sIgE needed for successful passive sensitization using soluble- or matrix-fixed Bet v 1.

Methods: Twenty-eight sera with Bet v 1 sIgE [7 sera within each allergy class (1: 0.1–0.70 kUA/L, 2: 0.71–3.50 kUA/L, 3: 3.51–17.50 kUA/L, and 4+: >17.50 kUA/L)] and a negative control serum pool were used to passively sensitize donor basophils, obtained from buffy coat blood ($n = 3$). The cells were incubated (30 min) with a soluble allergen (rBet v 1 from 0.2 to 50 ng/ml), matrix-fixed allergen (ImmunoCAP^{TM} containing recombinant Bet v 1), or phorbol 12-myristate 13-acetate (PMA)/ionomycin mixture (maximal HR) and released histamine was quantified fluorometrically.

Results: The lowest level of Bet v 1 sIgE generating a detectable HR (HR > 10% of maximal release) in all the 3 runs was found to be 1.25 kUA/L (corresponding to allergy class 2, 0.71–3.50 kUA/L). Furthermore, sera from allergy classes 3 and 4+ ascertained a significant reproducible HR: 42/42 vs. 5/21 in allergy class 1 and 15/21 in allergy class 2. Using ImmunoCAP^{TM}s containing Bet v 1 as a matrix-fixed allergen system, similar results were obtained where the lowest sIgE concentration mediating an HR was 1.68 kUA/L and 7/7 for both allergy classes 3 and 4+.

Conclusion: The results demonstrate that the IgE titer is strikingly robust in predicting the ability to sensitize basophils and produce a measurable HR.

Keywords: IgE, basophil, mast cell, histamine, passive sensitization, allergy

INTRODUCTION

Basophils are granulocytes found in the blood. They make up <1% of the white blood cells, but are often used in research allergy diagnostic tests due to their resemblance with mast cells, i.e., they release histamine and change surface receptor profile when activated *via* their high-affinity IgE receptors (FcεRI) due to allergen-IgE binding [(1), reviewed in (2)]. Such biological assays often require fresh blood (<24 h old) which can be a challenge in clinical settings. Furthermore, 10–20% of the population have non-releasing basophils (i.e., cells not degranulating), thereby excluding them from these kinds of tests (3).

Passive sensitization (PS) is a technique using basophils from a selected blood donor (confirmed releasing basophils) and serum (IgE) from the patient of interest (4, 5). The basophils have their autologous IgE removed by acidification, making all the FcεRI accessible for the patient serum IgE. These stripped basophils are then incubated with the patient serum allowing binding of IgE to occur. In this way, the IgE fingerprint from the patient will now be found on the donor basophil. PS is, however, not a replacement for serological IgE tests, but it adds a biological factor that can link IgE detection with clinical tests, such as skin prick test (SPT), or even oral food challenge (OFC) and it discriminates if the reactivity is IgE mediated or not (6–9).

Even though PS is a useful technique in advanced allergy diagnosis, it is in the field of allergen characterization where PS has its strength. By keeping the cell source constant, intrinsic mechanisms will not vary and the focus can be kept on the IgE and allergen interaction. Therefore, PS of basophils has been used in evaluating allergen extracts and recombinant allergens according to their biological activity (10–14), risk assessment of biotechnologically-derived products (15), and identification of biologically active food allergens in serum (16, 17).

Different cellular approaches have been used for sensitization besides human basophils (**Supplementary Table S1**). This includes human mast cells and the rat basophilic leukemia (RBL) cell line expressing the human FcεRI and sometimes also reporter genes (18–25). In addition, the readout can also be cell surface expression of activation receptors (i.e., CD63 and CD203c), as seen in BAT, instead of released histamine (2). However, whether all the sera can be used or if the allergen-specific IgE (sIgE) needs to be in a certain range, as suggested by some publications, is unclear (22, 26).

This study aimed to determine the concentration of sIgE needed to ensure an optimal PS. This was accomplished by the screening of 28 sera from birch pollen-sensitized individuals, employing rBet v 1 as the allergen and basophils from 3 different blood donors and using the basophil histamine release assay (BHRA).

MATERIALS AND METHODS

Selection of Sera

Based on the level of Bet v 1 sIgE, 28 sera [7 sera within each allergy class (1: 0.1–0.70 kUA/L, 2: 0.71–3.50 kUA/L, 3: 3.51–17.50 kUA/L, and 4+: >17.50 kUA/L)] were selected randomly from our serum bank at the Allergy Clinic (see **Table 1**). The selected sera originated from both mono- and polysensitized individuals. Pooled serum from 120 healthy non-allergic subjects was used as a negative control. This study was approved by the local ethical committee (De Videnskabsetiske Komiteer for Region Hovedstaden), protocol H-3-2010-090.

Measurement of Serum IgE

The level of Bet v 1 sIgE and total IgE was determined by the ImmunoCAP Specific IgE Assay t215 (14-5225-01) and the total IgE assay a-IgE;T (14-4509-01) both from Thermo Fisher Scientific (Uppsala, Sweden). Assays were conducted as described by the manufacturer.

Selection of Buffy Coat Blood

Fresh anticoagulated buffy coat blood was obtained from anonymous donors at the National University Hospital Blood Bank (Copenhagen, Denmark). To obtain a blood donor with highly responding basophils but without an allergic profile (no reaction to common allergens), the blood was screened as outlined: in a polypropylene tube, 1 ml of buffy coat blood was washed by adding 9 ml of pipes buffer (Hospital Pharmacy, 9.3 mM pipes, 0.14 M sodium acetate, 5.0 mM potassium acetate, 0.60 mM calcium chloride, 1.1 mM magnesium chloride, and adjusted to pH 7.4 with 1 M Tris) and centrifuged at 600 × g for 10 min at room temperature (RT). The supernatant was removed and the buffy coat was resuspended in pipes buffer to a total volume of 3 ml. The cell suspension was then added in duplicate to dilutions of stimulant and basophil histamine release (HR) was determined by the glass fiber method (RefLab, Copenhagen, Denmark): 25 µl of cell suspension was added to a 96 well glass fiber-coated plate containing 25 µl of dilutions of anti-IgE (KPL Inc., Gaithersburg, Maryland, USA, final concentrations: 1,000, 330, 110, 37, 12, and 4 ng/ml), recombinant Bet v 1 (Biomay AG, Vienna, Austria, final concentrations: 50, 17, 6, 2, 0.6, and 0.2 ng/ml), and phorbol 12-myristate 13-acetate (PMA)/calcium ionomycin mixture (Sigma-Aldrich, final concentrations: 1.6/6.7 µM, 0.53/2.2 µM, and 0.18/0.73 µM) or a standard panel of 10 inhalant allergens (grass, birch, mugwort, cat, dog, horse, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Cladosporium*, and *Alternaria*) and 10 food allergens (milk, egg, wheat, peanut, hazelnut, kiwi, cod fish, shrimp, celeriac, and soy) [Screening plate (RLA217), RefLab]. Cells were incubated at 37°C for 30 min. Subsequently, cells were removed from the plate by washing in deionized water followed by incubation with 0.4% sodium dodecyl sulfate solution (RefLab) at 37°C for 10 min. Plates were washed again and 75 µl 3.7 mM o-phthalaldehyde (RefLab) in 50 mM sodium hydroxide (Hospital Pharmacy) was added. After 10 min, 75 µl 0.59% perchloric acid (Hospital Pharmacy) was added and the released histamine was quantified fluorometrically using the HistaReader 501 (RefLab). Buffy coat donors were selected when basophils elicited an anti-IgE-induced HR of more than 50 ng/ml histamine released (>30% release), but with no HR response to the common allergens or rBet v 1. Buffy coat blood was added recombinant human interleukin 3 (R&D Systems Inc., Minneapolis, Minnesota, USA) to a final concentration of 10 pg/ml and kept at 8–10°C overnight.

TABLE 1 | Concentrations of Bet v 1 sIgE, total IgE, and % Bet v 1 sIgE in the 28 serum samples and the histamine release (HR) value obtained from the 3 runs of passive HR.

Sample ID	Bet v 1 sIgE (kUA/L)	Total IgE (kU/L)	% Bet v 1 sIgE	Calculated HR-value		
				Experiment 1	Experiment 2	Experiment 3
A1.1	0.34	4,853	0.01	0	0	43.70
A1.2	0.36	33.7	1.07	0	0	0
A1.3	0.37	46.4	0.80	0	0	1.13
A1.4	0.52	205	0.25	0	0	29.00
A1.5	0.54	37.1	1.46	0	0	0
A1.6	0.64	42	1.52	0	0	1.68
A1.7	0.7	98	0.71	0	0	0.46
A2.1	1.25	241	0.52	0.78	0.83	0.58
A2.2	1.62	29.1	5.57	0	0	0
A2.3	1.68	121	1.39	1.60	0	0.54
A2.4	2.34	339	0.69	3.92	0	0.11
A2.5	2.46	123	2.00	3.54	4.66	3.39
A2.6	2.63	104	2.53	2.13	0	2.47
A2.7	2.64	10.1	26.14	0.81	0.33	0.22
A3.1	6.25	91.6	6.82	15.54	4.74	5.65
A3.2	6.34	32.6	19.45	23.37	32.10	20.03
A3.3	7.25	244	2.97	35.78	16.00	22.73
A3.4	7.46	328	2.27	19.74	5.64	29.59
A3.5	10.3	29.8	34.56	18.72	5.64	12.50
A3.6	11.1	66.8	16.62	25.21	21.85	24.83
A3.7	13.9	70.7	19.66	69.59	28.15	32.99
A4.1	22.2	37.8	58.73	146.63	68.26	90.72
A4.2	26.8	149	17.99	186.52	74.68	164.92
A4.3	28.3	170	16.65	171.98	35.88	151.16
A4.4	33.8	64	52.81	148.60	32.73	129.93
A4.5	37.4	124	30.16	228.15	90.32	237.18
A4.6	45.8	486	9.42	188.39	69.83	82.93
A4.7	56.5	296	19.09	198.28	74.07	154.77

Passive Sensitization

The procedure of PS was carried out without isolation of basophils. In a polypropylene tube, 5 ml of buffy coat blood was mixed with 45 ml of physiologic saline and centrifuged at 1,000 × g for 10 min. at 11°C. The blood cells were resuspended in a 45 ml cold stripping buffer (0.14 M sodium dihydrogenphosphate and 5.0 mM potassium chloride, 4°C, pH 3.55) to remove autologous IgE and immediately centrifuged at 1,000 × g for 10 min at 11°C. Subsequently, the blood cells were resuspended in 45 ml pipes buffer, centrifuged at 1,000 × g for 10 min at 11°C, and cells were resuspended in pipes buffer to a total volume of 5 ml. In total, 1 ml of this cell suspension was added to 125 μl serum and allowed to incubate at 37°C for 1 h in a sealed polypropylene tube. After incubation, the cell suspension was diluted with pipes buffer to a total volume of 3 ml.

Passive Sensitization Basophil Histamine Release Assay

Basophil histamine release assay (BHRA) was performed using the glass fiber method and 25 μl of the cell suspension of the PS basophils which were stimulated with anti-IgE, recombinant Bet v 1 (rBet v 1) or PMA/ionomycin as described in "Selection of buffy coat blood." Basophil HR was expressed in percentage of the PMA/calcium ionomycin-induced maximal HR or as HR-value as indicated in the text.

Background values were calculated from the negative control serum pool as "mean HR + 3 × SD," where mean HR denotes the mean of all dilution points. Only HR values higher than background were used.

CDsens was calculated as the inverted rBet v 1 concentration eliciting 50% of maximum response (1/2max) times 100. If 1/2max could not be determined, CDsens was set to 0. Onset refers to the concentration corresponding to the intercept between the curve and the background. If the curve was above background for all the rBet v 1 concentrations, no value was given. Ymax was defined as the maximum HR. If Ymax was below the background, the value was set to 0. The area under the curve (AUC) was calculated using GraphPad Prism version 9.3.1 for windows. If no curve exists (HR below background), the AUC was set to 0.

Histamine Release Using Matrix-Fixed Allergen

ImmunoCAP™s containing recombinant Bet v 1 (t215), anti-IgE (assay control) (14-4417-01 (a_IgE;S), or streptavidin (background) (14-5320-01 (o212) (Thermo Fisher Scientific) was placed in a 96-well filter plate where the filter was removed ("ImmunoCAP plate"). ImmunoCAP™s were washed using 10 × 200 µl pipes buffer. Buffer was drained by suction, using an Aurum™ Vacuum Manifold (Bio Rad, Copenhagen, Denmark) and residual buffer was removed by centrifugation (600 × g for 10 s at RT). PS basophils were added to each immunoCAP™. For maximal HR, the cell suspension was first mixed with PMA/calcium ionomycin (1.6 /6.7 µM) and then added to a streptavidin immunoCAP™. The ImmunoCAP plate was then placed on top of a 96-well V-shaped microplate ("Collection plate") and this "sandwich" was incubated for 30 min at 37°C in a preheated moisture chamber. After incubation, 100 µl of pipes buffer was added to each immunoCAP™ and incubated for 5 min at RT. The ImmunoCAP plate + collection plate sandwich was centrifuged at 300 × g for 5 min at 20°C to recover all the cell suspension into the collecting plate. Released histamine was measured in 50 µl of the supernatant using the glass fiber method, as described in "Selection of buffy coat blood." Results were corrected for background and considered positive if HR > 5% (negative control serum pool).

Statistics

Pearson correlation coefficients were calculated using GraphPad Prism 6.00 and 9.3.1 for Windows, GraphPad Software, La Jolla, California, USA, www.graphpad.com.

RESULTS

Impact of Specific IgE Concentration for Optimal Passive Sensitization

The 28 sera were used for PS of blood donor basophils and the cells were subsequently stimulated with rBet v 1 or PMA/ionomycin. In total, three identical experiments were conducted using a new blood donor for each experiment. As seen in **Figure 1**, allergy class 1 sera were very poor at mediating an HR and we only saw a positive response from five sera in the third experiment (5/21 sensitizations with allergy class 1 sera). Using allergy class 2 sera only a weak response to the highest concentrations of rBet v 1 was found with 15/21. Allergy class 3 sera constitute a transition from the weak responses elicited by allergy class 2 sera to the strong responses seen with allergy class 4+ sera. Both with allergy classes 3 and 4+ sera 21/21 sensitizations mediated an HR response. Differences in curve patterns between experiments are associated with the different cell donors. Overall, the lowest concentration of sIgE to mediate HR was found to be 1.25 kUA/L, however, to obtain 100% success, sera from allergy classes 3 and 4+ should be used.

Correlation Between Histamine Release and Specific IgE Using the HR-value

To circumvent the need for subjective interpretation of the HR dose-response curves, we established the HR-value. This is the accumulated weighted area under the curve, as it takes into account both the curve height (reactivity) and the location on the x-axis (sensitivity). In brief, y-values (% HR) above background level (mean HR + 3 × SD of the negative sample) were multiplied by their corresponding inverted concentration and then added up. The HR value was calculated according to the formula:

$$HR-value = C_6^{-1}\times Y_6 + C_5^{-1}\times Y_5 + C_4^{-1}\times Y_4 + C_3^{-1}\times Y_3 + C_2^{-1}\times Y_2 + C_1^{-1}\times Y_1$$

Where C_6-C_1 denotes the rBet v 1 concentration in ng/ml (0.2–0.6–2–6–17–50) and Y_6-Y_1 is % HR. If the HR ≤ background, the HR value was set to 0. Calculated HR values for each experiment are given in **Table 1**.

To evaluate how the level of sIgE affects basophil HR using PS, the HR values were plotted against the level of sIgE and as shown in **Figure 2A**, the correlation is good. No correlation was found using total IgE (**Figure 2B**). Thus, a stronger HR is observed from basophils when using sera with higher concentrations of sIgE. Nevertheless, zooming in on the individual classes the correlation is less clear. Using results from the allergy classes 3 and 4+ sera, it is notable that the HR value does not follow the level of sIgE (**Figure 3**). This dissociation between sIgE and the HR value cannot be explained by the % of Bet v 1 sIgE out of total IgE (**Figure 3**; **Table 1**).

Histamine Release Induced by Matrix-Fixed Allergen

The ImmunoCAP™ system holds more than 500 allergen tests and it would, therefore, be a huge advantage if these matrix-fixed allergens could be used as an allergen source if they produce an HR similar to a soluble allergen. We used the 28 sera for PS of basophils which were then loaded in either Bet v 1 or anti-IgE containing ImmunoCAP™s and released histamine was extracted and quantified. The anti-IgE ImmunoCAP™s were used as an assay control to prove the reactivity of the basophils if the Bet v 1 response was absent. As seen from **Figure 4** and **Supplementary Figure S1**, a significant correlation was found ($p = 0.9298$) as Bet v 1 mediated HR is increasing with an increasing concentration of sIgE comparable with the results shown in **Figures 1**, **2**. No HR was found with the allergy class 1 sera, 2/7 of allergy class 2 where the lowest sIgE concentration mediating an HR was 1.68 kUA/L, and 7/7 for both allergy classes 3 and 4+. We, therefore, did not gain more sensitivity using the same allergen system by which the IgE was quantified. However, using sera in allergy classes 3 and 4+ it seems likely that a single concentration of matrix-fixed allergen can be used equally to serial dilutions of soluble allergen as the results obtained with PS-BHRA (HR-value) correlate with the CAP PS-BHRA (**Figure 5**).

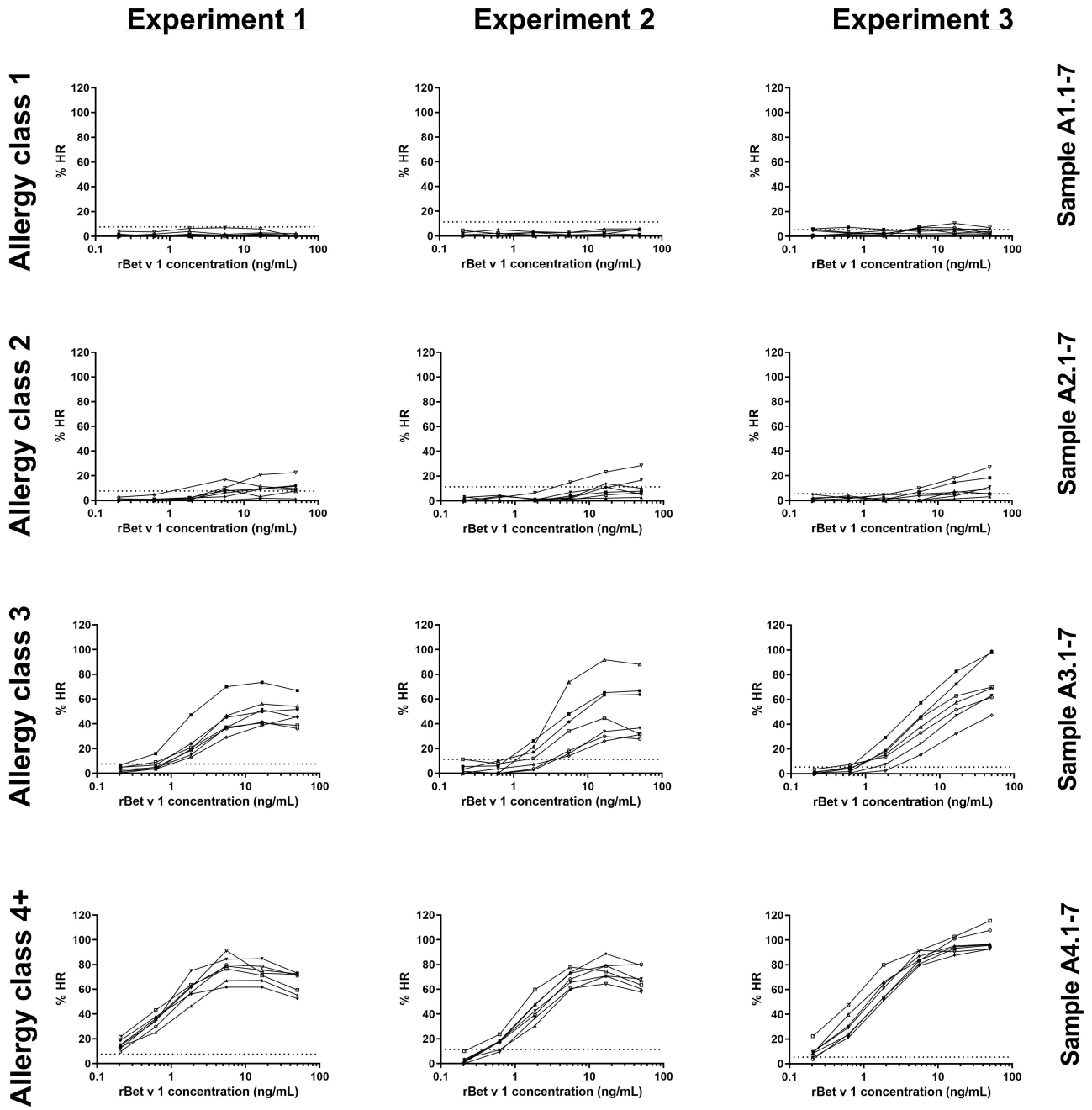

FIGURE 1 | rBet v 1-induced histamine release (HR) curves for 28 sera in experiments 1, 2, and 3. Histamine release is expressed as %HR of the PMA/ionomycin response. Sera are shown in their respective allergy class and each experiment represents a cell donor. Numbers refer to sample ID (**Table 1**). Dotted horizontal lines indicate background values obtained from the negative control serum pool (8, 11, and 6% for experiments 1, 2, and 3, respectively).

DISCUSSION

Passive sensitization of basophils may be used as a tool in allergy diagnosis when no sIgE test exists, fresh blood samples cannot be provided, or if the patient has non-releasing basophils (5). However, the real strength of PS lies within allergen characterization as PS takes into account both the IgE diversity using different sera, but keeps the cellular response uniform, which is useful, e.g., when screening for allergen candidates for immunotherapy (14). In 1977, MC Conroy et al. published that cell-bound IgE correlated with the IgE serum level; nevertheless, the releasability of the basophils was not entirely controlled by the IgE load as different donor basophils having approximately the same amount of IgE bound would respond differently to anti-IgE stimulation (27). Using passively sensitized basophils, this interdonor variation introduced by the cellular component

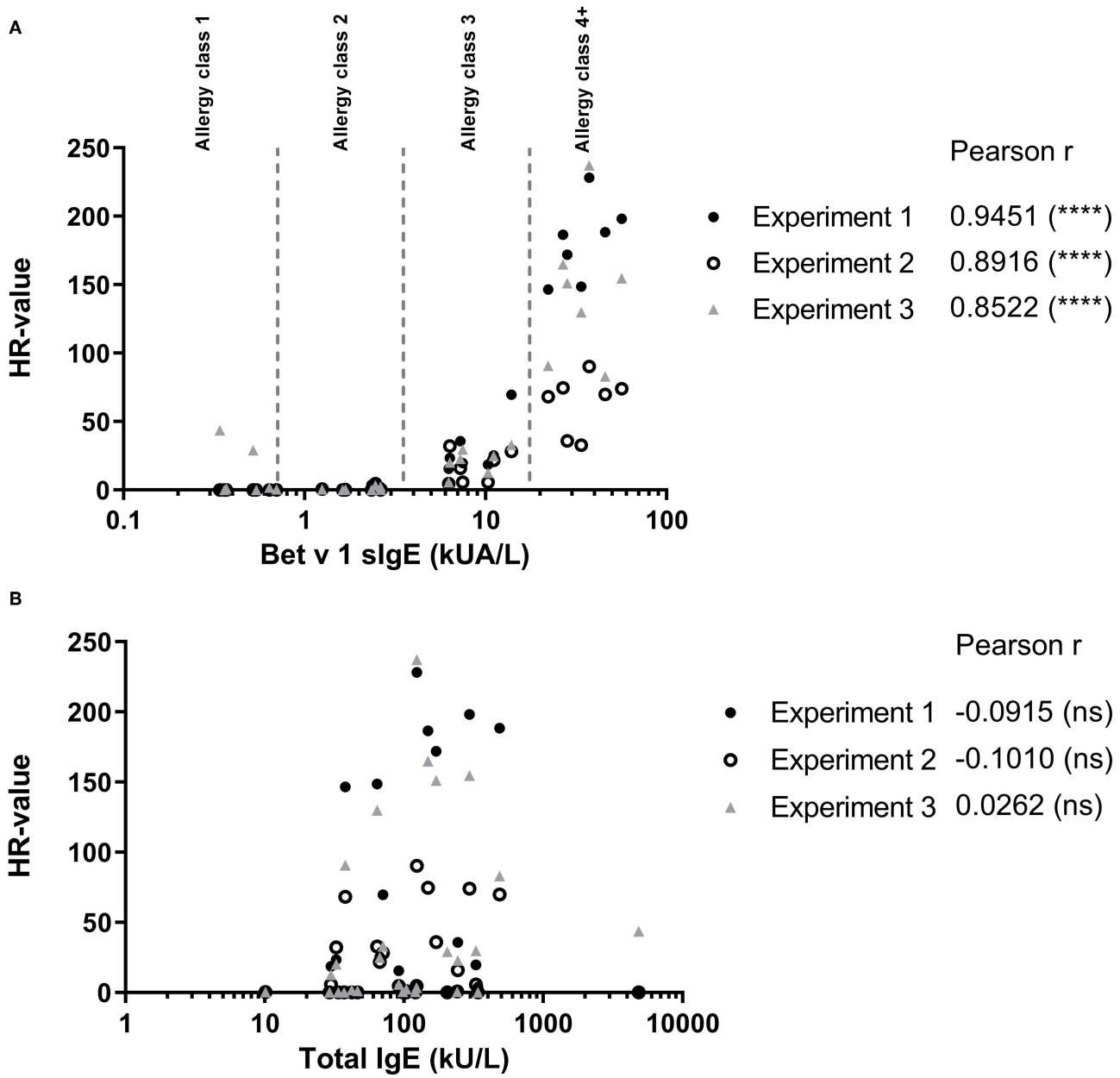

FIGURE 2 | (A) Results from the passive HR (expressed as HR-value) and the Bet v 1 sIgE concentration in serum used for passive sensitization. **(B)** HR-value plotted against the total IgE concentration in serum. • Experiment 1; ○ Experiment 2; and ▲ Experiment 3. Allergy classes are indicated in **(A)**. Pearson correlation.

is avoided as cells from one selected blood donor will provide basophils enough for multiple investigations. However, it is important to ensure optimal performance of the basophils which is why screening of blood donors is crucial for a successful PS. We used the criteria of more than 50 ng/ml histamine released (>30% release) after anti-IgE stimulation to ensure responding basophils (eliminating donors with non-releasing basophils) and a strong readout even if blood stored overnight would lose some activity. As the detection limit of the HR assay is 10 ng/ml, a 50% reduction of a 50 ng/ml HR response would still provide a feasible readout; however, the trend was more in the direction of an increase in the HR after storing the blood overnight, probably due to the addition of interleukin-3 (IL-3), which benefit basophil reactivity.

Another important aspect to consider before comparing HR data is in what way the HR data should be evaluated, as it often is given as a titration curve. We, therefore, introduced the HR-value to convert the full HR titration curve into a single number which considers both the location on the x-axis (sensitivity) and the y-axis (reactivity). Other parameters for sensitivity (½

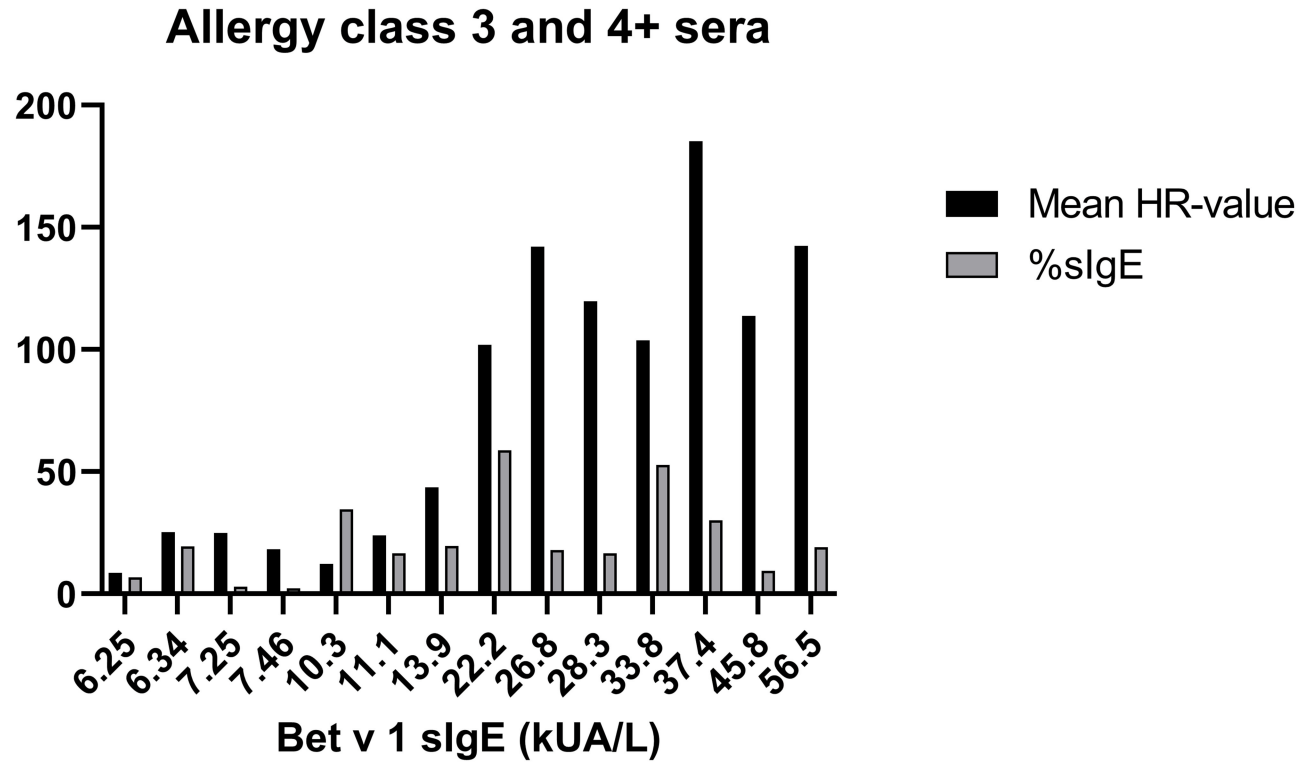

FIGURE 3 | Data from sera in allergy classes 3 and 4+ are shown; Bet v 1 concentration (kU/l) at the x-axes, mean of the HR-value from the 3 experiments in black column, % of Bet v 1 sIgE (%sIgE) in gray column.

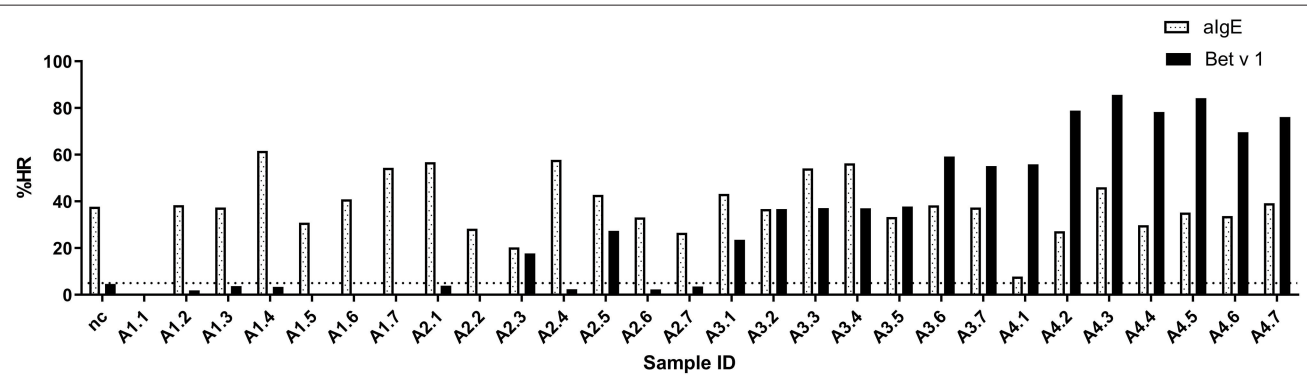

FIGURE 4 | PS basophils activated by matrix fixed allergen using Bet v 1 or anti-IgE (assay control) ImmunoCAP^{TM}. Released histamine was extracted and quantified. Dotted horizontal line at 5% indicates background; nc, negative control.

max HR/ED50/CDsens) and reactivity (Ymax/CDmax) have previously been used (28–30). In addition, a traditional area under the curve (AUC) calculation has also been suggested as a readout to compare basophil activity or quantification of allergen in serum (17). However, these curve parameters are often challenged, e.g., when weak responses might have a ½ max HR below the background (like allergy class 2 sera) or when the maximal response is not achieved (e.g., in allergy class 3). Furthermore, the sensitivity (the smallest allergen concentration needed to elicit an HR) is often omitted (AUC). Therefore, to evaluate the performance of the HR-value, we investigated how well AUC, CDsens, and Ymax correlated with sIgE and total IgE (**Supplementary Table S2**) and found that the Pearson correlation coefficient for sIgE was best with CDsens > AUC > Ymax, but that the HR-value was overall better than CDsens. None of the curve parameters correlated with total IgE. In addition, when looking at the number of tests that could be quantified, CDsens was poorer compared to AUC and Ymax, which performed equally well together with the HR-value (**Supplementary Table S3**). Therefore, taking

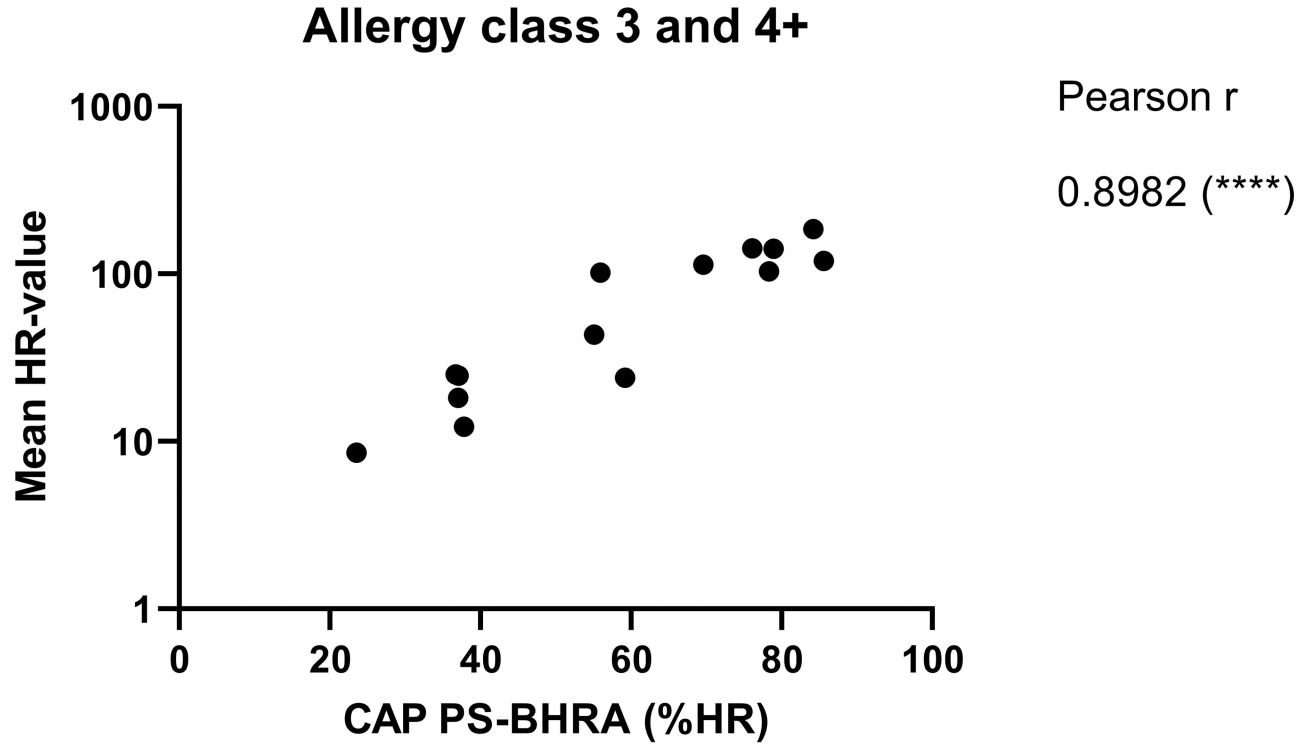

FIGURE 5 | Mean of the HR-value from the 3 experiments correlated with PS basophils activated by matrix fixed allergen (CAP PS-BHRA) for the allergy classes 3 and 4+ sera. Pearson correlation.

the above data into account, we suggest using the HR-value when comparing dose-response curves as it embraces both the sensitivity and reactivity and has the best success in quantifying the response.

The challenge of PS is allocated to the selection of sera as not all the sera perform uniformly, i.e., result in lgE-mediated activation of basophils. In this study, we tested 28 different sera distributed among allergy classes 1 to 4+ and found that the lower limit of sIgE to mediate a positive response in the PS-BHRA was 1.25 kUA/L (allergy class 2) even though few sera with less sIgE were found to sensitize 1 or 2 of the cell donors. Among the allergy class 1 sera, 5 resulted in weak sensitization in experiment 3. The background was very low in this experiment and if we had used the mean background of all the 3 experiments, only serum A1.4 would appear positive which also is the serum giving a borderline response in experiment 1. On the other hand, experiment 2 had a high background causing a lower HR-value in this run. Despite this variation, which cannot be avoided due to donor-donor variance, the overall pattern was similar in the HR-values among the 3 experiments (**Table 1**).

Looking at different studies using PS (**Supplementary Table S1**), it is notable that to elicit an HR, the sIgE concentration needs to be in the area of 1.25–3.5 kUA/L if human basophils are used but if the assay builds on RBL cells, which often is used as a basophil surrogate, the sIgE concentration needs to be higher (3.91–219 kUA/L) (22). New technologies have been used to improve the RBL system, e.g., by combining it with a luciferase reporter system. Nevertheless, as the serum has to be diluted at 1:100 only sera within the allergy classes 3 and 4+ can be used (23). To avoid serum cytotoxicity a suggestion has been made to heat the serum sample for 5 min at 56°C, however, due to the unstable nature of IgE at this temperature, this might change the success for sensitization (31). Lately, human mast cells, either blood-derived or the cell line LAD2, have been added to the PS toolbox. However, even though mast cells might be a more potent effector cell compared to the basophil, the sIgE concentration needed to perform PS is at the same level as seen for PS of basophils (**Supplementary Table S1**).

Using a pure system, with recombinant sIgE against Der p2, Christensen, LH et al. also find that sensitization proven by a positive basophil activation test (BAT) only takes place when the amount of recombinant sIgE is high enough (8–10 ng/ml = allergy class 3) (32). Therefore, no matter the type of the cellular system or the purity and source of sIgE, it seems that to obtain a successful PS you need sIgE in the range of allergy classes 3 to 4+ (**Supplementary Table S1**).

The performance of the BHRA has been challenged by the BAT as the BHRA might be less sensitive due to the limit in the quantification of histamine. Nevertheless, when performing PS the sIgE level still needs to be in the area of allergy classes 2 to 4+ when PS basophils are used for BAT (26, 33). Furthermore, we have experienced that incubating basophils with serum can affect the receptor expression of CD203c (unpublished data).

We introduced a matrix-fixed allergen system (ImmunoCAP™) which could have a positive impact on the IgE-allergen binding and, thereby, the cross-linking of

FcεRI and activation of the basophils. In contrast, replacing a dose-response curve with only one point might compromise the results as dose-response curves often are very broad and bell-shaped (28). Nevertheless, the reaction pattern and level of sIgE needed for activation were comparable between the two assays (1.25 vs. 1.65 kUA/L), again illustrating the level of sIgE necessary for a successful PS but also questing the need for an allergen titration curve. However, if one dilution of a soluble allergen would mediate the same result as one concentration of matrix-fixed allergen has to be further investigated.

We saw a strong correlation between the concentration of Bet v 1 sIgE and the HR-value but zooming on the individual allergy classes, this correlation was less clear. This is not explained by the concentration of total IgE as the percentage of Bet v 1 sIgE does also not follow the HR pattern. It has been described that sera containing > 10% sIgE perform well in PS but such sera are very likely to be allocated in allergy classes 3 and 4+ (**Table 1**) (21). An explanation for differences in response when using sera with approximately the same sIgE concentration could be the IgE clonality and affinity which seems to affect both the reactivity and sensitivity (32).

The type of allergen used when performing PS might also play a role. We had chosen birch pollen allergy as a "one-dimensional" model system since it has a single major allergen, Bet v 1, in contrast to, e.g., peanut, where Ara h 2/6 or Ara h3/h3.02 cross-reactivities may complicate interpretation of results. In addition, by using a small molecule as Bet v 1 (17 kDa), the findings might also be applicable to larger proteins, including more epitopes (34). Furthermore, according to the studies listed in **Supplementary Table S1**, sera from grass, house dust mite, or food allergic patients also perform well in PS when the level of sIgE is within allergy classes 3 and 4+, indicating that a successful PS is independent of the allergen system.

Overall PS is a useful technique embracing both diagnostic and allergen investigations. The limitation is within the concentration of sIgE needed to perform optimal PS. This is important to address especially if PS is used in diagnosis as false negative results can appear when the sIgE level is too low. Therefore, the concentration of sIgE has to be evaluated before using PS.

CONCLUSION

By using birch allergy and rBet v 1 as an allergen model system, we have demonstrated that the IgE titer is strikingly robust in predicting the ability to sensitize basophils and produce a measurable HR no matter if the allergen is in suspension or fixed to a matrix. We believe our results strengthen the selection of sera for future studies discriminating the ones with low potential for success in passive sensitization.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by De Videnskabsetiske Komiteer for Region Hovedstaden, protocol H-3-2010-090. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

BJ, PSS, and LP contributed to conceptualization and supervision. PS, BJ, and LP contributed to formal analysis. PS and BJ investigated the study, wrote the original draft, and visualized the study. LP contributed to resources. BJ contributed to project administration. All the authors were involved in validation, methodology, writing-review and editing, and have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/falgy.2022.875119/full#supplementary-material

Supplementary Figure S1 | Results from PS basophils activated by matrix fixed allergen using ImmunoCAP^{TM} (CAP PS-BHRA) and the log concentration of Bet v 1 specific IgE in serum used for passive sensitization. • Experiment 1; ○ Experiment 2; and ▲ Experiment 3. Allergy classes are indicated. Pearson correlation.

Supplementary Table S1 | Overview of passive sensitization models and the sIgE concentration in the serum sample eliciting a response.

Supplementary Table S2 | Pearson correlation coefficients between serological parameters and various titration curve algorithms.

Supplementary Table S3 | Number of quantitative tests within each allergy class when using different curve titration curve algorithms.

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Utility of the Basophil Activation Test Using Gly m 4, Gly m 5 and Gly m 6 Molecular Allergens for Characterizing Anaphylactic Reactions to Soy

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There are two major clinically described forms of IgE-dependent soy allergy: (i) a primary dietary form, linked to sensitization against soy storage proteins Gly m 5 and Glym 6, and (ii) a form included in birch-soy syndromes linked to Gly m 4, a PR-10-like allergen. This second form sometimes causes severe systemic reactions, even anaphylaxis, especially on consuming certain forms of soy such as soymilks or smoothies. Skin prick tests and specific IgE assays against soy whole extracts lack sensitivity. Assays of anti-Gly m 4, Gly m 5 and Gly m 6 specific IgEs have been developed to overcome this obstacle, but they unfortunately lack specificity, especially for anti-Gly m 4. We hypothesized that the basophil activation test (BAT) using molecular soy allergens Gly m 4, Gly m 5 and Gly m 6 would both remedy the lack of sensitivity of other tests and offer, through its mechanistic contribution, greater specificity than the assay of anti-Gly m 4 specific IgEs. This would enable the two types of soy allergy to be separately identified. In a characteristic clinical example of PR-10-induced anaphylactic reaction after consuming soymilk, we report preliminary results of Gly m 4-exclusive positivity of BAT supporting our hypothesis. It will be necessary to confirm these results on more patients in subsequent studies, and to specify the place of the BAT in an overall diagnostic strategy. Meanwhile, soy BAT using molecular allergens is a promising diagnostic tool for soy allergy and probably also for follow-up in specific immunotherapies.

Keywords: soybean allergy, basophil activation test (BAT), molecular allergen, Gly m 4, Gly m 5, Gly m 6, PR-10, anaphylaxis

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INTRODUCTION

Soybean has been consumed in South Asia since ancient times. The plant, *Glycine max*, belongs to the legume family and is widely used for its health and nutritional benefits in both humans and animals (1). Soybean is one of the "big eight" foods responsible for 90% of food allergies (2). However, the prevalence of food allergy to soy remains controversial, and varies greatly from one country to another, ranging in children from 0% in certain countries, such as Greece and Spain, to

around 0.4% in the USA (3, 4). Notably, soy is the fifth or sixth most common allergen found in children with atopic dermatitis, and 5–14% of children with an allergy to cow's milk protein develop an allergy to soy when exposed to soy-based formulas (5). More than half of children allergic to soy are cured by age 7, suggesting that this allergy is less prevalent in adults (6).

Soy allergy significantly impacts quality of life because soybean is present in many foods, often discreetly, making eviction difficult. Although soy seems to induce less severe forms of disease than the other priority allergens (7), severe or fatal soy anaphylaxis has been occasionally described in a long timeframe (8).

More than 16 soy allergens have so far been described at molecular level (2). Clinical relevance has not yet been demonstrated for most of them, but three main molecular allergens seem to be of special clinical interest and to correspond to two different forms of the disease. First are two highly abundant storage proteins in soybean seed, Gly m 5 and Gly m 6, respectively from the beta-conglycinin (7S globulin) and glycinin (11S globulin) families (9–11). These allergens, stable to heat and gastric digestion, may be responsible for anaphylactic reactions to all kinds of dietary soybean, including fermented and processed foods. They may be associated with forms of allergy of primary food origin with sensitization through the gastrointestinal tract, in particular linked to cross-allergies to molecular allergens from the same families of storage proteins, especially including those of peanuts (Ara h 1 and Ara h 3) (12, 13). Second is Gly m 4, another medically interesting allergen, which is a PR10-like protein, called starvation-associated message protein (SAM22), with a certain degree of homology with Bet v 1, the major allergen of birch pollen. IgE-dependent cross-reactions between Gly m 4 and Bet v 1 are thus involved in birch-soy syndrome, one of the most important pollen-food syndromes (PFS) to be medically investigated, through a common clinical pattern of severe oral allergy syndrome and anaphylactic reactions (14). Approximately 10% of highly sensitized patients allergic to birch present a cross-allergy to soy and nearly 50% of them have experienced systemic or even anaphylactic reactions (15). The PR-10 family of proteins is heat-sensitive, so these patients will not react to all types of soy foods. Moreover, it is known that the skin prick test (SPT), and the determination of specific IgEs (sIgEs) against whole natural extract of soy, both lack sensitivity when the soy allergy is mediated by the PR-10 family (16). This stems from the difficulty met in extracting the Gly m 4 allergen from whole extracts and the low content of this allergen in them (17).

Component-resolved diagnosis (CRD), based on the use of unit sIgE assays against molecular allergen, Gly m 4 (ImmunoCAP, Thermo Fisher Scientific®), can overcome this hurdle. CRD, additionally using Gly m 5 and Gly m 6, is also useful for differentiating between these two types of soy food allergy, whose clinical characteristics differ (9, 16, 18). The results of these tests are always interpreted in the light of medical history, and despite the limitations described above, with the results of the SPT and the determination of sIgE antibodies directed against whole extract of soy (f14).

When the results of molecular sIgE assays are dichotomous (i.e., sIgE against Gly m 5 and/or 6 positive with Gly m 4 sIgE negative, or the reverse), clinical and biological interpretation remains relatively straightforward to determine whether it is a soy allergy initially of food or respiratory origin. However, interpretation of the assays is often hampered by the fact that many patients highly sensitized to birch also have sIgEs that can recognize Gly m 4. For example, in the study by Mittag et al., about 71% of patients with anti-Bet v 1 IgE above 17.5 kU/L also had positive sIgE antibodies to Gly m 4, whereas only 9.6% of them described allergic symptoms after soy food consumption (15). The interpretation of the anti-Gly m 4 sIgE assay thus sometimes requires caution, especially for low values or when the anti-Gly m 5 or Gly m 6 sIgEs are also positive. This then raises the question of which allergens are implicated in a patient's clinical reactions. The DBPCFC (double-blind, placebo-controlled food challenge) is then sometimes the only way to make a reliable diagnosis (15, 19).

We hypothesized that the basophil activation test (BAT) using the soybean molecular allergens Gly m 4, Gly m 5 and Gly m 6, could be useful first to overcome the lack of sensitivity of the soy SPT and of the sIgE assay directed against the overall soy extract and second to highlight the type of soy allergy, according to whether it is linked to storage proteins or to PR-10. The BAT, by its functional aspect, should make it possible to prove *ex vivo* that the degranulation of polynuclear basophils is, according to the patients, specifically induced by stimulation by one type of molecular allergen family, thus providing important additional evidence of the mechanism underlying the allergy. In the case of PR-10-related soy allergies, this might offset the lack of specificity of the anti-Gly m 4 sIgE assay by differentiating, in terms of medical relevance, the patients positive for this assay according to the presence or absence of basophil degranulation in a BAT.

In initial support of our hypothesis, we report here a first confirmation using the basophil activation test of the involvement of PR-10/Gly m 4 in an anaphylactic reaction after ingestion of soymilk in a 27-year-old female patient.

ORIGINAL CASE STUDY

A 27-year-old female patient was referred to our center for anaphylaxis rated grade 2 in the Ring and Messmer classification. The reaction occurred in June 2018 during a vacation in Poland, immediately after breakfast. The meal was composed of one apricot and 200 mL of soymilk. Immediately after eating, the patient presented rhinitis, nasal congestion, cough, sneezing, skin rash of the trunk and upper limbs, abdominal pain, and palmoplantar pruritus. There was no laryngeal edema, and no voice modification. Neither pulse nor blood pressure were measured. The patient was not referred to any medical center and no tryptase assay had been performed. The symptoms disappeared spontaneously in a few hours with no treatment.

Detailed medical history revealed spring rhinitis for the previous 3 years, and an oral syndrome on eating raw apple for the previous 2 years. The patient reported episodic consumption of soymilk with no symptoms in 2007–2008, but not liking the taste, never consumed it again until the accident in June 2018. The patient changed her diet in March 2017 to become

TABLE 1 | Results for serum specific IgEs tested.

	sIgE (kUA/L)
Aeroallergens	
Birch	ND
Bet v 1	25.9
Bet v 2	<0.1
Trophallergens	
Soybean	0.15
Gly m 4	6.41
Gly m5	<0.1
Gly m 6	<0.1
Apple	ND
Mal d 1	3.2
Mal d 3	<0.1
Peach	ND
Pru p 1	8.24
Pru p 3	<0.1
Apricot	0.68

The sIgEs were tested using the ImmunoCAP method (Thermo Fisher Scientific®). The threshold of positivity was set at 0.1 kUA/L.
sIgE, specific IgE; ND, not done.

vegan. Since then, she had been eating soy almost daily, but in cooked forms, almost exclusively as tofu, and occasionally soy sauce after cooking, with no symptoms. The 2018 reaction did not change her eating habits. Since then, besides the previously described forms, she had consumed soy cream in boiled form but stopped because it triggered oral syndromes. She had also tried the lacto-fermented form of soy in cheese substitutes, but again, an oral syndrome quickly appeared. On the other hand, she fully tolerated tempeh (from the fermentation of soybean), but no longer consumed it (not liking the taste).

The patient's family history showed allergic diseases in both parents and one brother.

The diagnosis was confirmed by SPT and determination of sIgEs. Levels of sIgEs for selected allergens were determined using the ImmunoCAP® (Thermo Fisher Scientific®) system, using the Phadia 250 equipment according to the manufacturer's instructions (**Table 1**).

SPTs were positive for birch extract only. No skin reaction was observed for the other extracts tested (mites, cat, dog, grass, apple, native apple, soy, horse, plantain, herbaceous plants, almond, hazelnut). These negative results thus included both soybean and apple.

Notably, sIgE against whole soybean extract was very weakly positive (0.15 kU/mL). Regarding molecular soy allergens, only anti-Gly m 4 sIgEs were positive, unlike those directed against Gly m 5 and Gly m 6. In addition to anti-Gly m 4 sIgE, elevated sIgEs were found against Bet v 1 and Pru p 1, which also belongs to the protein family of PR-10. Elevated sIgEs were also found against apricot (and against cat and Fel d 1, data not shown).

Based on clinical history, SPT, and sIgEs, we considered the most likely diagnosis to be an anaphylactic reaction to soymilk, mediated by anti-Gly m 4 sIgE and subsequent to an initial birch pollinosis. However, given the negativity of the soy SPT and the quasi-negativity of the sIgE assay directed against the overall soy extract and in order to determine the reactivity threshold, we offered the patient an oral food challenge.

On the patient's refusal to take this test, we sought to confirm the diagnosis using the BAT with soy molecular allergens. We thus performed BATs against soybean extract (Bühlmann, Switzerland), and for the first time to our knowledge against Gly m 4, Gly m 5 and Gly m 6 (Indoor Biotechnologies, USA).

BATs were performed on whole blood using the Flow Cast® and B-CCR® kit (Bühlmann, Switzerland) according to the manufacturer's instructions. Briefly, EDTA whole blood was stimulated in an IL-3 containing buffer for 15 min at 37 °C with increasing concentrations of soybean extract (four concentrations tested in 10-fold dilution ranging from 22.5 to 0.0225 ng/mL for soybean extract, Bühlmann, Switzerland) or its major allergens Gly m 4, Gly m 5 and Gly m 6 (four concentrations ranging from 67.5 to 11.25 ng/mL, Indoor Biotechnologies, USA). Monoclonal antibody recognizing the high-affinity IgE binding receptor (FcεRI) and *N*-formyl-methionyl-leucyl-phenylalanine were used as positive controls. Before erythrocyte lysis, cells were stained with CD63-FITC, CD203c-PEcy5.5 and CCR3-PE. Basophils were gated as SSC-low/CCR3+, and among these, the CD63+ cells were termed activated basophils. Cells were acquired on an LSR II (Becton Dickinson). At least 300 basophils were analyzed using Flowlogic software (version 7.3, Miltenyi Biotec, Germany). Dead cells and doublet cells were excluded by a FSC/SSC gate and an SSC-A/SSC-H gate, respectively. Basophil activation was expressed as the % CD63 positive basophils (% CD63+) or % CD203c positive basophils (% CD203c+) among SSC-low/CCR3+ cells.

CDmax was defined as the maximal activation and corresponds to the maximum proportion of activated basophils (CD63 or CD203c) at any concentration of allergen.

The cut-off value for positive basophil activation in this study was set at >15% CD63 and CD203c basophils.

After stimulation with soybean extract (from 22.5 ng/mL, then with dilutions of 2.25, 0.225, 0.0225 ng/mL), no degranulation of the polynuclear basophils was found using CD63 marker, but a weakly positive activation was highlighted with CD203c marker (**Table 2**).

As expected, the BAT was very positive to Gly m 4 at 67.5 ng/mL for CD63 and up to 45 ng/mL for CD203c, but negative at all dilutions for Gly m 5 and Gly m 6.

An ImmunoCAP® ISAC® test (Thermo Fisher Scientific®) was also performed to obtain a whole sensitization profile and to explore the other PR-10 family allergens (data not shown). The results confirmed sensitizations against all PR-10 present on the biochip (Act d 8, Aln g 1, Api g 1, Ara h 8, Bet v 1, Cor a 1.0101, Cor a 1.0401, Gly m 4, Mal d 1, and Pru p 1).

In view of all these results, supported by those of the original BAT we developed, we established the diagnosis of anaphylactic reaction to soy, mediated by sensitization against PR-10. Subsequently, an immunotherapy against birch was implemented in the hope of treating the pollinosis and possibly the induced PR-10 soy allergy simultaneously (20). Further

TABLE 2 | Results of molecular soybean basophil activation test (BAT).

Allergen	Concentration (ng/mL)	Case	
		CD63 (%)	CD203c (%)
Negative control	-	1.26	3.06
FcεRI	-	70.7	74.3
fMLP	-	37.5	58.3
F14 (total extract)	22.5	2.11	**18.5**
	2.25	0.19	2.72
	0.225	0.58	2.51
	0.0225	3.02	9.06
Gly m 4	67.5	**51.2**	**77.6**
	45	3.38	**15.4**
	22.5	0.96	6.7
	11.25	0.38	6.9
Gly m 5	67.5	0.19	2.33
	45	0.39	2.91
	22.5	0.78	2.73
	11.25	0.4	3.78
Gly m 6	67.5	0.59	2.55
	45	0.78	2.72
	22.5	0.8	2.79
	11.25	0.84	4.63

CD63 and CD203c results are expressed as a percentage of the maximum basophil activation obtained (or CD max). The threshold of positivity corresponds to a minimum of 15% of activation.
Positive values appear in bold.

evaluation of all the results and particularly of the BAT activation threshold will now be necessary.

DISCUSSION

Initially, this case interested us because it was highly characteristic in some respects. In particular, it again illustrates the finding, first made by Kleine-Tebbe et al. in 2001, that severe oral allergy syndrome (OAS) and anaphylactic symptoms caused by a PR-10-related protein are likely to occur after consumption of a soy product in a patient with birch pollen allergy (14, 21–23).

Although most patients with PFS related to PR-10 have symptoms of moderate intensity, it is important to counsel them about the dangers of particular highly concentrated food forms such as dietary supplements, fresh fruit juices, smoothies and plant milks, like soymilk in our example (24). This seems to hold particularly for soybeans, probably due in part to a greater resistance of Gly m 4 to heat or gastric digestion than other PR-10 (15, 25).

This case is also interesting because it further illustrates that vegan diets may play a role in the development of food anaphylaxis (26). Our patient became vegan in 2017, and then started consuming much more soy, reacting for the first time to it in 2018. We can therefore legitimately suspect that the change in the patient's dietary habits may have played a role in the advent of the allergy.

Another characteristic point is the negativity of the soy SPT and the near negativity of the sIgE assay against total soy extract. This illustrates the weakness of the diagnostic tools classically at our disposal to diagnose soybean allergy when it is a form mediated by sIgE directed against PR-10.

In this example, the anti-Gly m 4 sIgE assay was already informative (27). However, when an oral food challenge cannot be performed, and since a very significant proportion of patients presenting birch pollinosis have anti-Gly m 4 sIgE without having a PR-10-mediated soy allergy, it is useful to have another confirmatory test for etiological purposes (15).

By demonstrating *ex vivo* the degranulation of basophilic polynuclear cells in contact with Gly m 4, the original BAT that we developed provides valuable mechanistic evidence that the anti-Gly m 4 sIgEs previously measured by the serum unitary assays have a functional activity and clinical relevance. The BAT mimics *ex vivo* what must have happened *in vivo* in the patient during the soy anaphylactic reaction. The BAT to soybean molecular allergens thus provides important evidence for the medical relevance of the sensitization measured against Gly m 4.

In our case, it is striking to see how closely the results of the sIgEs and BAT assays agreed. Thus, the sIgE assay directed against the overall extract was very weakly positive, as was that of the BAT against this same extract (CD63 negative and CD203c just above the threshold). Likewise, the anti-Gly m 4 sIgEs were quite high when the BAT against this allergen was very sharply positive for both CD63 and CD203c. Conversely, the anti-Gly m 5 and Gly m 6 sIgEs were fully negative, as was the BAT using these two allergens. This excellent agreement strengthens the relevance of the results of our original BAT based on soy molecular allergens.

Two very recent articles have focused on the use of BATs in the diagnosis of soy allergy. However, in both cases, the authors used only a total soy extract (soymilk proteins for one and natto extracts and soybean extract for the other, respectively) (28, 29). Although they both concluded that the BAT was of interest in this indication, their test method cannot distinguish between the two types of soy allergy at a molecular level and so does not seem to us to be able to answer all the questions raised by the diagnosis of soy allergy.

To conclude, in view of our preliminary results, we consider that in addition to clinical history, SPT and sIgE assays, BATs using soybean molecular allergens can be of use in the diagnosis of soy allergy in the near future. We have recently started to use BATs in practice, and will soon be working to define their place in the diagnostic tree, in particular in relation to oral food challenges, which they might even obviate. By following the basophil activation threshold over time, we can also envisage a place for BATs in the follow-up of possible oral soy immunotherapies, or in that of birch immunotherapies, in order to detect a possible concomitant induced effect on the allergy associated with PR-10 soy Gly m 4. These possibilities will be the subject of future studies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patient/participant provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BE designed the study and wrote the first draft of manuscript. BB and JC conducted the experiments. BE, JC, and BB analyzed the data and had access to and verified all underlying data. BB made the tables. All authors reviewed the manuscript and gave significant input. The final version of this paper was reviewed and approved by all authors.

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Basophil Activation Test With Aspergillus Molecules: The Case for ABPA

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Background: Allergic bronchopulmonary aspergillosis (ABPA) is an underestimated allergic disease due to *Aspergillus fumigatus* (AF). The main diagnostic criteria for ABPA rely on the evaluation of immunoglobulin (Ig) E and IgG responses to AF extracts, although these cannot discriminate AF-sensitization from ABPA.

Objectives: To evaluate the performance of cellular functional assays with extract and molecular AF allergens in ABPA.

Methods: A prospective cohort of 67 patients (6 ABPA) was investigated with basophil activation test (BAT) with AF extract. Twelve patients were further investigated for BAT responses to molecular AF components: Asp f 1, Asp f 2, Asp f 3, Asp f 4, and Asp f 6.

Results: BAT with AF extract with an optimized cutoff displayed 100% sensitivity and 77.6% specificity for ABPA diagnosis. Among patients with positive BAT to AF, BAT with Asp f 4 was significantly higher in ABPA patients at 10 ng/mL (mean basophil stimulation index 10.56 in ABPA vs. 1.24 in non-ABPA patients, $p = 0.0002$).

Conclusion: BAT with AF is a promising diagnostic biomarker in the context of suspected ABPA, which can be further improved with AF molecular allergens, especially Asp f 4.

Keywords: allergic bronchopulmonary aspergillosis (ABPA), basophil activating test (BAT), *Aspergillus fumigatus*, aspergillus molecular allergens, *ex vivo* technique

INTRODUCTION

Allergic bronchopulmonary aspergillosis (ABPA) is an underestimated allergic disease due to the ubiquitous mold *Aspergillus fumigatus* (AF). ABPA occurs mainly in patients with a chronic pulmonary disease, such as cystic fibrosis (CF), asthma or chronic obstructive pulmonary disease (COPD) (1). Its prevalence reaches 10 and 2% in CF and asthma patients, respectively (2, 3). Up to now, the reason why some AF-sensitized people stay free of subsequent AF-related disease whereas others develop ABPA with irreversible pulmonary lesions remains unknown. The main diagnostic criteria for ABPA, first established in 1977 (4), rely on the evaluation of humoral IgE and IgG responses to AF extracts, which cannot discriminate ABPA from AF-sensitization. Several new diagnostic tools have been evaluated, but none has overcome this limitation (2, 5, 6). More recently,

serum immunoglobulin (Ig) E responses to AF molecular components have been proposed, and the combination of specific AF molecular components showed promise for ABPA diagnosis (7–9). Meanwhile, diagnostic criteria based on the evaluation of functional cellular responses against allergens are increasingly cited in international guidelines (10, 11). Performance of the basophil activation test (BAT) with AF extract has been explored (12–16), but its relevance for ABPA diagnosis needs further evaluation. To our best knowledge, AF-induced basophil reactivity has not been deciphered at a molecular level, despite promising results of profiling IgE responses to such molecules. We report here the performance of BAT with AF extract and AF molecular allergens vs. usual diagnostic criteria of ABPA in a prospective cohort.

PATIENTS AND METHODS

Patient Cohort

We assessed adult patients ($n = 67$) followed at the CF reference care center and at the pulmonology department (Assistance Publique—Hôpitaux de Marseille, France) between January and September 2019. Patients were categorized as ABPA, AF-sensitized (AF-S), or control patients. These categories were defined as follows: ABPA met all the ISHAM criteria (2); AF-S displayed specific IgE (sIgE) to AF (0.1 kUA/L or greater) without fulfilling the ISHAM criteria for ABPA; and patients who were categorized in none of the two previous groups were considered as controls. According to ISHAM criteria, ABPA is made in patients with a lung predisposing condition who display AF sensitization and elevated total IgE levels and at least 2 of the following 3 criteria: (1) detection of AF specific IgG, (2) radiographic abnormalities, (3) total eosinophil count above 500 cells/μL.

Functional Cytometric Tests

The design of functional cellular assays is illustrated in **Figure 1**. BAT was firstly performed with AF extract (Bühlmann Laboratories®, Schönenbuch, Switzerland) with the Flow2CAST method (Bühlmann Laboratories®), using CCR3 (CD193) and CD63 as basophil identification and activation markers, following the manufacturer's instructions (17). Upon allergen contact, sensitized basophils degranulate and express CD63 as a cell surface activation marker, measurable by flow cytometry. For *in vitro* diagnosis, allergen-induced basophil activation is defined as a proportion of CD63+ basophils at least twice higher with the culprit allergen than with the reaction buffer. Positive controls were anti-RFcεI and the bacterial peptide fMLP. Fresh whole blood was incubated with AF extract or controls and staining antibodies for 30 mins at 37°C. After red cell lysis and washes, basophil responses were analyzed by flow cytometry. The proportion of CD63+ unstimulated basophils was lower than 5% in all patients, and no non-responder (anti-FcεRI-induced CD63 expression of 10% or less) was found in this cohort.

Samples with a positive BAT response to AF extract were subsequently assayed with BAT using each of the five following molecular AF: Asp f 1, Asp f 2, Asp f 3, Asp f 4 and Asp f 6 (a gift from Dr Jonas Lidholm, Thermo Fisher Scientific, R&D, Uppsala, Sweden). These molecular AF are the same used in the ImmunoCAP platform. Five concentrations of molecular components from 100 to 0.01 ng/mL by 10-fold dilution were used for each patient.

Flow cytometry was performed with a FACS Canto II platform (Becton Dickinson, Le Pont de Claix, France). At least 200 basophils per sample were acquired for BAT. Data were analyzed using FACS Diva software (TreeStar, Ashland, OR).

Clinical and Laboratory Data

White blood cell count (Sysmex, Villepinte, France) and serum total IgE and specific IgE (sIgE) and IgG (sIgG) to AF extract and molecular components Asp f 1, Asp f 2, Asp f 3, Asp f 4 and Asp f 6 (ImmunoCAP, Thermo Fisher Scientific, Uppsala, Sweden) were part of routine investigations. The quantification thresholds were 0.10 kUA/L for sIgE and 0.01 mgA/L for sIgG (18, 19). The patient's pulmonary function test results were retrieved from medical charts.

Data Analysis

Results were expressed as the basophil or lymphocyte stimulation index (SI), which is the ratio between the level of activation with the allergen and the level of activation with reaction buffer, with a threshold of 2 for allergy diagnosis. Statistical analysis was performed with the R statistical software (20). For BAT, optimal cutoff points were determined with the "OptimalCutpoints" package (21). The Youden index, which defines the maximum potential effectiveness of a biomarker to classify a disease status at a specific cutoff, was also calculated (22). Intertest correlation was estimated using Pearson's correlation coefficient. Mean SI of each group were compared *via* Wilcoxon's or Kruskal-Wallis tests as appropriate. A two-sided p-value < 0.05 was considered statistically significant.

Ethics Statement

All the experimental protocols were approved by the institutional ethics committee and GDPR commission with the reference number 2019–270. All methods used were carried out in accordance with relevant national guidelines. Written informed consent for participation was obtained for this study in accordance with the national legislation and the institutional requirements (23, 24).

RESULTS

Demography

The cohort was comprised of 20 CF patients, 25 asthmatic patients, 4 COPD and 18 patients with other chronic pulmonary diseases (6 pulmonary arterial hypertension, 4 idiopathic pulmonary fibrosis, 3 emphysema, 2 chronic bronchiectasis, 1 lymphangioleiomyomatosis, 1 idiopathic

Abbreviations: ABPA, Allergic Bronchopulmonary Aspergillosis; AF, *Aspergillus fumigatus*; AUC, Area under the curve; BAT, Basophil Activation Test; CF, Cystic fibrosis; COPD, Chronic Obstructive Pulmonary Disease; FcεR, Fc epsilon Receptor; fMLP, Formyl-methionyl-leucyl-phenylalanine; Ig, Immunoglobulin; ISHAM, International Society for Human & Animal Mycology; PHA, Phytohemagglutinin; SI, Stimulation Index.

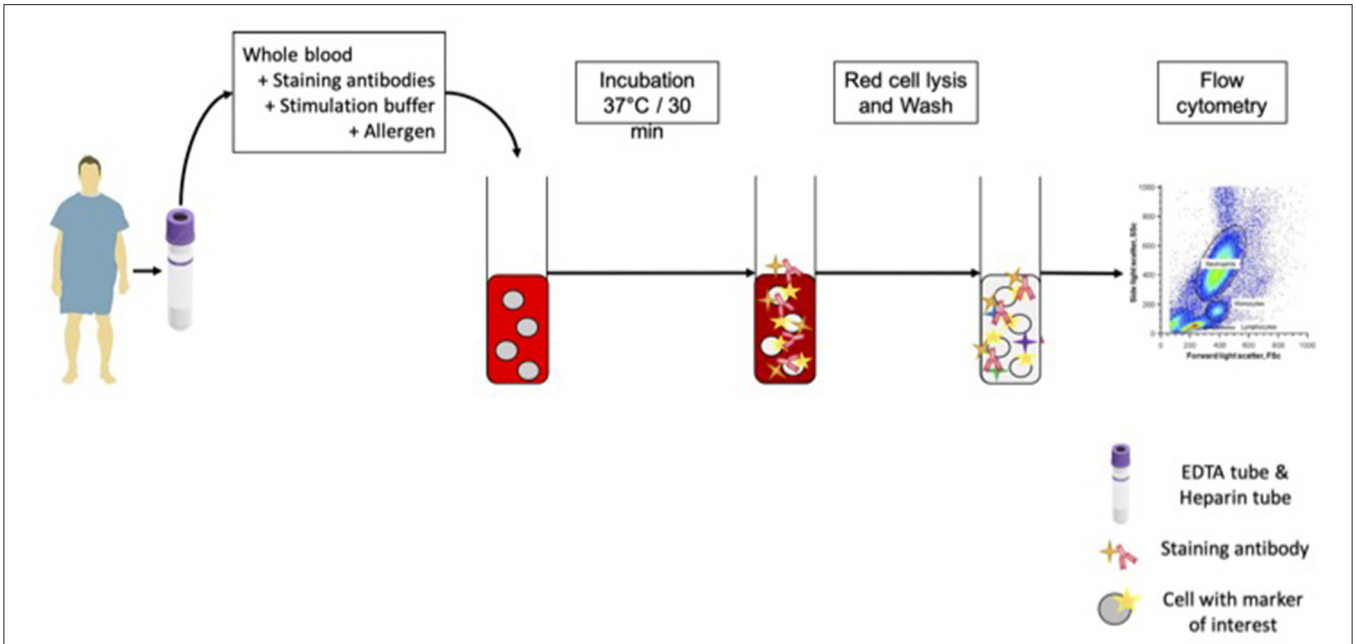

FIGURE 1 | BAT protocol. For each patient in the study, we sampled 1 EDTA tube for BAT. The whole blood was mixed with staining antibodies, stimulation buffer and allergen or controls. After incubation at 37°C, red cell lysis and wash, cells were analyzed on FACS.

TABLE 1 | Demographic and laboratory data of the study cohort.

(median +/−5–95 percentile)	**ABPA**	**AF-S**	**Control**	**Total**	***p***
n	6	25	36	67	
Age (years)	37.5 (16.3–61.8)	49.5 (25.0–75.0)	57.0 (26.7–78.0)	53.0 (24.3–76.4)	0.025
Male/Female	3/3	9/16	14/22	27/40	0.797
Cystic fibrosis	4	10	7	20	0.020
Asthma	1	8	15	25	0.514
COPD	1	2	2	4	0.509
Others	0	5	12	18	0.097
Lung transplantation	2/6	6/25	14/36	22/67	0.454
Time since transplantation (years)	8.6 (8.2–9.1)	7.7 (0.6–23.6)	1.7 (0.1–13.2)	4.5 (0.1–19.7)	0.294
Bacterial colonization	3/6	9/25	5/36	17/67	0.019
Fungal colonization	3/6	6/25	2/36	11/67	0.005
Total IgE (kIU/L)	1,132.0 (198.7–5,685.8)	197.0 (16.6–1,139.0)	27.5 (2.0–266.3)	66.7 (3.0–1,413.5)	$<10^{-3}$
IgE AF (kUA/L)	16.8 (0.5–66.3)	0.4 (0.1–15.8)	0.05 (0.01–0.09)	0.10 (0.01–19.9)	$<10^{-3}$
IgG AF (mgA/L)	46.8 (22.0–79.1)	18.2 (4.9–52.9)	12.3 (3.1–56.6)	16.6 (3.4–59.0)	0.002
Eosinophils (/mm^3)	300 (0–800)	100 (100–600)	100 (0–600)	100 (0–700)	0.554

ABPA, allergic bronchopulmonary aspergillosis; AF, Aspergillus fumigatus; AF-S, Aspergillus fumigatus sensitization; COPD, chronic obstructive pulmonary disease.

chronic eosinophilic pneumonia, 1 infectious pneumonitis). The ABPA group was composed of 6 patients, most of them were CF patients (4/6). The AF-S group included 25 patients, especially CF (10) and asthmatic (8) patients. The control group (n = 36) was composed of 7 CF patients, 15 asthmatic patients, 2 COPD and 12 patients with other chronic pulmonary diseases. Demographic data are summarized in **Table 1**.

BAT With AF Extract Displayed Good Performances for ABPA Diagnosis

BAT dose-response to AF extract showed that 50 ng/mL yielded a maximal response, and was therefore the optimal concentration to be used in further BAT (**Supplementary Figure 1**). BAT was positive in all ABPA patients (**Figure 2**). BAT discriminated both ABPA ($p = 0.0028$) and AF-S patients ($p = 0.00023$) from controls. However, BAT could not distinguish between ABPA and AF-S patients ($p = 0.72$). Comparing results in ABPA and the control groups, with the usual SI threshold of 2, BAT with AF extract displayed 100% sensitivity and 65.5% specificity. An optimized SI threshold allowing for the best Youden index was calculated as 6.55. This optimized SI retained 100% sensitivity but specificity increased to 77.6% (**Table 2**). The area under the curve (AUC) was 0.84 BAT with AF extract and 0.83 for sIgE to AF. Youden index was 0.78 for BAT with the optimized SI cut-off, but only 0.66 for sIgE to AF extract.

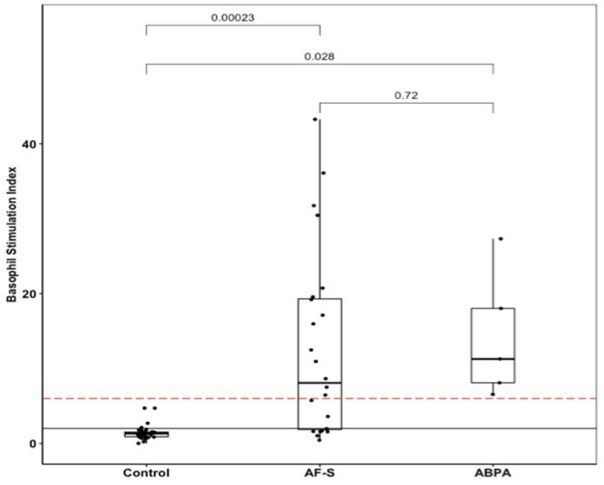

FIGURE 2 | BAT AF in ABPA, AF-sensitized (AF-S), or control patients. The solid black line shows the usual BAT positivity threshold (stimulation index = 2); the dotted red line shows the optimal BAT threshold (stimulation index = 6.55). ABPA, allergic bronchopulmonary aspergillosis; AF, Aspergillus fumigatus; AF-S, Aspergillus fumigatus-sensitized; BAT, basophil activation test.

BAT With Molecular Components Further Improved BAT Performance

In positive (SI $\geq$ 2) BAT with AF extract, a dose-response BAT was performed with each of the five AF molecular components. Six AF-S and 6 ABPA patients were tested (**Figure 3**). There was no significant difference in BAT AF responses for these patients (**Figure 3A**). The mean responses with each AF molecular component were higher in ABPA patients, but only BAT with Asp f 4 at 10 ng/mL reached the significance level (basophil SI of 10.56 in ABPA group vs. 1.24 in no ABPA group, $p = 0.0002$) (**Figures 3B–E**).

Matrix Correlation

As illustrated in **Figure 4**, total IgE were more strongly correlated with sIgE to AF ($r = 0.53$, $p = 0.01$) than IgG to AF ($r = -0.03$, $p = 0.001$), although sIgE were correlated with sIgG to AF ($r = 0.29$, $p = 0.003$). BAT AF was correlated with sIgG ($r = 0.45$, $p < 10^{-3}$) and IgE ($r = 0.35$, $p = 0.01$) to AF. Tiffeneau index was inversely correlated with the basophil count ($r = -0.36$, $p = 0.03$) and sIgE to AF levels ($r = -0.35$, $p = 0.01$). The FEV1 (Forced Expiratory Volume in 1 second) was strongly correlated with FVC (Forced Vital Capacity) ($r = 0.62$, $p = <10^{-3}$).

DISCUSSION

Data on BAT as a tool for ABPA diagnosis are scarce, with only five previous studies from our and other teams (12–16). Four of them have focused on CF patients, an underlying disease associated with the highest ABPA incidence (25) and the global conclusion was that BAT could improve ABPA diagnosis in CF patients. The study of Prasad et al. displayed more contrasted results on asthmatic patients. The present study brings further insights, from a clinical viewpoint, into the usefulness of BAT for ABPA diagnosis in asthmatic patients.

First, we demonstrated the absence of a plateau phase in basophil responses to high AF concentrations. This finding suggests that the basophil signaling switch induced by supraoptimal allergen concentrations does occur with AF extract, similarly to experimental conditions of highest response induced by equivalent amounts of allergens and IgE bound

TABLE 2 | Performance analysis of total and specific immunoglobulins and cellular functional assays with the optimized cutoff.

	Optimized cutoff	AUC (5–95 percentile)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Youden index
Total IgE	90	0.68 (0.54–0.82)	66.7	66.7	59.3	73.3	0.33
IgE AF	0.24	0.83 (0.71–0.94)	75.0	90.9	85.7	83.3	0.66
IgG AF	27	0.69 (0.53–0.84)	76.2	65.5	61.5	79.2	0.42
BAT with AF extract	6.55	0.84 (0.74–0.94)	100	77.6	27.8	100	0.78

Optimization consisted in the identification of the cut-off value associated with the best Youden index.
AF, Aspergillus fumigatus; AUC, Area under curve; BAT, Basophil activation test; NPP, Negative predictive value; PPV, Positive predictive value.

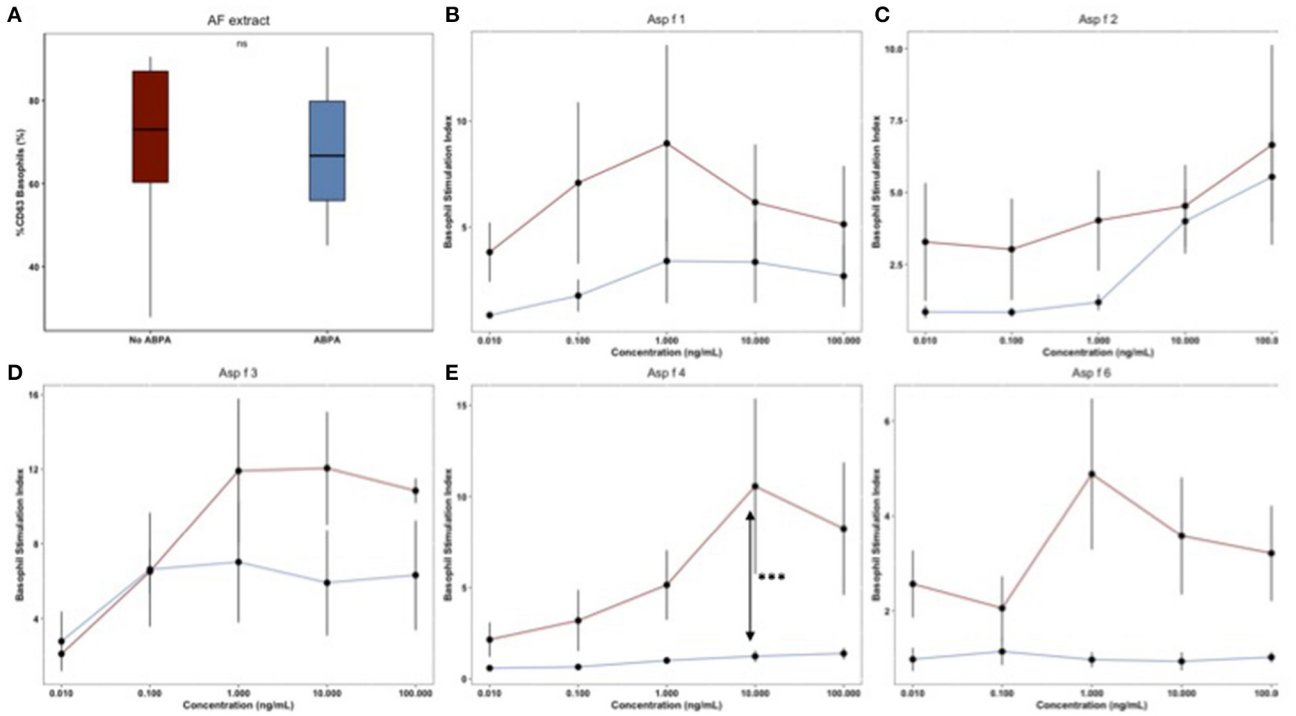

FIGURE 3 | (A–E) Mean BAT responses for AF molecular components: Asp f 1, Asp f 2, Asp f 3, Asp f 4, Asp f 6. Mean (SD) responses for AF extract and each AF molecular component's concentration were calculated for ABPA ($n = 6$, red lines) and AF sensitized ($n = 6$, blue lines) patients. Significant difference was calculated at each point.

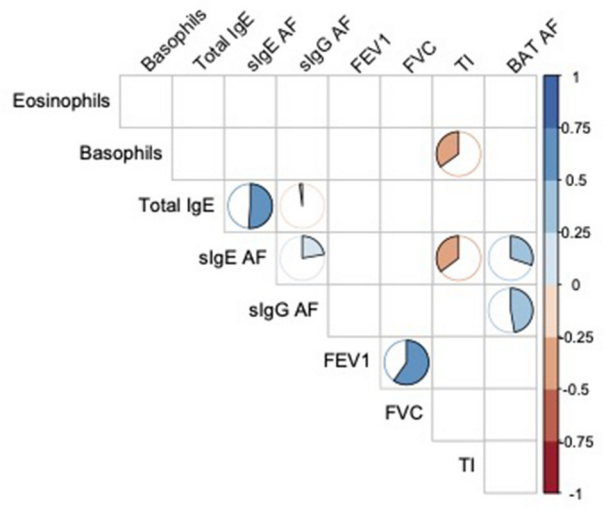

FIGURE 4 | Spearman's correlation matrix of eosinophils and basophils count, total IgE, specific IgE and IgG AF levels, pulmonary function tests results and functional cellular assays. Only significant correlations (a = 0,05) are illustrated by a pie chart. The fill rate of pie chart is proportional to correlation coefficient. AF, Aspergillus fumigatus; FEV1, Forced expiratory volume in 1 sec; FVC, forced vital capacity; TI, Tiffeneau index.

to mast cell and basophil FcεRI (26, 27). The bell-shaped curve of AF-induced basophil activation indicates the need for standardized concentrations of AF extract for BAT, and its potential use as a follow-up test through the monitoring of CD-sens and EC-50 (28, 29).

Secondly, we confirmed that BAT with AF extract performs better than humoral markers (total IgE, IgE to AF and IgG to AF) for ABPA diagnosis. With a 100% negative predictive value, BAT could assist with ruling out ABPA in clinical settings.

In order to improve ABPA diagnostic accuracy, we performed BAT with AF molecular components in BAT AF-positive patients from our cohort. The soluble form of ImmunoCAP® antigens was employed. Overall, ABPA patients displayed higher BAT responses to all the molecular components as compared to AF crude extract. Asp f 4 BAT significantly discriminated ABPA from mere AF-sensitization. Serum sIgE to Asp f 4 has been reported as a better discriminant than AF extract and other AF molecular components for ABPA diagnosis in CF patients (9, 30–32). Hence, IgE immunization against Asp f 4 appears as a relevant marker of ABPA pathophysiology. Functions of this protein in *Aspergillus sp* have not been described yet. Ramachandran *et al* have studied the structure and immunogenicity of this protein, providing evidence of the key role of C-Terminal cysteine residues for IgE binding (33).

Basophil activation has been correlated with a strong and effective allergic immune activation, lung damage and pulmonary symptoms (34). We speculate that strong BAT responses in ABPA patients are related to their complex molecular sensitization profile (35).

The main limitations of our study were its monocentric design and the relatively small size of the cohort, hence calling for

confirmation through larger studies. The main novelty of this study is the evidence of an improved discrimination between AF-S and ABPA, by using AF molecular components. In conclusion, cellular functional assays are easy to implement in the routine clinical laboratory for direct and personalized evaluation of each patient's functional responses to AF extract and proteins. They might thus be the next first-line test for ABPA diagnosis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MM, MG, CG, and JV contributed conception and design of the study. MM, YS, and FM performed the experiments. CC, SR, and MR-G performed the statistical analysis. J-LM wrote the first draft of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/falgy.2022.898731/full#supplementary-material

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Immediate hypersensitivity to COVID-19 vaccines: Focus on biological diagnosis

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Soon after the release of the new anti-COVID mRNA vaccines, reports came in from the US and the UK of anaphylactic reactions. Fueled by the necessary caution toward these new vaccine platforms, these reports had a great impact and were largely commented upon in the scientific literature and global media. The current estimated frequency is of 5 cases per million doses. Very little biological data are presented in the literature to support the anaphylaxis diagnosis in these patients in addition to skin tests. Allergic reactions to vaccines are rare and mostly due to vaccine excipient. Therefore, the poly-ethylene-glycol (PEG) present in both mRNA formulation, and already known to be immunogenic, was soon suspected to be the potential culprit. Several hypersensitivity mechanisms to PEG or to other vaccine components can be suspected, even if the classical IgE-dependent anaphylaxis seems to be one of the most plausible candidates. In the early 2022, the international guidelines recommended to perform skin prick tests and basophil activation tests (BAT) in people experiencing allergic reaction to the first dose of COVID-19 vaccine or with a history of PEG allergy. The aim of this review is to discuss the main potential mechanisms of immediate allergy to COVID19 vaccines based on published data, together with the various techniques used to confirm or not sensitization to one component.

KEYWORDS
COVID-19 vaccine, anaphylaxis, basophil activation test, IgE, complement

Introduction

In the context of COVID-19 pandemic, several vaccines have been developed in a few months, and the number of companies involved in vaccine development is increasing. These vaccines are presented in Table 1. Their effectiveness in reducing severe cases is remarkable. However, the existence of adverse events in particular potential allergic reactions has been rapidly reported. Indeed, severe immediate allergic reactions to the COVID-19 vaccines were described very early after the

TABLE 1 Composition of the vaccines approved by the European medical agency (**potential allergens in bold**).

	BNT162B2 Pfizer/BioNTech Cominarty	BNT162B2 bivalent Pfizer/BioNTech Cominarty Original/BA	mRNA-1,273 Moderna Spikevax	mRNA-1,273.214 Moderna	ChAdOx1-S AstraZeneca Vaxzevria	NVX-CoV2373 Novavax Nuvaxovid	Ad26.COV2-S Janssen-Cilag Jcovden	VLA2001 Valneva Valneva
Type of vaccine	mRNA coding for SARS-CoV2 spike glycoprotein	Bivalent vaccine: addition of mRNA coding for spike from BA1 omicron variant to the initial vaccine	mRNA coding for SARS-CoV2 spike glycoprotein	Bivalent vaccine: addition of mRNA coding for spike from BA1 omicron variant to the initial vaccine	Chimp adenovirus vector encoding SARS-CoV2 spike glycoprotein	Recombinant adjuvanted SARS-Cov2 spike protein	Adenovirus type 26 encoding SARS-CoV2 spike glycoprotein	Inactivated adjuvanted adsorbed SARS-Cov2 virus
Active substance **Potential allergens**	mRNA (30 μg) **polyethylene glycol 2,000 tromethamine and tromethamine hydrochloride (only in ready to use vials)**	mRNA (30 μg booster dose)) **polyethylene glycol 2,000 tromethamine and tromethamine hydrochloride**	mRNA (100 μg) **polyethylene glycol 2,000 tromethamine and tromethamine hydrochloride**	mRNA (50 μg booster dose) **polyethylene glycol 2,000 tromethamine and tromethamine hydrochloride**	recombinant ChAdOx1-S, produced by HEK 293 cells **polysorbate 80**	Recombinant adjuvanted spike protein produced in Spodoptera frugiperda Sf9 insect cells **polysorbate 80**	Recombinant Ad26. COV2-S produced in PER.C6 Tet R cells **Polysorbate 80**	Wuhan strain hCoV-19 produced on Vero cells, adsorbed on Aluminium hydroxide

beginning of vaccination in the United States and the United Kingdom, and then all over the world. The more recent reports estimate that anaphylaxis cases for both Pfizer BNT162b2 and Moderna mRNA-1273 vaccines exhibit an estimated frequency of 11.1 to 12.4 and 2.5 to 20.4 cases per million doses administered, respectively (1, 2). Altogether, the number of doses given in the European Union as of June 2022 are the following : 649 million of Comirnaty, 155 millions of Spikevax, 69 millions of Vaxevria, 19 millions of Jcovden and 216,000 of Novavax. The existence of poorly understood severe reactions indirectly contributed to limiting vaccine access by fueling some reluctance to vaccination in the early 2021. To address this issue, a better knowledge of these reactions and of their mechanisms was urgently needed and led to several studies. Beside the identification of the mechanism(s) involved in allergic reactions, the identification of the culprit allergen(s) has also been evaluated.

The mechanisms of drug-induced anaphylaxis can be immunological, involving IgE-mediated basophil and mast cell activation, or IgG-mediated with activation of neutrophils and possibly monocytes and platelets; in other cases, it mainly relies on pharmacological activation of mast cells *via* complement activation or engagement of MRGPRX2 (3). All these pathways have been investigated in COVID-19 vaccine-induced anaphylaxis by preliminary studies, sometimes controversial, that will be discussed in the present review. These recent information on the potential immediate hypersensitivity mechanisms led to the establishment of clinical (skin testing) and biological guidelines to (1) evaluate the risk of a second vaccine dose and propose a safe alternative for at-risk patients, and (2) identify at-risk patients with an history of a previous allergic reaction to one of the vaccine components.

Beside these immediate hypersensitivity reactions, some delayed reactions have been reported in less than 0.3% which were mostly mild and did not contraindicate subsequent vaccinations (4). These reactions will not be discussed in this review.

Potential mechanisms of COVID 19 vaccine-induced immediate hypersensitivity

The hypotheses regarding the mechanisms of anaphylactic reactions induced by mRNA vaccination against SARS-CoV-2 are multiple, and probably correspond, at least in part, to the classic mechanisms of drug anaphylaxis (5). Moreover, their rate is close the anaphylaxis rate to other vaccines (6). The first hypothesis is an IgE- or IgG-dependent mechanism linked to the presence of allergenic substance(s) in these vaccines which implies prior exposure and sensitization. However, the clinical reactions could also be linked to pseudo-allergic phenomena such as complement activation (complement activation-related pseudo-allergy or CARPA) without prior exposure, or the Mas-related G protein Receptor X2 receptor (MRGPRX2) engagement (7, 8) (**Figure 1**).

IgE-mediated basophil and mast cell activation

IgE-mediated anaphylaxis implies a first exposure to an allergen leading to the production of specific IgE. These IgE bind to the high affinity receptors FcεRI on mast cells and

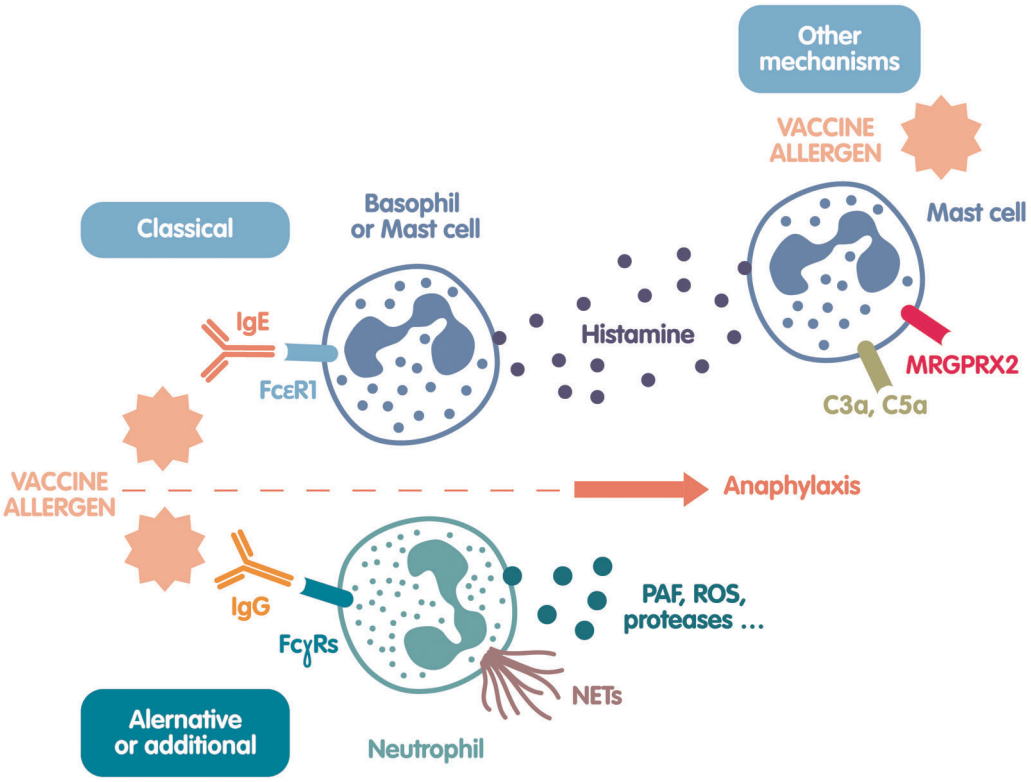

FIGURE 1
Legend. Main mechanisms of potential COVID-19 vaccine-induced hypersensitivity. The classical mechanism involves specific IgE-dependent mast cell and basophil activation leading to histamine/tryptase release. The alternative or additional mechanism involves specific IgG-dependent neutrophil activation leading to the release of reactive oxygen species (ROS), proteases such as elastase or neutrophil extracellular traps (NETs). Finally, several other mast cell activation mechanisms are suspected to play a role *via* C3a or C5a fixation to their receptors, or *via* the direct activation of MRGPRX2 by the vaccine.

basophils. Upon a new encounter, the allergen or a closely related substance activates mast cells and basophils by surface IgE cross-binding, which triggers degranulation of various mediators such as histamine or tryptase. This mechanism is the basis for routine anaphylaxis biological diagnosis, which encompass degranulated tryptase and histamine measurement, as well as specific IgE assessments. A true IgE-mediated allergic reaction to COVID-19 vaccines is possible, mainly based on documented PEG-mediated reactions in the literature, but seems very rare, as we'll see below.

IgG-mediated anaphylaxis

Up to 30% of patients with clinically proven drug anaphylaxis do not have any sign of an IgE-dependent mechanism (9). Our group has demonstrated in various mice models that anaphylaxis can be triggered by a pathway involving specific IgGs that activate neutrophils (10, 11). Activated neutrophils release platelet-activating factor (PAF), a potent vasoactive lipid with effect similar to histamine. In a multicentric clinical study, we were able to confirm this mechanism in human, and showed that signs of neutrophil activation (in particular degranulation of neutrophil elastase and production of neutrophil extracellular traps) were correlated with severity in perioperative anaphylaxis patients (9).

Complement activation and mast cell degranulation

Besides these two mechanisms, other pathways have been proposed to explain anaphylaxis that do not rely on the adaptive immune response. Since they do not require previous sensitization, these mechanisms may explain reactions observed to the first allergen exposure. Most of these mechanisms involve pharmacological activation of mast cells by the allergen. Some allergens have been described to activate the complement system, releasing C3a and C5a cleavage fragments that are able to trigger mast cell degranulation through specific receptors. These adverse effects known as CARPA have been documented with nanomedicines

in experimental models but evidence in human are lacking (12). Moreover, Szebeni group also reported anti-PEG IgG-triggered complement terminal complex-mediated damage to PEGylated nanomedicines, that could decrease the efficacy of the nanomedicine and increase the toxicity *via* this complement activation (13).

Mas-related G protein-coupled receptor X2 (MRGPRX2) engagement on mast cells

Mast cell direct activation by positively charged substances like iodinated contrast media, quinolones, or some neuromuscular blocking agents has been described through the Mas-Related G Protein coupled Receptor X2 (MRGPRX2) (14). Interestingly, mRNA stabilization with PEG induces also a positive charge that could make this mechanism possible during COVID19 vaccine reaction. Whether basophils can also express MRGPRX2 at their surface upon activation remains controversial, but would be of great interest in assessing COVID19 vaccine-related hypersensitivity (15). However, it was recently shown that tryptase release by activated mast cells cannot discriminate between IgE- and MRGPRX2-related mechanisms, leaving yet unanswered questions concerning this interesting receptor (16).

Release of other active mediators

Finally, many mediators like prostaglandins, bradykinin, serotonin or nitric oxide could mimic anaphylaxis symptoms by inducing vasodilation or bronchoconstriction, and their potential contribution to anaphylaxis is only beginning to be investigated.

Potential allergens in COVID 19 vaccines

Allergic reactions to vaccines are mostly due to excipients or contaminants, and exceptionally to the antigens themselves (3). The potential allergens contained in the vaccines that are available in the European Union are listed in **Table 1**.

Both mRNA vaccines (Cominarty and Spikevax) have a similar structure: they contain no protein or adjuvant, but only the mRNA which is packed with stabilizing lipids inside a lipidic nanoparticle covered with polyethylene glycol (PEG) to increase water solubility. While PEG has been the first suspected candidate, other components must be evaluated (17).

PEG or macrogol is an ether polymer with a molecular weight ranging from 200 to 35,000 g/mol. It is used in many industrial products, either pure in preparation for colonoscopy and laxatives, or as an excipient in some food, cosmetics, topical drugs, or therapeutic proteins. Anaphylaxis to PEG-containing products remains rare but have been reported (18). These reactions were mostly with high molecular weight PEG (>2,000 g/mol), both with oral route (19) or injected drugs (20). Positive skin tests have been reported in PEG allergic patients, and specific IgG and IgE have been recently reported in some patients with severe reactions to injectable drugs and therapeutic protein (21, 22). This shows that PEG can be recognized by the immune system and can trigger the classical IgE pathway mechanism (23). The role of PEG IgG is less clear in this context. It has been suggested that specific IgG could activate the complement *via* the classical pathway, which in turn could activate mast cells *via* the anaphylatoxins. However, the prevalence of these IgG is high in patients exposed to PEG without any allergic reaction. Very recently, a time-course study of anti-PEG IgG did not evidence any increase in concentrations after each dose of mRNA vaccine, regardless of the vaccine used (24) A more detailed analysis of IgG subclasses involved, and the measurement of their affinity could help to distinguish harmful IgG susceptible of triggering a reaction. Moreover, it has been demonstrated that PEG itself can directly activate the complement system *via* the lectin and the alternative pathway (13, 25–27) and that lipid-conjugated PEG could be involved in the allergic reactions rather than PEG alone (28).

In addition to PEG, Moderna mRNA-1273 vaccine (Spikevax) also contains **tromethamine (or trometamol)**, a widely used buffering agent. Some cases of anaphylaxis have been published to injectable drugs where tromethamine was identified as the culprit agent (29, 30). In the second version of Cominarty vaccine (ready to use vials), tromethamine has also been added. Very recently, bivalent mRNA vaccines from Pfizer (Cominarty Original/BA) and Moderna (mRNA-1273.214) have been approved by the EMA. mRNA coding for spike from BA1 omicron variant have been added to both original vaccines. However no other modification of the vaccine composition can be noticed, in particular concerning potential allergens.

A third vaccine, widely used in Europe, is a viral vector from a chimpanzee adenovirus coding for SARS-CoV2 spike protein (ChAdOx-1-S, AstraZeneca). It does not contain adjuvant either, but contains **polysorbate 80 (or Tween 80)**, a non-ionic detergent with poly(ethylene oxide) side chains that are similar to the PEG structure. Anaphylaxis to polysorbate 80 has also been observed, with cross-reactivity to PEG components (25, 31).

Two vaccines consisting in recombinant spike proteins are also available in the European Union : Nuvaxovid (recombinant adjuvanted spike protein produced in Spodoptera frugiperda Sf9 insect cells) and JCovden (Recombinant Ad26. COV2-S produced in PER.C6 Tet R cells). They both contain **polysorbate 80.**

Finally the Valneva vaccine, composed of inactivated adjuvanted adsorbed SARS-Cov2 virus does not contain any component suspected to induce allergic reaction.

In summary, most of COVID-19 vaccines contain a few potential allergens able to trigger anaphylaxis *via* several mechanisms incompletely understood (32). In addition to the clinical evaluation by allergologists and the use of skin tests in a stepwise fashion (33, 34), a biological evaluation can be done to get more information and determine the risk for vaccination or re-vaccination.

Biological evaluation of COVID19 vaccine-induced allergy

Anti-PEG antibodies

The few studies carried out on the presence of anti-PEG of the IgE isotype but also IgG and IgM have been done using "in-house" techniques (21) A recent commercial ELISA was studied in 20 patients known to have experienced clinical reactions to drugs containing PEG; in this work, 4 out of these 20 patients had anti-PEG 2,000 IgE, and all had positive PEG skin tests (35). On a technical level, it is important to note the possible interference of bovine serum albumin and Tween 20, often used in ELISA; skimmed milk and an alternative detergent would probably be more appropriate reagents (35). Flow cytometric methods have also been described to assay anti-PEG IgE (36). Interestingly, Zhou *et al.* (21) found anti-PEG IgE and IgG in patients who had an anaphylactic reaction to products for colonoscopy preparation containing PEG 3350. It seems that some of these antibodies preexist in the general population, with a frequency of anti-PEG IgG of 5 to 9%, which could explain the manifestations observed at first administration (37).

The recent results on the frequency of anti-PEG antibodies during post-vaccination reactions are contradictory. This may be partly due to a lack of standardization of assay methods and of the gradation of the severity of allergy to PEG (38, 39). Some authors detected neither anti-PEG IgE nor IgG in post-vaccination reactions (34, 40), others found IgE and IgM but their control population was small. One of the questions is whether it would not be preferable to develop techniques to search for antibodies directed against PEG in the form of nanoparticles, or even against the vaccine itself (41). New robust tests are needed.

Proteins from complement activation

When hypothesizing CARPA-type mechanism, different complement activation parameters can be measured at the time of the reaction: anaphylatoxins C3a and C5a and the soluble fraction of the membrane attack complex C5b-9. In a pig experimental work, increased soluble C5b-9 levels correlated with the presence of anti-PEG IgM, after stimulation with PEGylated liposomes (32). Lim *et al.* (42) found increased C3a levels just after the clinical reaction in 3 patients, persisting from 48 h to one month. However, this increase was not confirmed by our group in 5 patients sampled at the time of the reaction (43). These preliminary results do not make it possible to conclude on the interest of these markers. Moreover, it is difficult to obtain a blood sample at the time of the clinical reaction, particularly in patients vaccinated outside a hospital.

Mast cell activation and -derived mediators

To assess a possible mast cell degranulation in favor of an anaphylactic reaction induced by mRNA vaccines, histamine and tryptase assays could be informative. Very few studies report the measurement of tryptase at the time of the reaction, and they do not show any increased levels (26, 38, 42, 44, 45). Warren et al. study is the only one reporting elevated tryptase levels (between 14 and 25 μg/l for a basal tryptase between 2 and 6 μg/L) in 8 patients at time of the reaction (34). Our group reported increased histamine levels in 1 patient out of 5, within 30 min of the post-vaccination reaction, while tryptase levels were not modified (43).

Basal tryptase levels could also be of interest, even if no increased risk for reaction has been described in patients with mastocytosis (3). A few studies have shown a subnormal concentration in some patients: median of 8.5 to 12.8 μg/l, i.e. above the 95th percentiles described in the general population (46, 47). This could be in favor of gene duplication-related hyper-alpha – tryptasemia that needs to be better documented in the future (48). Moreover, the KIT D816V mutation research in the blood can be done to document mastocytosis, even in the presence of normal baseline tryptase (49).

The basophil activation test

The basophil activation test (BAT) using CD63 and/or CD203 as activation markers by flow cytometry was developed as early as January 2021 to explore immediate hypersensitivity to mRNA vaccines. Various authors tried to determine its place in the management of patients who reported reactions to drugs containing PEG before the first dose (50), or experienced reactions just after the first dose. In both cases there was an urgent need to secure vaccine injections (46).

Most of the published studies have been done on small patient series. Troelnikov *et al.* (50) performed BAT with PEG 2,000 nanoparticles in 3 patients known for PEG allergy and evidenced basophil activation. Labella *et al.* (46) found a positive BAT to PEG 2,000 and to the vaccine in 5/16

patients. Warren *et al.* (34) reported a positive BAT in 10/11 patients tested in the presence of PEG 2,000 DMG in the form of nanoparticles and vaccine. The frequency of patients with positive BAT is therefore very variable and could depend on the patients (already known to react to PEG or not for example) and the stimuli used *ex vivo*, whole vaccine and PEG nanoparticles seeming to give the highest positivity. Different allergens can be used in BAT. PEG 2,000 and PEG 2,000 DMG have been recently marketed for this test. However, as PEG contained in the vaccines is in the form of nanoparticles conjugated with lipids, some authors carried out BAT in the presence of the vaccine and/or PEG in the form of lipid nanoparticles approaching the truly potential immunogenic form (34, 50). However, in the early 2022, some authors evidenced that BAT was positive in response to vaccine alone in 50% of the patients who had COVID, and did not react during the vaccine injection (46). This information, that remains to be confirmed, must encourage to interpret BAT results with caution, in particular in patients who experienced SARS-Cov 2 infection. However, most authors agree in concluding that in the event of an anaphylactic reaction after injection of an mRNA vaccine, BAT is more frequently positive than skin tests confirming an activation mechanism which would not necessarily be IgE dependent (34, 40, 50). In our group in Paris, preliminary data in 30 patients with anaphylaxis after the first injection of a mRNA vaccine confirm that BAT can be positive while skin tests are negative (Nicaise-Roland *P*, Soria A *et al.*, unpublished results). A recent Review by Eberlein *et al.* concluded that BAT helps elucidate allergic reactions to COVID-19 vaccines, but defining exact threshold of positivity is still needed (51).

We can thus assume that BAT is a quite simple and well-known test that needs to be further evaluated in larger well-characterized patients, with appropriate and standardized stimuli.

The histamine release test

This test is only documented in two studies in this setting. The first one evidenced transient positive results in 3 patients who experienced a reaction (52), and the other one described positive results in 2/10 patients with positive skin tests to PEG (53).

Conclusion

Eighteen months after the first vaccinations against COVID-19, the present real-world cohort survey can suggest that serious adverse effects are extremely rare. For instance, an analysis of 20,000 participants revealed that the adverse effects observed in 0.3% of the subjects were associated with full vaccination dose, vaccine brand, young age and COVID-19 (54). Research improved our understanding of COVID-19 vaccine allergy mechanisms, and made available some biological tools to an adequate management of the suspected patients (55, 56). Some tests, such as BAT, are now available to help the diagnosis in addition to skin tests. We can assume that BAT is the best biological tool to evaluate the *ex vivo* reaction to both whole vaccine and each excipient. The identification of the culprit agent even led to a safe and successful desensitization in a recent series of 6 patients (57). Conversely, the quantification of anti-PEG IgE or IgE cannot be recommended so far. Finally, lessons learned from nanomedicines need to be applied (58). There is a need to safely immunize patients who are at risk or who experienced immediate vaccine reactions, using antihistamines for example. Several studies are still ongoing in order to increase our knowledge and make large-scale vaccination safe and successful.

Author contributions

All authors made substantial direct contributions to writing the manuscript, and approved it for publication. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Towards an FDA-cleared basophil activation test

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Food allergy is a global health problem affecting up to 10% of the world population. Accurate diagnosis of food allergies, however, is still a major challenge in medical offices and for patients seeking alternative avenues of diagnosis. A flawless test to confirm or rule out a food allergy does not exist. The lack of optimum testing methods to establish precise clinical correlations remains a major obstacle to effective treatment. Certain IgE measurement methods, including component testing, have received FDA clearance, but they have been used primarily as an analytical tool and not to establish clinical correlations. Most allergy tests are still carried out within the laboratory, and skin tests outside a laboratory setting that are used for food allergy diagnosis rely on non-standardized allergens, according to the FDA definition. Epitope mapping and basophil activation test (BAT) have recently been proposed as a means of establishing better clinical correlations. Yet neither have received FDA clearance for widespread distribution. Of the two methods, the BAT has the advantage of being a functional assay. Over the past few years, several large private practice groups in the United States, have developed BAT as a clinical assay and have started using it in patient care. Given this clinical experience, the vast number of papers published on BAT (more than 1,400 as of 2022) and the trend toward increasing FDA regulation, it is essential to understand the roadmap for regulatory clearance of this assay.

KEYWORDS

basophil activation test, food and drug administration, peanut allergy, food allergy, laboratory developed test

1. Introduction

The prevalence of food allergies in the United States is between 4% and 10%. Milk, tree nuts, peanut, egg, shellfish, fish, soy, and wheat make up approximately 95% of the total (1, 2). A major challenge in identifying food allergies stems from the lack of readily available and accurate in-vitro clinical laboratory tests (IVCT) that correlates with patients' clinical presentations (3, 4). The first steps in the work-up of food allergies is establishing a good clinical history and conducting skin testing and allergen specific IgE measurements (5). Although these testing procedures have demonstrated good sensitivity for detecting allergic individuals, their specificity is low, and they lack reliable threshold values (6, 7). For this reason, physicians treating food allergies often base their decisions on personal experience, which can vary significantly, and on anecdotal information.

At the present time, an oral food challenge (OFC) is the gold-standard for confirming a food allergy. This method of confirming food allergy diagnosis, however, has serious disadvantages. It can be labor intensive, costly and carries the risk of allergic reaction in an office setting (8). Furthermore, the test can be a source of anxiety for patients and their families because of the risk of such a reaction. Therefore, a new test is needed, especially for the most common and important food allergies (**Figure 1**).

Peanut allergy is an important health problem because it is among the most common food allergies. Depending on the geographic location, peanut allergy has a prevalence of 0.1%–1.5%. Currently, peanut allergy is the only one for which an FDA approved drug is available (9–12). The basophil activation test (BAT) is well suited for detecting peanut allergy. BAT can very effectively distinguish various clinical phenotypes of peanut allergy (e.g., anaphylactic vs. non-anaphylactic reactions) (13–15). In the context of cross-reactivity syndromes (e.g., wheat and grass pollen), results of BAT can overestimate clinical allergy. For peanut allergen, this cross-reactivity is less of a problem for seed storage proteins, but not for lipid transfer proteins (LTP) in certain geographical areas (16).

A significant clinical challenge is differentiating between clinical food allergy and sensitization, the latter of which can be seen in up to 10% of the population (17). Given advances in managing food allergies, it has become more important to identify those with real clinical allergy versus sensitization (positive test but no clinical reaction) and to predict the type of allergic reaction. These issues are important in decision-making for treatments. Tests that can separate sensitivity from clinical allergy with clear cut-off values are in great need (18).

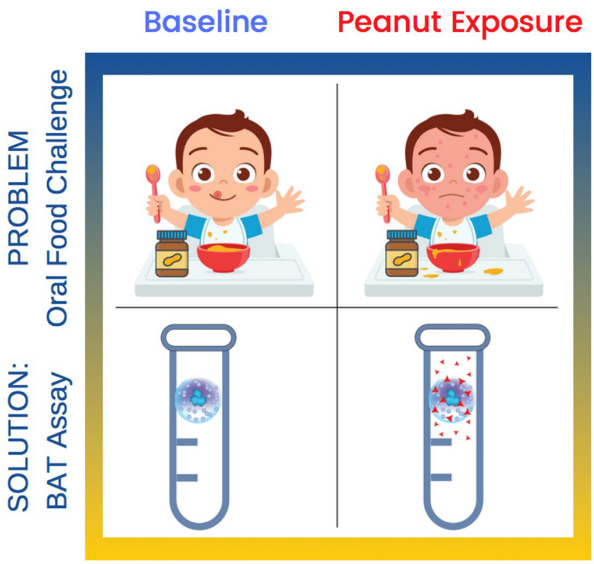

FIGURE 1
Basophil activation test is a good measure to assess clinical reactivity to prevent unnecessary food challenges.

2. Basic methodological aspect of BAT

BAT is a flow cytometry assay which measures the expression of activation markers on the basophil surface and the basophil activation process through IgE cross-linking. The hallmark of BAT, detection of CD63 on the basophil cell surface, was first discovered by Edward Knol in 1991 (19). In his report, human basophils were activated with anti-IgE and chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (fMLP). Both these methods of stimulation induced a distinct increase in expression of the CD63 on the surface of basophils. Cell surface CD63 was detected by the monoclonal antibody (MAb) 435. Time dependent kinetics of CD63 up-regulation as detected by Mab 435 binding to basophils correlated strongly with histamine release. This indicates degranulation. A comprehensive review of the historical, technical, and clinical aspects of BAT has recently been published (20).

The first step in performing a BAT is identification of basophils in whole blood. Two approaches to identify basophils in whole blood is shown in **Figure 2**. It is possible to combine the two approaches for increased stringency. Once basophils are identified, spontaneous activation and the effect of an inert antigen on basophils is tested. For spontaneous activation, basophil surface markers are stained in the absence of any allergens. To assess basophil response to an inert antigen one that humans are not sensitized or allergic to is needed. For this purpose, we have implemented the use of keyhole limpet hemocyanin (KLH). This antigen is a metalloprotein found in deep sea giant keyhole limpet, off the coast of California. There is very little cross-reaction with any

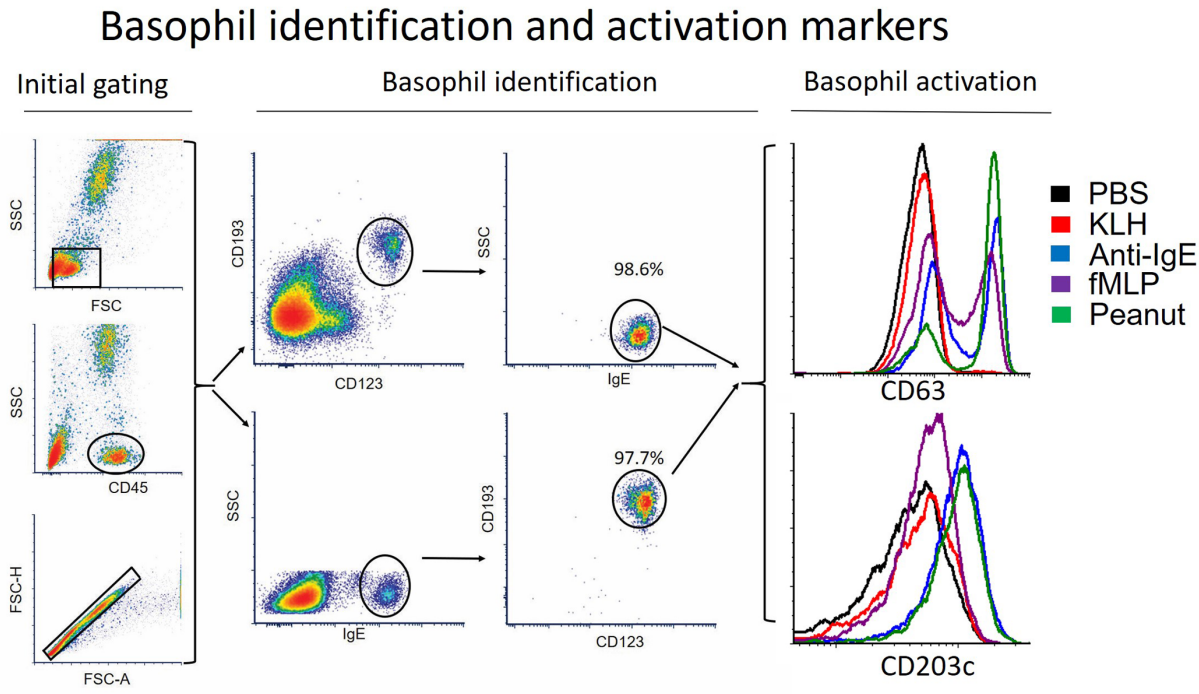

FIGURE 2
Determine basophil activation by flow cytometry. The initial gating aims at isolating singlets and removing eosinophiles, neutrophils and to some extent monocytes and DC's. The subsequent basophil identification where basophils are identified based on their surface markers. Here two approaches are shown, CD123/CD193 and IgE/SSC. Lastly the activation determined by CD63 and CD203c are shown for the two negative controls (PBS and KLH), the two positive controls (Anti-IgE and fMLP) and the peanut allergen.

other allergen and humans are rarely sensitized to it, making it a perfect negative control allergen (21, 22).

The second step is the use of a positive control that verifies the viability and responsiveness of the basophils. As positive controls, both fMLP and anti-IgE are used. Activation of basophils independently of the IgE-FcεRI pathway by fMLP is important to verify whether basophils in the blood sample are healthy to go through BAT (4, 23). These controls are critical in evaluating degraded/expired allergens, interference with basophil surface receptors or signaling, inhibition by various plasma proteins, and poor response due to baseline activation of the cells as well as non-responsive (anergic) basophils (4, 24).

The third step in BAT is to perform the allergen dose (concentration)-response curves. These dose-response curves can be interpreted with metrics such as basophil sensitivity, median effective concentration, area under the curve (AUC) and basophil reactivity (25). Each of these reporting methods have been validated with proper cut-off values based on the patient population tested and detailed summaries of these methods have been published (26, 27). Each clinical laboratory establishes optimal allergen-specific cutoff limits for the specific question the test is being used in the clinic. Clinical relevance of different types of dose-response curves (i.e., bell shape, linear, bimodal and plateau) still need further investigation.

Additional basophil activation markers have also been identified, including CD203c, diamine oxidase measurement of intracellular histamine, CD107a, CD13 and CD164, among others. However, CD63 has remained the most widely used market (28–30). In contrast to CD63, many of these other activation markers may be up-regulated in response to non-degranulation stimuli, hence limiting their clinical utility (31). It is possible that these additional markers may have value in defining clinical desensitization or basophil tolerance induction (32, 33).

2.1. The use of BAT in clinical practice and its interpretation

Although BAT is a well-established, robust, and reproducible assay with great potential for physicians in identifying allergies, its use in clinical practice has been limited by several factors. Differences in the infrastructure and expertise of the laboratories where tests are performed, the diversity of clinical reporting methods, differences in preparation and sources of the allergens, and a lack of clear clinical guidelines in how to use BAT in the diagnostic algorithms are some reasons for the limited use. Each clinical

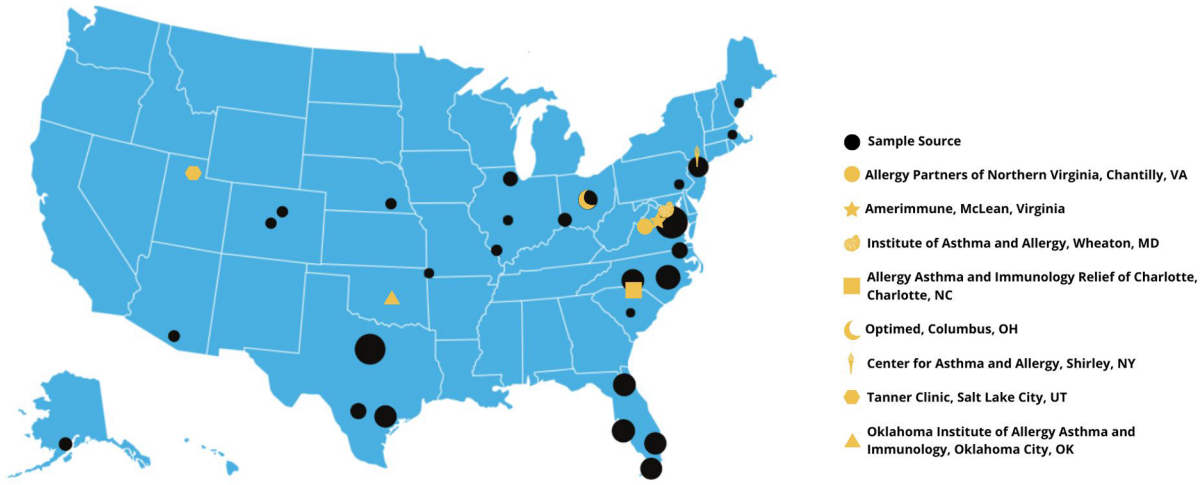

FIGURE 3
Sample sources and laboratory location for BAT in the United States. Dark circles represent sources for the blood samples. Size of each circle represent sample size from each location. Total samples are over 1,000 between year 2018 and 2021. The yellow symbols represent CLIA/CAP accredited laboratory locations capable of performing BAT as an LDT.

laboratory has developed its own methods and reporting protocols. Currently, there are eight laboratories in the United States that perform BAT as a CLIA (Clinical Laboratory Improvement Amendments) approved CAP (College of American Pathologists) accredited assay (**Figure 3**). All these laboratories are within private Allergy/Immunology practices. There is no country-wide standardization because the FDA allows regulations to be devised by the states. In other countries, the use of BAT is standardized because it is regulated by national-level authorities. BAT has been widely used as a research test and a clinical diagnostic tool in Sweden, Spain, Germany, Denmark, Italy, and South Africa under such a regulatory system (20).

BAT has the potential to be a more effective diagnostic tool in the U.S. if a consistent nationwide standard under the FDA could be established. To develop such a standard, it is important to determine at which point of the diagnostic process this test should be performed. The most practical utility of BAT is to guide a food challenge decision (20). BAT can also help determine appropriate candidates for oral food immunotherapy, natural resolution of food allergy and monitoring response to immunotherapy. Since BAT is a functional assay and uses multiple allergen concentrations to obtain dose response curves, the information obtained from this test allows for a much more detailed picture of the response to the food allergen.

In a recent and well documented study, basophil allergen threshold sensitivity (the lowest concentration of peanut allergen activating basophils) and IgE antibodies to peanut allergen were compared to double-blind placebo-controlled food challenge, (DBPCFC). Over 90% of children who failed DBPCFC also showed reactive BAT after stimulation with peanut or Ara h 2, indicating excellent sensitivity. Of those with a negative DBPCFC, approximately 70% were negative in basophil activation with peanut and Ara h 2. Three children with negative food challenges with positive serum peanut specific IgE and Ara h 2 demonstrated positive BAT to both allergens. All children with negative basophil activation passed DBPCFC to peanut indicating excellent specificity of the test (34, 35). Larger studies with similar clinical design are needed to further validate peanut—BAT before regulatory clearance can be obtained (34). Such studies will most likely require several hundred subjects to obtain robust sensitivity and specificity data.

In certain cases, BAT can predict the severity of clinical reactions as well as the prognosis of the patient's food allergy (36). In cases of oral food immunotherapy, BAT can predict thresholds of reactivity to help determine dosing of the patients as well as degrees of tolerance. The reporting of these recommendations, however, will require controlled clinical trials and the establishment of a federal standard that is country wide.

Although BAT for peanut has been studied the most, there is also data on foods such as cow's milk, egg, wheat, tree nuts, shellfish, apple carrot and celery among others. For example, the current diagnostic tests for cow's milk allergy include sIgE (sensitivity 87%, specificity 48%) and SPT (sensitivity 88%, specificity 68%). BAT has a higher sensitivity of 89% and a specificity of 83% and a positive predictive value of 81% and negative predictive value of 96% in identifying true cow's milk allergy (37).

For egg allergy, BAT has a sensitivity of 63% and a specificity of 96% for CD203c expression and a sensitivity of

77% and a specificity of 100% for CD63 expression. These numbers are much better compared to performance of skin testing or sIgE for egg (38).

Although these studies show that BAT has potential clinical utility, not all used OFC as the comparator and results are very variable due to variations used in allergen preparations. The use of allergen components may lead to better performance of BAT for these allergens and their clinical correlation.

In tests where basophils do not respond to anti-IgE stimulation, negative results to allergens should generally be considered un-interpretable (39). If basophils are not reactive to the anti-IgE control but show response to the allergen, BAT can be considered positive as long as there is no non-specific activation in the KLH control or other non-allergic control individuals (40).

2.2. BAT as a laboratory developed test (LDT) in the United States

The current system of state-regulated testing is known as laboratory developed testing (LDT). In the United States FDA defines an LDT as "(a) laboratory developed test (LDT) is a type of *in vitro* diagnostic test that is designed, manufactured and used within a single laboratory (41). LDTs can be used to measure or detect a wide variety of analytes (substances such as proteins, chemical compounds like glucose or cholesterol, or DNA), in a sample taken from a human body. Some LDTs are relatively simple tests that measure single analytes, such as a test that measures the level of sodium. Other LDTs are complex and may measure or detect one or more analytes" (42). BAT has been developed and used in clinical care as a LDT in the United States.[1]

The FDA further indicates that "while the uses of an LDT are often the same as the uses of FDA-cleared or approved *in vitro* diagnostic (IVD) tests, the FDA does not consider diagnostic devices to be LDTs if they are designed or manufactured completely, or partly, outside of the laboratory that offers and uses them". This also implies that LDTs made in an individual laboratory are not sold. Inter-state commerce is an important variable in determining the level of regulation (**Figure 4**).[1]

The FDA in principle has the authority to intervene in cases in which patient safety is jeopardized. The FDA in most cases has not enforced its authority (enforcement discretion) on LDTs because LDTs have generally been simple laboratory assays or have been used in a very controlled fashion. Laboratory testing personnel and clinicians have also often operated within the same institution or clinical practice (43). This had provided a safety net in clinical utility and potential adverse events related to the testing outcomes.

However, advances in technology and new business models, the FDA has noted, has resulted in more complicated LDTs that present greater risks, and which are more similar to other FDA approved tests that have undergone premarket review. In 2010, the FDA announced its intention to reconsider its enforcement discretion for LDTs. More recently, a bill called the VALID Act has been proposed to increase FDA oversight on LDTs.

There are three components to LDTs in clinical care (44) (**Table 1**). The first, which is the pre-analytical part, is determined and initiated by the ordering physician. The final post-analytical part is prepared by the clinical pathologist who interprets the results in the context of clinical condition. The analytical part of the test is performed by the laboratory personnel. The analytical steps of laboratory testing are a complex process that starts with the draw to the finalizing of the results for interpretation. Since LDTs, by definition, are developed and used in a single laboratory, regulatory requirements and inspections by the state focuses solely on the analytical part of the testing process (20). In contrast, FDA approved tests can be distributed widely and sold across state lines, and for this purpose need very strictly defined clinical indication and reporting—two important points that will need to be clearly defined for BAT in the process of FDA clearance of an LDT.

The LDT state-level process has reduced cost as well as the speed such tests are entered into clinical practice. It has also enabled innovation by the rapid identification of new biomarkers facilitating the development of novel therapies. One example is the speedy identification of the role of caspases in COVID-19 pathogenesis, development of this as a laboratory developed test, verification of the findings in clinical samples which led to the completion of a Phase 1 study in COVID in 2021 using a pan-caspase inhibitor (45). Innovations in diagnostics have helped advance many therapies.

3. History of flow cytometry-based diagnostics and the FDA

BAT is a flow cytometry-based diagnostic test. The FDA has only approved one such test. Its record of reviewing such tests offers lessons for what is needed for BAT approval.

A major event in the 510(k) regulatory history of clinical flow cytometry occurred in 1997, when the FDA issued the Analyte Specific Reagent (ASR) Rule to provide "assurance that reagents distributed to clinical laboratories by manufacturers for use in clinical assays (in this case LDTs) developed by the laboratories were made under current Good Manufacturing Practices (cGMP)". Manufacturers of these

[1]https://www.fda.gov/medical-devices/in-vitro-diagnostics/laboratory-developed-tests

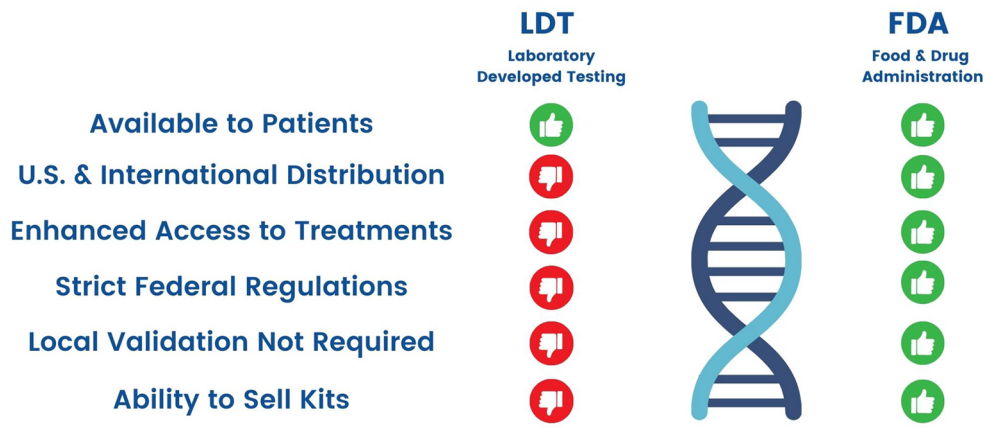

FIGURE 4
Differences between Laboratory Developed Tests and FDA-Cleared Tests.

TABLE 1 Total testing process.

- **Pre-Analytical**: Test selection
  - Accurate test selection is based on the questions formulated by the ordering physician.
  - Asking the right question for the right issue
  - Interference with various immune targeting medications should be considered
  - This is the part of the test selection that is most prone to errors
  - Right laboratory for the right test should be selected
- **Analytical:**
  - Only part of the test that takes place in laboratory proper
  - Only part of the testing that's covered by certification (e.g., CLIA) and accreditation (e.g., CAP) process.
- **Post-Analytical**
  - Interpretation of the assay data
  - Interpretation of the assay results
  - Action plan by the ordering physician
  - Similar to the pre-analytical phase, part of the total testing process most prone to errors

reagents were required to register with the FDA and list such reagents. FDA also required the reporting of malfunctions, injuries and deaths related to these reagents.[2]

After the publication of the ASR rule in 1997, some manufacturers started bundling individual ASRs together to form reagent cocktails. This conflicted with the definition of the single reagent ASRs rule that the FDA had defined. In 2007, the FDA clarified the intentions of the ASR rule in the Guidance for Industry and FDA Staff on Commercially Distributed Analyte Specific Reagents (ASRs). In the 2007 guidance, the FDA states that "bundling of ASRs into a panel of multi-analytes is inconsistent with the definition of an ASR". After this guidance, most multi-analyte reagents/cocktails were withdrawn from the market to comply with this new ASR ruling.[2]

The next events in the regulation of clinical flow cytometry were three CDER FDA sponsored public workshops in 2013, on minimal residual disease (MRD) in leukemias and Clinical Flow Cytometry and Hematologic Malignancy. Ultimately, this same approach was applied to the standardization of MRD in plasma cell neoplasms (MM) and resulted in a Special Issue of Clinical Cytometry (46).

A Flow Cytometric Devices Guidance Document was released *via* the Federal Register on October 14, 2015. After several unfavorable comments to the docket, it was withdrawn on February 21, 2015. The major criticism was that it did not address the issues of hematologic malignancies and that it was outdated. Prior to the publication and withdrawal of this second FDA flow cytometry guidance document, there was a consensus document prepared and published by two professional organizations: the International Council for Standardization in Hematology (ICSH) and the International Clinical Cytometry Society (ICCS). These Practice Guidelines (2013) consisted of the following: preanalytical issues; analytical issues; post analytic considerations and assay performance criteria. These Practice Guidelines were submitted to the FDA for review as a recognized standard. The decision was out on hold due to an announcement that Congress was going to pass the Valid Act. A decision concerning these guidelines is still pending.

On June 29, 2017 the FDA approved Beckman Coulter's ClearLLab Reagents, making this the first flow cytometry test

[2]https://www.fda.gov/regulatory-information/search-fda-guidance-documents/commercially-distributed-analyte-specific-reagents-asrs-frequently-asked-questions

that detects leukemias and lymphomas. These reagents were approved so they could be used to screen malignant cells in peripheral whole blood, bone marrow, and lymph node samples. The test has the capability to distinguish among chronic leukemia, non-Hodgkin lymphoma, and myeloma. In an official statement the FDA said that this was "a major step forward for the hematology-oncology community." That assessment was provided by Alberto Gutierrez, Ph.D., Director of the Office of *In Vitro* Diagnostics and Radiological Health in the FDA's Center for Devices and Radiological Health. "Laboratories and health care professionals now have access to an FDA-validated test that provides consistent results to aid in the diagnoses of these serious cancers," it added.[3]

FDA evaluated the ClearLLab Reagents through their *de novo* premarket pathway and cleared the test and the reagents based on the tests performance in a clinical trial. The clinical study was performed on 279 patient samples using other detection methods of malignancy as a comparison. The results of this study showed that the assay correctly identified a cancer presence 84.2% of the time which agreed with the clinical trial site's diagnosis in over ninety percent of the cases.

Finally, a proposed down classification for clinical flow cytometers was posted in the Federal Register on March 6, 2019. However, it was put on hold with the onset of the COVID-19 pandemic.[4]

A new Clinical and Laboratory Standards Institute (CLSI) document entitled H62 Validation of Assays Performed by Flow Cytometry was released on October 27, 2021 (47). This document has been submitted to the FDA for consideration as a recognized standard in clinical flow cytometry.

Taking the BAT test through the FDA pathway will ensure reproducibility across laboratories by standardizing the test reagents (antibodies, allergens, etc.). The FDA approval process will ensure standardization of basophil identification in peripheral blood samples, analysis of the data, and a specific indication for the use of BAT. The validation will happen through a multicenter clinical trial.

4. VALID act

Since the approval of FDA's Medical Device Amendments in 1976, the agency has tightened its stance on enforcing LDTs, starting to target how certain laboratory tests are used and marketed. The FDA has used its discretion in certain scenarios where it felt the safety and the accuracy of the tests were impacted but not take broad steps to regulate LDTs in general as of now.

There was a significant change in FDA's perspective in 2014 when a draft guidance was published that described the plan to phase out FDA enforcement discretion and to fully regulate LDTs. This guidance led to debate raising concerns about the FDA regulating LDTs, as that is currently performed at the state level. There was also concern that changing a longstanding regulatory policy might result in decrease in innovation and patient care. Lastly, some have questioned whether FDA has the necessary infrastructure to regulate the complex LDT market. Considering these issues, this draft guidance was withdrawn in 2015.

Despite this back and forth, bipartisan support for the VALID has continued to grow, particularly with regard to developing a new statutory authority that would address concerns raised by various stakeholders on FDA's approach. After various legislations failed to advance through Congress, the Senate recently attached the "Verifying Accurate Leading-edge IVCT Development Act of 2022" (the "VALID Act") to the first draft of a "must pass" user fee legislation. Once the user fee reauthorization draft was introduced in May 2022, the United States Senate prepared a new bill that would re-write FDA regulation of clinical testing. VALID was introduced to the United States Senate by Senators Patty Murray (D-WA) and Richard Burr (R-NC) as a part of the bipartisan FDA Safety and Landmark Advancements Act. Once enacted into law, the plan was for VALID to take effect in October 2027. This would provide the FDA time to transition into the new clinical diagnostics regulation environment.[5]

In a summary statement "The Senate Committee on Health, Education, Labor, and Pensions (HELP) on June 14, 2022 approved a package of bills to reauthorize existing Food & Drug Administration (FDA) user fees and included new legislation (the VALID Act) which would authorize the FDA to regulate *in vitro* diagnostics (IVDs) including laboratory developed tests (LDTs)". A summary of the key components of the VALID Act is shown in **Table 2**.[6]

At the time of writing of this manuscript, deliberations in both the Senate and the House resulted in a decision to delay the authorization of the law to a later date.

[3]https://www.fda.gov/news-events/press-announcements/fda-allows-marketing-test-aid-detection-certain-leukemias-and-lymphomas

[4]https://www.federalregister.gov/documents/2019/03/06/2019-03967/medical-devices-exemption-from-premarket-notification-class-ii-devices-flow-cytometer-instruments

[5]https://www.cap.org/advocacy/latest-news-and-practice-data/june-15-2022

[6]https://www.help.senate.gov/hearings/s-4348-s-958-s-4353-hr-1193-and-s-4053

TABLE 2 Key components of the VALID Act.

- Exempting all laboratory-developed tests currently in use through the VALID Act's "grandfather" exemption.
- Laboratories may still introduce LDTs without undergoing premarket review between the VALID Act's passage and October 1, 2027.
- The VALID Act would not be implemented for 5 years with an effective date of October 1, 2027, allowing time to further refine the regulatory framework.
- Requires the FDA to conduct public hearings 1 year from date of enactment and publish formal regulations which are subject to public comment within 2 years of enactment.
- Directing the FDA to avoid issuing or enforcing regulations or guidance that are duplicative of CLIA.
- Offering several exemptions from FDA pre-market review, including those LDTs that are low-risk, low volume, modified tests, manual tests, and humanitarian tests.
- Authorizing the FDA to collect user fees and establish a process by which the FDA must negotiate with the laboratory industry to set user fees, including future approval by Congress.
- Establishes mitigating measures, such as labeling, performance testing, and clinical studies, to shift higher-risk LDTs to lower tiers of regulation.
- It would create a risk-based system of oversight utilizing tiers (low-, moderate-, and high-risk) to target FDA oversight.
- It would utilize mitigating measures to shift LDTs into lower tiers of regulation. These measures would include such practices as appropriate labeling, performance testing, submission of clinical data, clinical studies, and posting information on a website.
- It would prohibit the FDA from infringing on the practice of medicine.

Even though the VALID act may not pass during the current United States administration, it is clear that the discussion will continue. Engaging with the FDA to approve tests such as BAT, will allow for a better understanding of the process of the clearance of the use of flow cytometry for different indications as the regulatory landscape for laboratory testing goes through changes in the United States.

4.1. Where do we go from here? A path to FDA cleared BAT

The best guidance that is currently available for developing FDA cleared flow cytometry based testing comes from the September ICCS 2020 virtual meeting. There were two presentations, which addressed minimal residual disease (MRD) detection.

The first was by Doug Jeffery, PhD of IVDx Consulting, LLC, titled "Flow cytometry-based minimal residual disease analysis assays submitted for FDA Clearance: Regulatory Perspective". The presentation outlined three regulatory pathways for clinical flow cytometry assays: (1) LDTs, (2) Investigational Device [Exemption (IDE)] and (3) the IVD 510(k)/*de novo*. LDTs were and remain under enforcement discretion. Of these, the IVD pathway is more demanding in that it must be determined to be substantially equivalent to a predicate device. If there is no predicate device, then the *de novo* pathway is necessary.

The second presentation by Horatiu Olteanu, MD, PhD, Professor and Medical Director, Cell Kinetics Laboratory, Mayo Clinic, Rochester, MN was titled "Flow Cytometry-Based Minimal Residual Disease Analysis Assays Submitted for FDA Clearance: A Laboratory Perspective". It was a personal assessment from his perspective as medical director of the flow cytometry laboratory, based on two MRD flow assays submitted for FDA IDE clearance as part of two different clinical trials. The same flow cytometric assay, the consensus EURO Flow two tube 8-color assay was used in both studies. One clinical study involved a treatment decision in treated MM with or without MRD. The second clinical trial involved patients with high risk CLL, and continued treatment was determined by the presence of MRD. The FDA determined that there was significant risk in the MM study thereby necessitating the submission of an IDE. In the CLL study, the FDA determined that there was a non-significant risk. The FDA did note that they thought the recently published European Research Initiative Consortium (ERIC) single tube, 10 color MRD assay was superior to the two tube Euro Flow panel and less costly. FDA recommended that in a future submission, the sponsor should consider using the ERIC panel over the EuroFlow panel. If the future submission were to contain a therapeutic indication, banked specimens were recommended (55–57). The recommendations of these two speakers point to a do list for the clearance of BAT through the FDA (summarized in **Table 3**).

4.1.1. Summary of LDT data

For a CLIA or CAP certified laboratory currently performing BAT as an LDT, including a summary of the results of the LDTs for potential FDA review will be critical. The data includes basophil identification (manuscript in preparation), activation, as well as the performance of the testing with clinical correlation. Such a summary along with an SOP should already be in place for both CLIA and or CAP inspection of the LDTs. This will provide the FDA information on the performance of the test and help in the design of the study for FDA clearance.

4.1.2. Discussions with the FDA

Any FDA clearance path will also require a clinical trial to support the indication for the test. Such a trial should be designed only after discussions with the FDA on the technical aspects, indication for the use of the test as well as patient size of the clinical study.

TABLE 3 Regulatory clearance pathway for a BAT kit.

- **Generating summary of LDT data on peanut BAT performed in laboratories across the United States**
- **Discussions with the FDA:**
  - Regulatory pathway; *de novo*, 520 (k), analytical, exemption, registration
  - Assay specific endpoints
  - Clinical trial design
  - Sample size determination
  - Flow cytometry device down-regulation
- **Standardization of Allergen Extracts**
- **Analyte Specific Reagent generation**
- **Transportation logistics of blood samples**
- **Securing intellectual property on preparation of BAT samples and assay preparation**
- **Securing funding**
  - Crowd-funding; medical and non-medical community
  - Other conventional sources; angel, venture capital, pharma

4.1.3. Generation of a standardized allergen extract

Good Manufacturing Practice (GMP) to standardize allergens for use in diagnosis and treatment is a critical regulatory requirement by the FDA. Allergens are derived from natural sources. Their manufacturing may involve roasting, grinding, defatting, extraction, clarification, and sterilization that results in allergen heterogeneity. A consistency within the manufacturing process will improve the efficacy and the safety of the BAT. Sourcing of peanut flour for BAT from a food-grade peanut manufacturer will be the starting point for test substance manufacturing.

The clinical correlation of recombinant molecules (i.e., Ara h 1, Ara h 2, etc.) versus native peanut allergen has not been studied as extensively, but may provide added value in clinical correlations.

4.1.4. Generation of analyte specific reagents to be used in BAT

ASRs are raw materials and components that are used to develop a laboratory assay. By definition, the key characteristic of each component of an ASR is its ability to attach to or react with a substance whose detection is clinically meaningful.

ASR rule requires that manufacturers list proprietary name, common name, and quantity or concentration of the reagent; the source and a measure of its activity; and the name and place of business of the manufacturer. There also needs to be an establishment of registration, device listing, and compliance with FDA's quality system regulation, medical device reporting requirements, and ASR labeling and distribution requirements.

The ASRs for BAT will include antibodies, antigens (e.g., peanut and control allergen) as well as reagents that stimulate basophils (e.g., fMLP, anti-IgE).

4.1.5. Transporting the blood sample

This can be a potential hurdle for clinical BAT. Temperature control boxes, choice of anticoagulant (heparin is the preferred option), time frame before the test would become invalid will need to be part of the clinical trial readout. In a recent paper, we demonstrated very minimal impact of transport on blood samples (33).

4.1.6. Securing intellectual property

For patents related to diagnostic subject matter, U.S. case law stipulates there are several types of claims to try to meet eligibility. These include (a) method of preparing samples for analysis, (b) method of diagnosing + treating, (c) A set of assay samples, (d) a kit and (e) a method of diagnosing for ex-US filings are important to consider.

4.1.7. Securing funding

Recent developments in fundraising options (e.g., crowdfunding) allowing the greater allergy and patient community to invest will facilitate such a testing process to go through the clinical trials and regulatory process, in the absence of pharmaceutical, government or device manufacturer backing. With the Jumpstart Our Business Startups (JOBS) Act of 2012, signed into law by President Barack Obama on April 5 of that year, equity crowdfunding has emerged as a viable source for early-stage seed capital. Under Title III of the JOBS Act of 2012, early stage ventures could raise a maximum of $1.07 million in a 12-month period from both accredited and non-accredited investors, so long as the funding round in question is hosted on a Financial Industry Regulatory Authority (FINRA)-approved crowdfunding portal. On March 15, 2021, this maximum was increased to $5 million per 12 month period. A total of $486.8 million was raised in 2021through 1,448 individual regulation crowdfunding (also referred to as RegCF) rounds. The crowdfunding investment market has held firm despite recent economic weakness, with $235.1 million investing *via* regulation crowdfunding (RegCF) in the first half of 2022, compared with $219.4 million in the same period in 2021.

5. Conclusion

BAT has been used as a research test now for over 30 years. Over the past 4 years, this test has been validated for use in diagnosing and monitoring food allergies as a laboratory developed test in the United States. Given the increase in demand for BAT from clinics treating patients with food allergies as well as many centers looking to develop their own LDTs for this test, it is time for its standardization and FDA clearance. FDA acceptance of the first peanut OIT in 2017

and increasing use of BAT in clinical trials of emerging food allergy therapeutics are additional reasons for pursuing agency approval for this test. Establishing BAT as a platform to test many food allergens and the standardization of the reagents and food antigens used in this assay will improve patient care as well as research in food allergies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

OA, GM contributed to conception and design of the study. OA and GM wrote the first draft of the manuscript. RW, TK, AD, AS, DJ, DM, HL, LI, MR, EP, DS, MO'C, MP, KR, CP, SO'R, and SS wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved manuscript version.

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Conflict of interest

SO'R was employed by New Columbia Capital, LLC. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Towards standardizing basophil identification by flow cytometry

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Background: Basophils normally make up <2% of the white blood cells (WBC). There is no clear consensus for basophil identification by flow cytometry. The increased demand for basophil activation test (BAT) to identifying and monitoring allergic patients highlights the need for a standardized approach to identify basophils.
Methods: Using flow cytometry we analyzed whole blood stained with antibodies against: IgE, CD123, CD193, CD203c, CD3, HLADR, FcεRI, CRTH2 and CD45. We examined unstimulated blood as well as blood stimulated with Anti-IgE and fMLP. Finally, we compared the results to a complete blood count (CBC) from an FDA approved hematological analyzer.
Results: Basophil identification relying on just one surface marker performed worse than approaches utilizing two identification markers. The percentage of basophils from WBC determined by flow cytometry results had a good correlation with the CBC results even though the CBC results were generally higher. Stimulating whole blood with the basophil activators did not interfere with the basophil identification markers.
Conclusion: In flow cytometry assays, two surface markers should be used for identifying basophils and if a very pure basophil fraction is desired a third marker can be considered. In our hands the approaches that included CD123 in combination with either CD193, HLADR^{negative} or FcεRI performed the best.

KEYWORDS

basophil activating test (BAT), basophil identification markers, basophil actication test, CBC (complete blood count), flow cytometry

Introduction

The basophils make up <2% of the white blood cells (WBC) in healthy individuals. Historically basophils have not been given much attention. The rapid development in flow cytometry combined with the discovery that CD63 is translocated to the basophil surface after activation lead to the development of the flow cytometry-based basophil activation test (BAT) and has revitalized the interest in basophils (1–5). The BAT is increasing in popularity because it offers a safe alternative to the oral food challenge when diagnosing food allergy (6). Measuring CD63 by flow cytometry has been established as the best marker for basophil activation in the BAT assay. Basophil identification is done with a varsity of markers and no clear standard has been established. It is possible to identify basophils using only one surface marker combined with FSC/SSC but these methods do not always give the best result (7–13). Many clinical and research laboratories use a combination of markers to identify basophils including but not limited to: CD123$^+$/CD193$^+$, CD123$^+$/HLADR$^-$, CD3$^-$/CD193$^+$, CD3$^-$/CRTH2$^+$, CD193$^+$/CD203c$^+$ (3, 6, 8, 12, 14–16). In the BAT, basophils go through various stimuli such as anti-IgE and N-Formylmethionyl-leucyl-phenylalanine (fMLP) which are also considered as positive controls for this test, as well as different allergens that are being tested. These stimulations have been reported to affect the expression of several basophil identification markers including CD203c (increased expression), CD123 and CD193 (reduced

expression) (16–20). Furthermore, it might not always be possible to process a sample immediately which makes it critical that the identification markers that are chosen to be used in this test are stable *in vitro*. In this study we stain whole blood with an antibody cocktail containing all the above-mentioned antibodies and analyze them by flow cytometry. The aim of this study is to compare the different methods of identifying basophils by flow cytometry. We compare the impact of different gating methods. Furthermore, we compare the expression of the markers after activation with anti-IgE antibody and fMLP. We assessed the post collection stability of the identification markers. Finally, we compare the result for % basophils of WBC with the result of a CBC test obtained using an FDA approved hematology analyzer.

Methods

Donors

All clinical investigations were conducted according to Declaration of Helsinki principles. All human studies were approved by the Western Institutional Review Board (IRB 1285028). A total of 79 donors, age range 5 to 87, were utilized for the study. The donors were recruited from patients and employes at Amerimmune LLC, CBC was done by Quest Diagnostics using the FDA approved Sysmex XN11 automated hematology analyzer (Kobe, Japan).

Basophil phenotyping

Whole blood was collected in a heparin and an EDTA tube. The EDTA tube was used for external CBC analysis. The heparin tube was kept at 18–25°C. Basophil identification was done using unstimulated blood (PBS) as well as blood stimulated with either Anti-IgE-FITC (Thermo Fisher, Waltham, MA) or fMLP (Sigma, St. Louis, MO). The samples were incubated for 20 min at 37°C followed by 10 min at 4°C (4, 14, 21–24). Each sample was stained with the following antibodies anti-IgE-FITC (Clone Ige21), anti-CD193-PE (Clone 5E8), anti-CD123-PerCPCy5.5 (Clone 6H6), anti-CD203c-PECY7 (Clone NP4D6), anti-CRTH2-APC (Clone BM16), anti-CD3-AF700 (Clone UCHT1), anti-CD45-EF506 (Clone HI30) anti-FcεRI-SB600 (Clone AER-37) (all Thermo Fisher, Waltham, MA) and anti-HLADR-Pacific blue (L243) (Biolegend, San Diego, CA) for 30 min at 4°C. Each antibody was titrated to obtain the best separation (25). The red blood cells were lysed using BD FACS lysis solutions (BD Bioscience, San Jose, CA) and resuspended in 400 µl PBS before acquisition.

Instrumentation

The samples were acquired on a 3 laser/10 color BD FACSCanto. CS&T beads (BD Bioscience, San Jose, CA) were acquired daily to ensure consistent performance of the cytometer. The instrument has been CAP and CLIA validated for clinical diagnostic studies. All samples were acquired for 5 min at the highest acquisition speed setting.

Data analysis

Data analysis was performed using FCS Express software (De Novo software, Glendale, CA). The gating strategy is to gate on singlets using FSC-A/FSC-H plot. A CD45/SSC plot is used to identify WBC. This is followed by an FSC/SSC gate to gate out eosinophils and majority of neutrophils. The basophils are subsequently identified in 13 different plots: CD123/CD193, IgE/FcεRI, HLADR/CD123, CD203c/FcεRI, FcεRI/CD193, CD3/CD294, CD3/CD193, FcεRI/CD193, CD123/FcεRI, IgE/SSC, FcεRI/SSC, CD193/SSC and CD203c/SSC (**Figure 1** and **Table 1**). The % basophil result is the gated population in each of these plots as percentage of the WBC population.

Graphs were generated as scatter plots, and statistical analysis was performed using GraphPad Prism. All data comparisons were analyzed as paired, two tailed, two-sample unequal variance using the students *t*-test to determine significance. A *p*-value less than 0.05 is considered significant, $^{*}p < 0.05$, $^{**}p < 0.01$. Correlation and Bland-Altman analysis and plots were performed using GraphPad Prism (32, 33).

Results

Basophil marker stability

Recent published papers by us and other groups show that the BAT is stable up to 28 h post collection (14, 22). The efficacy of this assays as well as other flow cytometry assays involving basophil identification is dependent on stable expression of the chosen markers on the basophils. **Table 1** summarizes the different markers and parameters utilized in the study, and the gating strategy is shown in **Figure 1**.

We started by testing the stability of the different gating strategies to see if the expression of the markers that help identify basophils would change over time. Whole blood was collected in heparin tubes and the expression of the markers were measured by flow cytometry 0–4 h post collection and again after 22–26 h. The blood was stored at room temperature (18–25°C).

The results show a slight reduction in the % basophils to 89%–92% at Day 1 compared to Day 0. The absolute number of basophils collected dropped to 83%–87% of the value at Day 0. The results are very similar for all the tested gating combination with no method detecting a significant different percentages or absolute number of basophils (**Figure 2**). Based on this experiment we conclude that we can accurately evaluate the percentages of basophils within 22–26 h post collection.

Frequency of problematic gating

We examined if it was possible to gate on a distinct basophil population with all gating approaches in all the samples. We recorded the instances where the basophil population does not

10.3389/falgy.2023.1133378

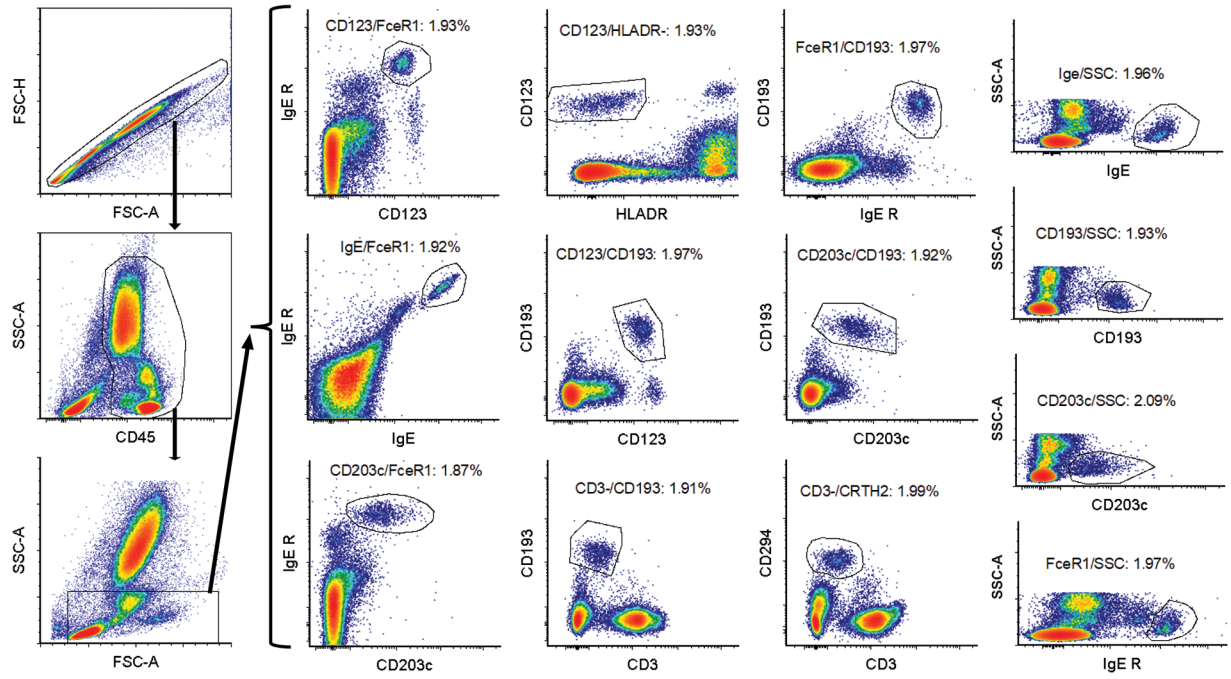

FIGURE 1
Gating strategy for basophil identification. First an FSC-A/FSC-H gate was used to gate on singlets. Subsequently a CD45/SSC gate was drawn around the white blood cells. This was followed by an FSC-SSC gate to gate out some non-basophils. The basophils were then identified using 13 different gating strategies: CD123/CD193, IgE/FcεRI, HLADR/CD123, CD203c/FcεRI, FcεRI/CD193, CD3/CD294, CD3/CD193, FcεRI/CD193 and CD123/FcεRI, IgE/SSC, FcεRI/SSC, CD193/SSC, and CD203c/SSC.

TABLE 1 Basophil identification markers and flow cytometry parameters used to identify basophils in whole blood samples.

	Marker	Cell expression	Description and gating strategy	References
Basophil identification markers expressed on the cell surface	CD123	Basophils, eosinophils, dendritic cells,	Highly expressed on basophils. Is normally used in combination with another basophil marker or HLADR^{negative}.	(8, 17, 26)
	CD193	Basophils, eosinophils, mast cells, Th2 lymphocytes	Solid marker for basophil identification. Have been used alone, in combination with other basophil markers or CD3^{negative}.	(8, 10, 12, 27)
	CD294 (CRTH2)	Basophils, eosinophils, T-lymphocytes	Basophils can be differentiated from T-lymphocytes by CD3 and from eosinophils by side scatter	(28, 29)
	IgE	Basophils, monocytes, dendritic cells	Expressed as both a soluble molecule and bound to the FcεRI on basophils.	(9, 13, 30)
	FcεRI	Basophils, mast cells, dendritic cells, monocytes in patients with allergic disorder	Bound to IgE. Crosslinking receptors with a relevant allergen of anti-IgE activates the basophil.	(7, 26)
	CD203c	Basophils, CD34+ progenitor cells, mast cells	Used as both an identification and an activation marker for basophils. Is expressed at low levels on resting basophils.	(31)

form a separate population and the instances where it was impossible to identify a basophil population at all. Our results show that CD123/CD193 and CD123/HLADR^{-} gave a clear separate population in all analysis. CD203c/FcεRI, CD193/FcεRI and CD123/FcεRI worked for all but one donor. In the other end of the spectrum are IgE/SSC, FcεRI/SSC and CD3^{-}/CDTH2 which frequently could not detect any basophils or often did not give a clear separate population (Table 2). In the patients where it was impossible to identify basophils at all with a given gating combination then the specific combination from that patient was removed from the subsequent analysis.

Correlation between methods the different flow-based methods

Next, we used linear correlation to see if the % basophils of WBC results are similar using the different gating approaches. The R

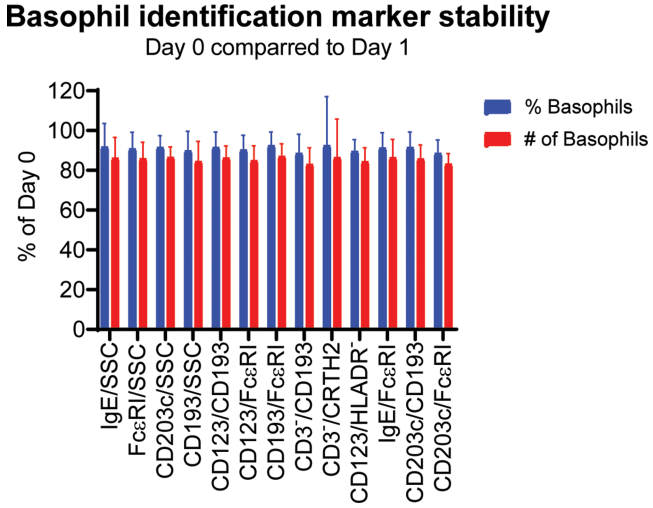

FIGURE 2
Stability of the different basophil identification markers. Whole blood from 9 donors was collected in heparin tubes and the expression of the markers were measured by flow cytometry after 0–4 h (Day 0) and again after 22–26 h (Day 1) using the gating strategy shown in Figure 1. The blood was stored at room temperature (18–25°C). The % basophils of WBC as well as the total number of basophils identified were measured. The results for Day 0 are set to 100% and the results for Day 1 are normalized accordingly. Student's *t*-test paired, $n = 9$.

TABLE 2 Problematic basophil identification.

Issue	Impossible to gate on basophils			Basophils form a shoulder rather than a detached population		
Stimulation	PBS	Anti-IgE	fMLP	PBS	Anti-IgE	fMLP
IgE/SSC	10	10	10	0	0	0
FcεRI/SSC	7	14	6	7	8	6
CD203c/SSC	1	1	1	3	2	1
CD193/SSC	1	0	1	3	5	8
CD123/CD193	0	0	0	0	0	0
CD123/FcεRI	0	0	0	0	1	0
CD193/FcεRI	1	0	0	2	7	4
$CD3^-$/CD193	2	0	0	5	8	9
$CD3^-$/CRTH2	3	3	2	10	11	14
$CD123/HLADR^-$	0	0	0	0	0	0
IgE/FcεRI	1	1	1	5	3	3
CD203c/CD193	1	1	1	2	1	3
CD203c/FcεRI	0	0	0	1	0	1

The table show the numbers of samples where it was not possible to identify a basophil population as well as the number of samples where the identification can be prone to error because the basophils does not form a separate population but rather a shoulder on the negative population.

square value ranges from 0.69 (FcεRI/SSC vs. FcεRI/IgE) to 0.99 (IgE/SSC vs. CD123/CD193; Ige/SSC vs. CD123/$HLADR^-$; IgE/SSC vs. IgE/FcεRI; and CD123/CD193 vs. CD123/$HLADR^-$). To easier appreciate the performance of each gating strategy we calculated the average for the R square values for each gating strategy, The average R square ranking are (high to low): IgE/SSC, CD123/CD193, CD123/FcεRI, CD123/$HLADR^-$ and CD203c/CD193 > CD203c/FcεRI > IgE/FcεRI > CD203c/SSC, CD193/FcεRI, $CD3^-$/CRTH2 > CD193/SSC > $CD3^-$/CD193 > FcεRI/SSC. The graphs are all shown in **Supplementary Figure S1**. The values for the *R* square and the slope are summarized in **Table 3**.

Specificity and inclusiveness of the different gating strategies

An essential question is if the basophils identified by one gating strategy will also be identified as basophils by the other gating strategies. We addressed this by displaying the basophils obtained by one gating strategy in each of the 12 other strategies and recording the percentages of the cells that were then identified as basophils. The individual box plots are shown in **Supplementary Figure S2** and the mean values are shown in **Table 4**. The highest specificity defined as the gating method where most of the identified basophils were also identified as basophils in the 12 other approaches is $CD123/HLADR^-$ followed by (high to low, as shown in the bottom row in **Table 4**): CD123/CD193 > IgE/SSC > CD203c/CD193 > CD123/FcεRI > CD193/FcεRI > IgE/FcεRI > CD203c/FcεRI > CD193/SSC > $CD3^-$/CD193 > $CD3^-$/CRTH2 > CD203c/SSC > FcεRI/SSC. This ranking shows how pure the initial captured basophil population is. The summary column on the far right of the table shown the inclusiveness, defined as how well the different gating options are at capturing all the basophil events with the best being IgE/SSC followed by (high lo low): $CD123/HLADR^-$ > CD193/FcεRI > CD123/CD193 > CD123/FcεRI > $CD3^-$/CD193 > CD193/SSC > CD203c/CD193 > IgE/FcεRI > FcεRI/SSC > $CD3^-$/CRTH2 > CD203c/FcεRI > CD203c/SSC.

Comparing flow cytometry results to CBC

A CBC with differential test is a well-established method that among other results provides the percent basophils of whole blood. For each donor we collected a tube for CBC analysis at an external reference laboratory. The CBC were all run within 24 h of sample collection on an FDA approved hematology analyzer. The linear correlation analysis between the CBC and each of the flow methods show some degree of correlation with an R square value between 0.59 to 0.8. The highest being IgE/SSC, CD193/SSC, CD123/CD193 followed by CD123/FcεRI, CD123/HLADR- > IgE/FcεRI, CD203c/FcεRI > CD203c/CD193 > CD193/FcεRI > CD203c/SSC > CD3-/CRTH2 > FcεRI/SSC. The sloop is between 0.74–0.84 indicating that the flow values generally are lower than the CBC values. To better understand the difference between the methods we visualized the data in two different Bland-Altman plots, Difference vs. Average and Ratio vs. Average (32, 33). The results for all flow methods except FcεRI/SSC show that the CBC systematically are higher than the flow cytometry results. The most pronounced difference is for CBC results between 0.3%–1.3% basophils but even for the higher values the CBC result is higher. The Ratio vs. Average plot show that the CBC values are generally higher by 40%–50% percentages rather than fixed value. The

TABLE 3 Linear correlation between different flow methods.

		R square													
		IgE/ SSC	FcεRI/ SSC	CD203c/ SSC	CD193/ SSC	CD123/ CD193	CD123/ FcεRI	CD193/ FcεRI	$CD3^-$/ CD193	$CD3^-$/ CRTH2	CD123/ $HLADR^-$	IgE/ FcεRI	CD203c/ CD193	CD203c/ FcεRI	Average R square
Slope	IgE/SSC	-	0.73	0.94	0.92	0.99	0.98	0.91	0.95	0.92	0.99	0.99	0.96	0.97	0.94
	FcεRI/SSC	0.96	-	0.72	0.75	0.74	0.79	0.81	0.79	0.78	0.74	0.69	0.79	0.74	0.75
	CD203c/ SSC	0.92	0.72	-	0.88	0.94	0.93	0.88	0.91	0.88	0.94	0.90	0.93	0.94	0.90
	CD193/ SSC	0.97	0.72	0.99	-	0.95	0.93	0.86	0.90	0.85	0.93	0.89	0.92	0.88	0.89
	CD123/ CD193	0.97	0.74	0.99	0.95	-	0.98	0.91	0.95	0.92	0.99	0.95	0.97	0.96	0.94
	CD123/ FcεRI	0.98	0.78	1.01	0.96	1.01	-	0.96	0.95	0.94	0.98	0.93	0.97	0.95	0.94
	CD193/ FcεRI	0.96	0.80	0.98	0.92	0.97	0.99	-	0.91	0.91	0.91	0.89	0.93	0.91	0.90
	$CD3^-$/ CD193	0.98	0.79	1.00	0.94	0.99	0.98	0.96	-	0.94	0.95	0.92	0.98	0.94	0.85
	$CD3^-$/ CRTH2	0.94	0.77	0.88	0.90	0.96	0.95	0.93	0.95	-	0.93	0.89	0.93	0.91	0.90
	CD123/ $HLADR^-$	0.98	0.75	0.96	0.95	1.01	0.98	0.94	0.96	0.97	-	0.96	0.97	0.96	0.94
	IgE/FcεRI	0.97	0.73	0.98	0.93	0.99	0.96	0.93	0.94	0.96	0.98	-	0.93	0.94	0.91
	CD203c/ CD193	0.95	0.76	0.98	0.92	0.97	0.96	0.93	0.96	0.96	0.96	0.94	-	0.96	0.94
	CD203c/ FcεRI	0.91	0.72	0.95	0.87	0.93	0.91	0.89	0.90	0.91	0.93	0.91	0.94	-	0.92

A summary of the linear correlation analysis between the different flow-based basophil identification methods shown in Supplementary Figure S1. Light red background are the R square values, and the light blue background is the slope. The average R square values are the average of the 12 *R* square values where the gating combination on the right is involved.

10.3389/falgy.2023.1133378

TABLE 4 Linear correlation between different flow methods.

		Initial gate													
		IgE/ SSC	FcεRI/ SSC	CD203c/ SSC	CD193/ SSC	CD123/ CD193	CD123/ FcεRI	CD193/ FcεRI	$CD3^-$/ CD193	$CD3^-$/ CRTH2	CD123/ $HLADR^-$	IgE/ FcεRI	CD203c/ CD193	CD203c/ FcεRI	Average (Inclusiveness)
% Back-gated	IgE/SSC		74	81	88	94	94	93	86	80	96	98	91	91	88.8
	FcεRI/SSC	88		75	80	83	86	84	77	72	84	88	81	86	82.0
	CD203c/SSC	82	65		78	81	81	79	74	70	83	79	83	87	78.5
	CD193/SSC	90	70	78		93	88	91	88	79	91	86	91	85	85.8
	CD123/ CD193	92	71	80	90		92	92	87	81	95	89	92	88	87.4
	CD123/ FcεRI	94	73	80	86	93		91	84	79	95	92	89	91	87.3
	CD193/ FcεRI	92	73	78	89	94	92		88	81	92	90	92	90	87.6
	$CD3^-$/ CD193	88	69	76	91	93	88	91		83	90	86	94	85	86.2
	$CD3^-$/ CRTH2	81	65	71	80	84	82	82	82		84	80	84	79	79.5
	CD123/ $HLADR^-$	94	72	81	88	95	94	92	86	81		91	91	90	87.9
	IgE/FcεRI	92	73	75	81	86	88	87	80	76	88		84	87	83.1
	CD203c/ CD193	86	67	78	87	90	85	88	86	79	88	84		84	83.5
	CD203c/ FcεRI	84	68	78	77	82	84	82	75	71	83	85	84		79.4
	Average (Specificity)	88.6	70.0	77.6	84.6	89.0	87.8	87.7	82.8	77.7	89.1	87.3	88.0	86.9	

A summary of the linear correlation analysis between the different flow-based basophil identification methods shown in Supplementary Figure S2. Last row with light red background is the average of the 12 values in the column and is a measurement of how specific the gate is for basophils when compared to the 12 other gating methods. The last blue column is the average of each row is the inclusiveness which represents how big percentages of the basophils fall within the given gate.

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TABLE 5 Correlation between the different flow cytometry methods of identifying basophils and the results of a CBC obtained using the XN11 automated hematology analyzer at an accredited reference laboratory.

	Correlation analysis		Bland-Altman analysis (Difference vs. Average)		Bland-Altman analysis (Ratio vs. Average)	
	R square	Sloop	Bias	SD	Bias	SD
IgE/SSC	0.80	0.82	0.14	0.19	1.5	0.8
FcεRI/SSC	0.59	0.84	0.02	0.29	1.2	0.6
CD203c/SSC	0.71	0.74	0.14	0.23	1.4	0.6
CD193/SSC	0.80	0.83	0.13	0.18	1.4	0.6
CD123/CD193	0.80	0.81	0.14	0.18	1.5	0.8
CD123/FcεRI	0.79	0.81	0.13	0.19	1.5	0.8
CD193/FcεRI	0.74	0.80	0.13	0.21	1.5	0.8
$CD3^{-}$/CD193	0.73	0.79	0.11	0.22	1.4	0.8
$CD3^{-}$/CRTH2	0.67	0.74	0.13	0.24	1.4	0.6
CD123/$HLADR^{-}$	0.79	0.81	0.14	0.19	1.5	0.8
IgE/FcεRI	0.78	0.81	0.15	0.19	1.6	0.9
CD203c/CD193	0.77	0.78	0.15	0.20	1.5	0.9
CD203c/FcεRI	0.78	0.76	0.18	0.19	1.6	0.8

The graphs are shown in Supplementary Figure S3.

results for the FcεRI/SSC gating method the diversion between flow cytometry and CBC seems more of a random nature (Table 5 and Supplementary Figure S3).

Identification of activated basophils

The BAT involves stimulating whole blood with positive controls that activates the basophils in both a IgE/FcεRI dependent and independent manner. The activation of basophils might affect the expression of the identification markers.

We stimulated whole blood with Anti-IgE-FITC or fMLP before staining for basophil identification and compared it to unstimulated blood (PBS control) to see if stimulation affects our ability to identify basophils.

We observed that fMLP activation decreases CRTH2 expression slightly. Anti-IgE activation reduce the FcεRI signal. The antibody used for stimulation was the same used for detection which result in an increase in IgE signal after Anti-IgE stimulation. Both anti-IgE and fMLP activation increases CD203c expression. The rest of the markers were unaffected by stimulation (Supplementary Figure S4).

Next, we performed a linear correlation to determine if the percentages of basophil are similar before and after stimulation. The results show an R square above 0.95 for Anti-IgE and fMLP for IgE/SSC, CD123/CD193, CD123/FcεRI, CD193/FcεRI, and CD123/$HLADR^{-}$. The lowest correlation with an R square below 0.9 was seen for FcεRI/SSC, CD193/SSC, CD193/CD3-, CD294/CD3- and CD203c/CD193 (Figure 3A).

Finally, we compared the absolute number of basophils identified to show whether basophil events are gained or lost by stimulating in any of our basophil identification approaches. The results are similar in stimulated and unstimulated samples (Figure 3B).

Discussion

In this study we examined 13 different gating strategies for basophil identification in a whole blood by flow cytometry. For each gating strategy we looked at (1) Reliability/easiness of gating, (2) Specificity vs. inclusiveness, (3) Correlation between the different approaches, (4) Correlation with CBC results, (5) Stability of the markers, (6) Effect of stimulation.

All the strategies used in this paper to identify basophils utilizing published markers and marker combinations. We started by examining if we get similar results between the different methods. The linear correlation analysis showed the highest correlation between gating strategies utilizing two surface markers unless one of the markers is $CD3^{negative}$ in which case the correlations were low. The single surface markers approaches did not perform well except for IgE/SSC that was among the best. It is worth noting that for 10 of the samples IgE/SSC could not identify any basophils at all. All these patients have elevated levels of circulating IgE that will compete with the surface bound IgE for anti-IgE antibody binding. This approach does not provide us information on the extent the gates captures all the basophils or how basophil specific they are. To understand which strategies are best at capturing the highest percentages of basophils with the least contamination we investigated to what extend a population identified by a given gate would also be identified as basophils using the other gating strategies. The results of this approach are very similar to what we saw in our initial correlation analysis, and it confirms that best gating strategies utilizing two surface markers unless one of the markers is $CD3^{negative}$. It is important to remember that this form of comparison has a bias towards higher specificity and inclusiveness when the same marker is present in both strategies such as comparing CD123/$HLADR^{negative}$ vs. CD123/CD193 or IgE/SSC vs. IgE/FcεRI.

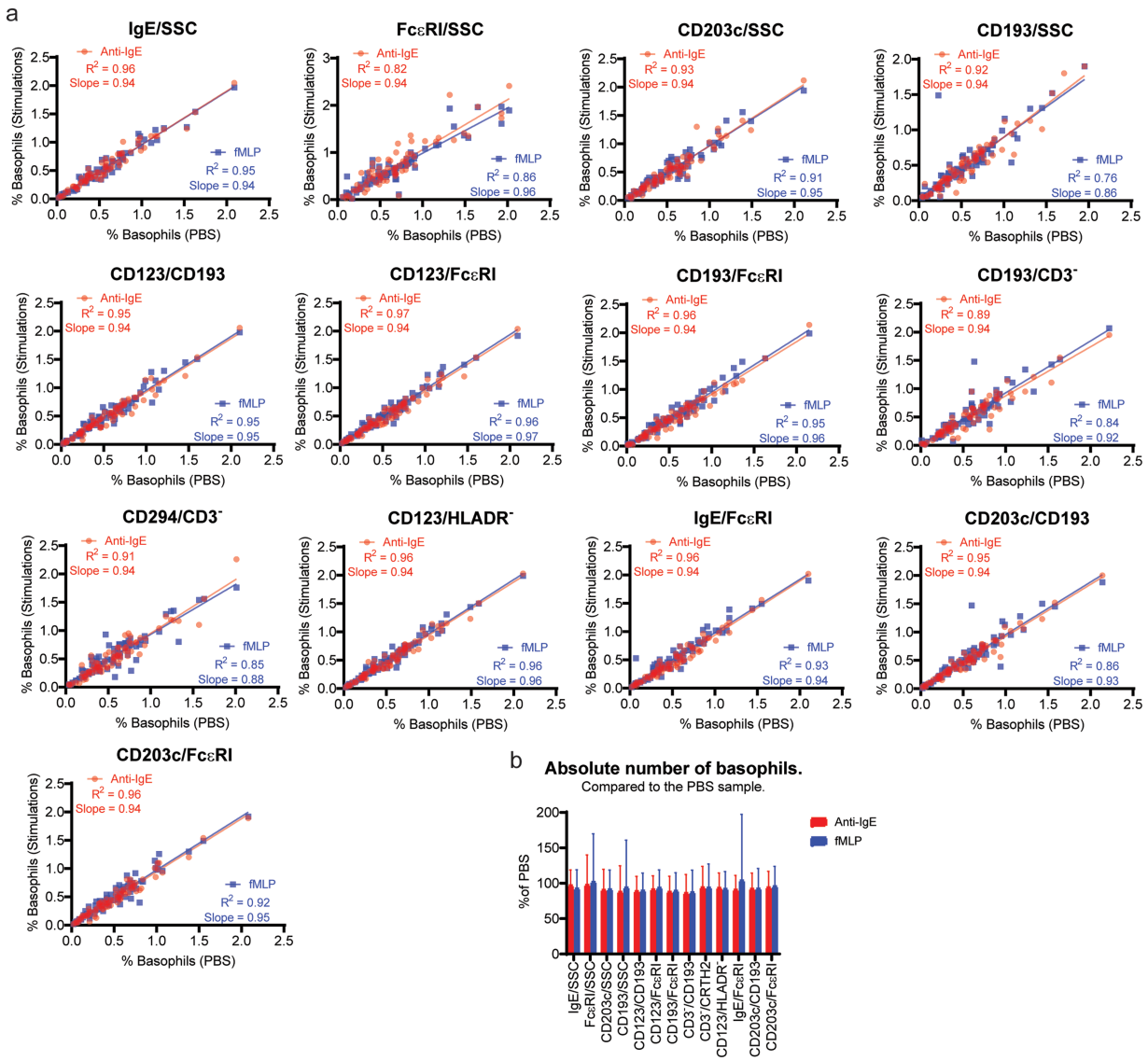

FIGURE 3
Identification of activated basophils. Whole blood was stimulated with either Anti-IgE FITC or fMLP for 20 min before staining for basophil identification together with the unstimulated control sample (PBS). (A) Linear regression analysis shows the comparison between the PBS and Anti-IgE FITC or fMLP for each of the 13 identification methods. The basophils number shown is as % of WBC. (B) The absolute number of basophils collected in the Anti-IgE and fMLP stimulated sample using the different gating strategies are shown as percentages of the absolute number of basophils in the PBS sample.

A selection of hematolytic analyzers is approved by FDA and used at almost all reference laboratories. No flow method for identifying basophils has been FDA approved and we tested to see how well the two approaches compare. Several other studies have compared % basophils from a CBC with flow cytometry and each time the correlation is mediocre with the best *R* square value at 0.68 (8, 27, 34). The correlation in our experiments has an *R* square ranging from 0.59 to 0.80 with seven of our gating strategies having an R square of at least 0.75. The slope ranged from 0.74 to 0.84 showing that the flow result was generally lower than the CBC result. This was confirmed by the Bland Altman plot that showed the CBC to be 40%–50% higher than the flow results with the difference being lower as the basophil percentages increase. The fact that basophil count in a CBC can be overestimated rather than underestimated especially for when the basophil percentages is in or below the normal range has been described previously (34–36).

It has, for the longest time, been a dogma that basophils were unstable cells and that the BAT should be run within 4 h of collecting the sample. Recently studies have shown that the BAT results are stable up to 1 day (20–28 h) post collection (14, 22). Our results show that no matter which gating strategy is used the percentage and absolute number of basophils identified in a 22–26 h old sample is very similar to what can be identified within 4 h post collection. An essential part of the BAT is the stimulation. We did not see any systematically drop in the identified basophils for any of the gating strategies after stimulation with either fMLP or anti-IgE showing that the markers are not shredded or internalized after stimulation to an extend that makes utilization impossible. The gating strategies with the lowest correlation between

stimulated and control samples are also the strategies where there were most instances of difficult to identify basophils due to the population being an attached rather than a separate population (Table 2). The strategies with few or no instances of shoulder population had both R square and sloop values very close to 1.

A recurring dilemma in designing a BAT flow panel is on its simplicity and cost-effectiveness. We included CD45 because it is essential for a good WBC gate. We would recommend including it rather than relying solely on FSC and SSC to narrow in on the basophil population.

We do not recommend relying on just one marker for identifying basophils as those strategies did not perform very well in our study. Three of them were not very precise or accurate. IgE/SSC works very well when it does not completely fail which it did more than 13% of the time. Among the gating strategies utilizing two surface markers $CD3^{-}$/CRTH2 and $CD3^{-}$/CD193 performed the worst with respect to inclusiveness and specificity. The IgE/FcεRI gate has too many instances difficult/impossible to gate. This is typically observed in individuals where the IgE staining does not work. The CD193/FcεRI approach also have some instances of difficult to gate issues, especially after anti-IgE stimulation, even thou the specificity and inclusiveness is among the best. Not having a well-defined population can cause variation between technologists analyzing the samples and problems if using automatic gating. CD203c expression is low in resting cells and increase after basophil stimulation. This can cause the gate used for identification to shift between samples. Our identification was mainly done on resting cells and for both CD203c/CD193 and CD203c/FcεRI we saw a high specificity but the inclusiveness we low reflecting that is a problem capturing all the basophils. The remaining three approaches, CD123/CD193; $CD123/HLADR^{negative}$; and CD123/FcεRI all performs as the best in all our tests, and we recommend choosing one of these. If the flow panel allows for one more parameter, it is possible to combine two methods such as CD123/CD193 and $CD123/HLADR^{negative}$. All three methods include CD123 as one of the parameters and CD123 expression has been reported to be reduced after stimulation (12, 17). This statement has been rebuked by others (37–39). We did not see any reduction in CD123 after stimulation in our experiments. CD123 gave a clear separation in all our experiments but there might be instances where it is not the case, and it will make gating that includes it impossible. Another approach could be to combine one of the CD123 strategies with IgE/SSC. This gate has a very high inclusiveness and specificity but should be excluded from the analysis when a basophil population cannot be identified. If the flow panel allows for four markers to identify basophils it is possible to combine several of the approaches shown here. The advance of using more than one approach is that the gates can be more inclusive, and the specificity can at the same time be increased.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Western Institutional Review Board (IRB 1285028). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

SUS and OA contributed to project conceptualization. DL and OA coordinated the sample collection and project administration. SS and MP contributed to formal analysis. SU did the experimental work and wrote the original draft. All authors contributed to the article and approved the submitted version.

Conflict of interest

Author SUS, MP and DL are employed by Amerimmune. OA is the owner of Amerimmune. This study was funded by resources internally at Amerimmune. The funder (OA) had the following involvement with the study: Approved study design and the final manuscript. All authors declare no other competing interests.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/falgy.2023.1133378/full#supplementary-material.

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Case Report: A family history of peanut allergy and hereditary alpha-tryptasemia

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Context: Hereditary alpha-tryptasemia (HαT) is associated with elevated basal serum tryptase (bST) and is associated with a higher risk of severe anaphylactic reactions in patients with clonal mast cell disorders or IgE-mediated Hymenoptera venom-induced anaphylaxis. The consequence of this genetic trait remains to be determined in other allergic diseases and food allergy in particular.
Objectives: Here, we describe three cases of peanut allergy among siblings from a single family of four: two of them were associated with HαT, and the third one was associated with the tryptase wild-type genotype.
Methods: *TPSAB1/TPSB2* genotypes were determined by digital PCR. After the case description, we provided a review of the literature regarding bST levels and tryptase genotypes in anaphylaxis, with a particular focus on food allergy.
Results: Compared to the sibling with the conventional tryptase genotype, the two siblings with HαT presented a lower peanut threshold at the initial oral food challenge, higher peanut skin prick test reactivity, higher levels of specific IgE to peanut, Ara h 2, and Ara h 6, and a lower IgG4/IgE ratio after 10 years of oral immunotherapy.
Conclusion: The tryptase genotype and HαT status might modify the clinical presentation and biological features of food allergy.

KEYWORDS

food allergy, anaphylaxis, tryptase, hereditary alpha-tryptasemia, case report

Introduction

Elevated basal serum tryptase (bST) has been described as a risk factor for severe anaphylactic reactions, particularly in Hymenoptera venom anaphylaxis (1). One of the main determinants of bST levels is the tryptase genotype (2). In particular, hereditary alpha-tryptasemia (HαT) corresponds to additional copies of the TPSAB1 gene encoding α-tryptase and is associated with elevated bST levels, almost exclusively >8 µg/L (3). This genetic trait is present in about 5% of the population of Caucasian descent and is overrepresented among patients with clonal mast cell disorders including systemic mastocytosis (4). When associated with clonal mast cell disorders, HαT has been associated with a higher risk and severity of anaphylactic reactions to

hymenoptera venom (4, 5). In addition, among patients with severe Hymenoptera venom anaphylaxis or idiopathic anaphylaxis, HαT is also present at a higher rate than in the general population, even in the absence of overt mast cell clonal disorder (4).

In food allergy, alpha-tryptase-positive genotypes, in general, have been associated with more severe food reactions (6). Here, we describe three cases of peanut allergy among siblings from a single family of four, two of them were associated with HαT, and the third one was associated with a conventional α-tryptase-positive genotype.

Case description

We report three cases of peanut allergy among siblings from a family of four children. Two of them were associated with HαT. The mother reported atopy but no food allergy. The father and the remaining daughter did not report any history of allergic disease.

Patient A

The 31-year-old brother (patient A) presented with a history of atopic dermatitis, remitting asthma, and allergic rhinitis to house dust mites, cats, and birch and grass pollens during childhood. In his childhood, he also experienced abdominal pain and vomiting after drinking soymilk and oral pruritus and abdominal pain after eating pasta containing cashew nuts. However, he can now consume bean curd, stem beans, and cashew nuts without experiencing any hypersensitivity reactions. In addition, he had reactions to peanuts during childhood.

In 2011, at age 20, he underwent an oral food challenge (OFC) to peanuts, which revealed a positive result at a threshold of 650 mg. Since then, he has been receiving peanut oral immunotherapy (OIT), currently with six peanut M&M's 3 days per week. He once ate 15 peanuts without experiencing any hypersensitivity reactions. In 2018, peanut prick tests were positive for native roasted peanuts (7 mm wheal; histamine positive control: 4 mm). In 2015, the total serum IgE level was 124 kUI/L. In 2019, polysensitization to peanut molecular allergens was found, with a serum peanut-specific IgE level of 39.5 kU_A/L, a serum Ara h1-specific IgE level of 23.6 kU_A/L, a serum Ara h2-specific IgE level of 13.9 kU_A/L, a serum Ara h3-specific IgE level of 9.36 kU_A/L, and a serum Ara h6-specific IgE level of 16 kU_A/L. Biological results after 10 years of oral immunotherapy are summarized in Table 1. In 2021, the serum peanut-specific IgG4/IgE ratio was 24.2, the serum Ara h2-specific IgG4/IgE ratio was 105.1, and the serum Ara h6-specific IgG4/IgE ratio was 83.7. Sensitization to soy molecular allergens was also investigated, with a serum soy-specific IgE level of 3.18 kU_A/L, a serum Gly m4-specific IgE level of 5.91 kU_A/L, a serum Gly m5-specific IgE level of 0.96 kU_A/L, and a serum Gly m6-specific IgE level of 4.61 kU_A/L. Of note, the serum lupine-specific IgE level was 1.19 kU_A/L.

Recently, he experienced three exercise-induced anaphylaxis-like reactions soon after peanut consumption: one with urticaria, one with asthma and urticaria, and then one after climbing stairs with abdominal pain and flush, which improved within 4 h with anti-histamine and corticosteroid treatment. Percritical serum tryptase levels at the time of reactions were not investigated.

TABLE 1 Clinical and biological characteristics of patients after 10 years of peanut oral immunotherapy.

Patient	A	B	C
Peanut anaphylaxis	Yes	Yes	Yes
Initial peanut OFC threshold (mg)	650	1,000	1,500
Basal serum tryptase (μg/L)	14.3	9.5	3.9
Tryptase genotype *TPSAB1/TPSB2*	ααβ:ββ	ααβ:ββ	αβ:ββ
Hereditary alpha-tryptasemia	Yes	Yes	No
KIT D816V mutation in peripheral blood (>0.01%)	No	No	No
Peanut OIT maintenance dose (3 per week)	6	4–5	5–7
Peanut skin prick test (wheal in mm)	12	12	6
Peanut-specific IgE (kU_A/L)	38.3	30.3	0.71
rAra h 2-specific IgE (kU_A/L)	11.8	27.6	0.57
rAra h 6-specific IgE (kU_A/L)	16	30.4	0.9
Peanut-specific IgG4/IgE ratio	24.2	18.4	163.0

The basal serum tryptase level was 14.3 μg/L. Tryptase genotyping of *TPSAB1* and *TPSB2* was performed using droplet digital PCR, as described by Lyons et al. (3). Patient A presented HαT with *TPSAB1* duplication and ααβ:ββ genotype (Figure 1). Research of *KIT* D816V mutation on peripheral blood by digital PCR was found negative.

Patient B

The 25-year-old sister (patient B) presented with a history of atopic dermatitis, persistent asthma, and allergic rhinitis with sensitization to house dust mites, cats, and birch and grass pollens. In 2005, at 9 years old, she presented with airborne conjunctivitis, labial angioedema, and pruritus after carrying peanuts and nuts. In 2011, an oral food challenge to peanuts revealed positive results at a threshold of 1,000 mg. In April 2012, after eating half a pad Thai on top of which she had

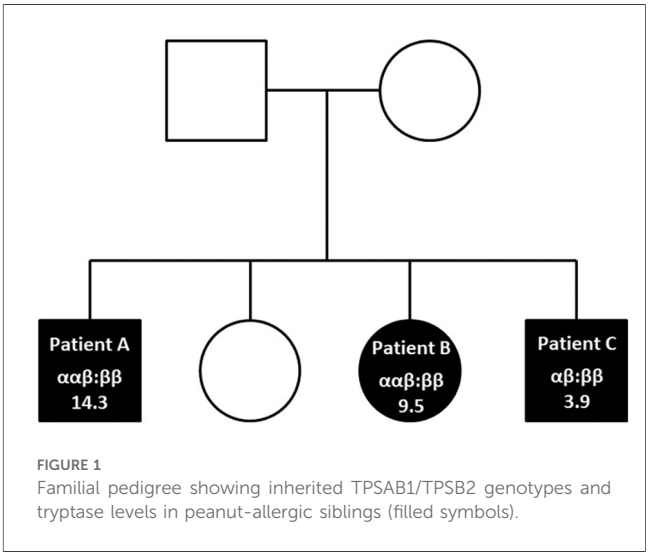

FIGURE 1
Familial pedigree showing inherited TPSAB1/TPSB2 genotypes and tryptase levels in peanut-allergic siblings (filled symbols).

removed peanuts, she presented with labial and pharyngeal pruritus, abdominal pain, and vomiting. Since 2011, patient B has been receiving peanut oral immunotherapy with 4–5 peanut M&M's 3 days per week, as well as budesonide/formoterol 400 b.i.d., montelukast 10 mg once a day, ebastine once a day, and azelastine 1,000 µg/fluticasone propionate 365 µg b.i.d.

In 2016, peanut prick tests were positive for native roasted peanuts (8 mm wheal; histamine positive control: 3 mm). In 2019, polysensitization to several peanut molecular allergens was found, with a serum peanut-specific IgE level of 54.9 kU_A/L, a serum Ara h1-specific IgE level of 4.73 kU_A/L, a serum Ara h2-specific IgE level of 38 kU_A/L, a serum Ara h3-specific IgE level of 1.84 kU_A/L, and a serum Ara h6-specific IgE level of 41.4 kU_A/L. In 2021, after 10 years of oral immunotherapy, the serum peanut-specific IgG4/IgE ratio was 18.4, the serum Ara h2-specific IgG4/IgE ratio was 16.6, and the serum Ara h6-specific IgG4/IgE ratio was 20.1. Of note, the serum lupine-specific IgE level was 14.4 kU_A/L. Other biological results after 10 years of oral immunotherapy are summarized in Table 1.

The basal serum tryptase level was 9.5 µg/L. Tryptase genotyping revealed HαT with *TPSAB1* duplication and αα**β**:**β**β genotype (Figure 1). Research of *KIT* D816V mutation in peripheral blood was found negative.

Patient C

The 23-year-old brother (patient C) presented with a history of remitting asthma and allergic rhinitis. After reactions to peanuts during childhood, an oral food challenge was performed in 2011 and revealed positive at a threshold of 1,500 mg. Oral immunotherapy was initiated and is still ongoing. Patient C is now receiving peanut oral immunotherapy with 5–7 peanut M&M's once a week. In 2014, after a 3-month discontinuation of a 20-mg-peanut-dose oral immunotherapy, he presented with abdominal pain after peanut exposure, which was treated with corticosteroids and phloroglucinol, and resumed oral immunotherapy. Later, he reported having eaten once 10 peanuts without allergic reactions.

In 2018, peanut prick tests were positive with a 6-mm wheal for native roasted peanuts and a 5-mm wheal for histamine control. In 2019, sensitization to serum peanut molecular allergens was evaluated, with a serum peanut-specific IgE level of 1.16 $_A/L$, a serum Ara h1-specific IgE level of <0.10 kU_A/L, a serum Ara h2-specific IgE level of 0.80 kU_A/L, a serum Ara h3-specific IgE level of <0.10 kU/L and a serum Ara h6-specific IgE level of 1.21 kU_A/L. Biological results after 10 years of oral immunotherapy are summarized in Table 1. The total serum IgE level was 214.6 kU/L. The serum peanut-specific IgG4/IgE ratio was 18.4, the serum Ara h2-specific IgG4/IgE ratio was 16.6, and the serum Ara h6-specific IgG4/IgE ratio was 20.1.

The basal serum tryptase level was 3.9 µg/L. Consistent with tryptase levels, no HαT was found but the conventional α**β**:**β**β tryptase genotype (Figure 1). No cKit D816V mutation was found in peripheral blood.

Discussion

To the best of our knowledge, this is the first description of HαT in a family with peanut allergy. Interestingly, the two siblings with HαT presented a lower peanut threshold at the initial oral food challenge, higher peanut skin prick test reactivity, higher levels of specific IgE to peanuts, Ara h 2, and Ara h 6, and a lower IgG4/IgE ratio after 10 years of oral immunotherapy compared to the third sibling who displayed a conventional genotype.

Although limited to the description of siblings from a single pedigree, this study allows the unique comparison of HαT status and clinical or biological variates in individuals with similar parental history, overall genetic background, and personal history of anaphylaxis to the same food. All were treated with oral immunotherapy for the same duration, underwent serological evaluation at the same follow-up time point, and were evaluated by the same physician. No bone marrow studies were performed due to the absence of argument suggesting a clonal mast cell disorder, despite slightly elevated tryptase levels in two siblings, later explained by the presence of HαT. Evaluation of the *KIT* D816V variant allelic fraction in the peripheral blood was performed, but the result showed a negative finding.

Several studies have underlined the relationship between bST levels and the risk and severity of anaphylaxis in patients with Hymenoptera venom allergy (7–10), in children with food allergy (11, 12), and in adults with cofactor-dependent wheat allergy (13). Most conditions associated with higher bST levels, such as male gender, older age, cardiovascular conditions, or clonal mast cell disorders, are also risk factors for severe anaphylaxis (1, 7, 14). In the case of HαT, the vast majority of $HαT^+$ patients exhibit bST levels ≥8 µg/L, with some falling in the range between 6 and 8 µg/L (15). Initial studies revealed that bST levels in HαT follow a gene–dosage effect, meaning that a higher gene copy number is associated with higher bST levels (3). However, tryptase over-expression in HαT now appears mainly related to an enlarged over-active promoter element co-inherited with additional *TPSAB1* copies (2). In exceptional cases, individuals with numerous additional *TPSAB1* copies might even exhibit bST levels above 100 µg/L (2). In clonal mast cell disorders, increased HαT prevalence was consistently reported in several cohorts compared to the general population (4, 5, 16–18). In addition, there is a strong consensus that in clonal mast cell disorders, HαT is a modifier of the frequency and severity of anaphylaxis to hymenoptera venom and likely idiopathic anaphylaxis (4, 5, 16, 17). Conversely, the prevalence of HαT appears elevated in patients with a history of grade IV Hymenoptera venom anaphylaxis or idiopathic anaphylaxis, even in the absence of clonal MC disorder (4).

It still remains unknown whether HαT is also overrepresented among patients experiencing severe drug or food anaphylaxis in the absence of clonal mast cell disorder. However, food intolerances were reported as a frequent complaint in patients with HαT, affecting up to 40% of patients with HαT referred for elevated bST (19). Moreover, in seminal papers about HαT, out of 10 anaphylaxis triggers reported in 33 patients, two were foods, two were Hymenoptera stings, and one was an idiopathic reaction (20). Similarly, in a cohort of 101 patients with HαT referred for mast

cell activation-related symptoms and without clonal mast cell disorders, 57.4% presented doctor-diagnosed anaphylaxis: drugs were the most frequent trigger (52%), followed by foods (29%), venoms (17%), and idiopathic reactions (14%) (21). Recently, Lang et al. reported that not only HαT but all α-allele-bearing genotypes, including conventional αβ:ββ and αβ:αβ genotypes, were associated with a higher risk of anaphylaxis among children with food allergy compared with the ββ:ββ genotype. Children with food allergy and an α-tryptase$^+$ genotype also tended to present more severe reactions. In a second cohort of children with peanut allergy, individuals with α-tryptase$^+$ genotypes had higher total severity scores during oral food challenge than those with the ββ:ββ genotype. Moreover, symptom severity scores in this group positively correlated with the α-tryptase copy number (6). The specific properties of α/β-tryptase heterotetramers present in individuals expressing α-tryptase, such as EMR2 pre-activation or protease-activated receptor-2 (PAR2) activation, provide the conceptual basis for differences in allergic phenotypes according to the HαT status or even the conventional tryptase genotype (22).

In conclusion, within the context of scarce data regarding the relationship between HαT status and food allergy, this study provides more insight into the serological and clinical correlates of HαT. Siblings with HαT presented with a lower reaction threshold at the initial challenge, but after 10 years of oral immunotherapy, they displayed higher sensitization levels and lower IgG4/IgE ratios compared to the sibling with a wild-type genotype. Cohort studies are needed to confirm this association.

Patient perspective

All three patients declared a satisfying quality of life under oral immunotherapy. Regarding the two patients with tryptase elevation, concordant tryptase genotypes and negative *KIT* D816V in peripheral blood without other manifestations of systemic mastocytosis were comforting for the patients. The uncertainties related to the HαT status in the context of food allergies were explained to the patients, as well as the reassurance about the very low risk of severe allergic reactions in their progeny.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author yannick.chantran@aphp.fr.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YC: Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing – original draft. HR: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft. MA: Methodology, Supervision, Writing – review & editing. TG: Conceptualization, Data curation, Investigation, Methodology, Supervision, Validation, Writing – original draft. AN: Conceptualization, Data curation, Investigation, Methodology, Supervision, Validation, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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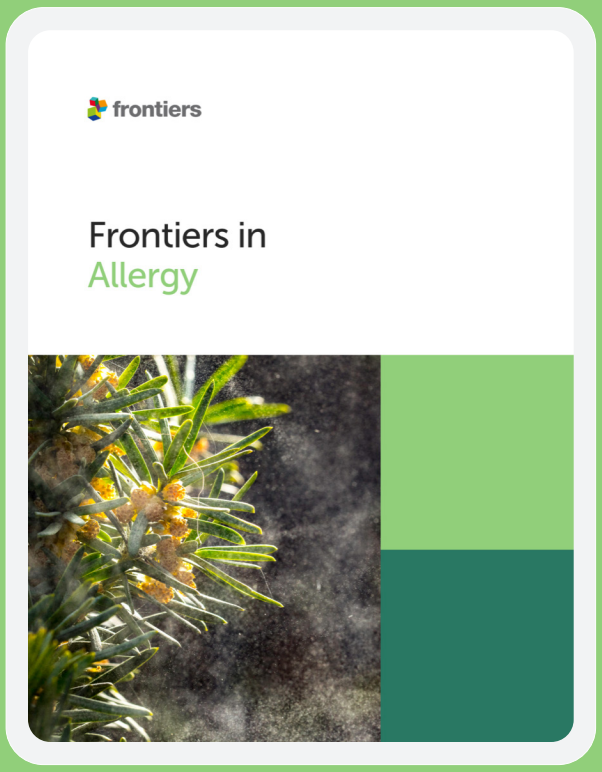

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