

# Human Basophils: A Unique Biological Instrument to Detect the Allergenicity of Food

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## ■ Abstract

**Background:** Labeling of major food allergens is mandatory for the safety of allergic consumers. Although enzyme-linked immunosorbent assay, polymerase chain reaction, and mass spectrometry are sensitive and specific instruments to detect trace amounts of food proteins, they cannot measure the ability of food constituents to trigger activation of mast cells or basophils.

**Aim:** We evaluated the basophil activation test as an instrument to determine the allergenic potential of trace amounts of food allergens in complex matrices. Peanut (*Arachis hypogaea*) allergy was selected as a proof-of-concept model.

**Methods:** The study population comprised 5 severely peanut-allergic patients (3 males/2 females; median age, 12 years) all sensitized to 3 major peanut allergens (Ara h 1, Ara h 2, and Ara h 3) and 5 peanut-tolerant individuals (2 males/3 females; median age, 8 years). Basophils from patients and controls were stimulated with pure peanut extract and blank and peanut-spiked (0.1, 0.01, and 0.001 ppm) biscuits (baking time 11, 16, 21, 26 minutes) and chocolate extracts.

**Results:** Blank biscuits and chocolate did not induce cell activation in patients or controls. A comparison between patients and controls showed significantly higher activation of basophils after stimulation with 0.1 and 0.01 ppm of peanut-spiked biscuit at all baking times and peanut-spiked chocolate ( $P < .05$ ).

**Conclusions:** The basophil activation test is a highly sensitive and specific tool to detect traces of functionally active food allergens. For biscuits, its accuracy seems independent of baking time. Furthermore, it allows even the most sensitive patients to be included in study protocols.

**Key words:** Food allergy. Trace allergens. Food safety. Food labeling. Basophil activation test.

## ■ Resumen

**Antecedentes:** El etiquetado de los principales alérgenos alimentarios es obligatorio para la seguridad de los consumidores alérgicos. Pese a que el ensayo de inmunoadsorción, la reacción en cadena de la polimerasa y la espectrometría de masas son instrumentos específicos y sensibles para detectar cantidades mínimas de proteínas alimentarias, no pueden determinar la capacidad de los componentes de los alimentos para desencadenar la activación de basófilos o mastocitos.

**Objetivo:** Se evaluó el test de activación de basófilos como instrumento para determinar el potencial alergénico de cantidades mínimas de alérgenos alimentarios en matrices complejas. La alergia al cacahuete (*Arachis hypogaea*) se seleccionó como modelo de prueba de concepto.

**Métodos:** La población en estudio incluyó a 5 pacientes altamente alérgicos al cacahuete (3 hombres/2 mujeres; mediana de edad: 12 años), todos sensibilizados a 3 alérgenos principales del cacahuete (Ara h 1, Ara h 2, y Ara h 3), y a 5 individuos tolerantes al cacahuete (2 hombres/3 mujeres; mediana de edad: 8 años). Los basófilos de los pacientes y los controles se estimularon con extracto puro de cacahuete y con extractos de chocolate y galletas sin y con cacahuete adicionado (0,1; 0,01 y 0,001 ppm) (tiempo de cocción: 11, 16, 21, 26 minutos).

**Resultados:** Las galletas sin cacahuete adicionado y el chocolate no causaron activación celular en los pacientes ni en los controles. Una

comparación entre pacientes y controles mostró una activación significativamente más elevada de los basófilos después de la estimulación con 0,1 y 0,01 ppm de galleta con cacahuete adicionado en todos los tiempos de cocción y chocolate con cacahuete adicionado ( $p < 0,05$ ). **Conclusiones:** El test de activación de basófilos es una herramienta muy sensible y específica para la detección de cantidades mínimas de alérgenos alimentarios funcionalmente activos. En las galletas, su exactitud es independiente del tiempo de cocción. Además, permite incluir a los pacientes más sensibles en los protocolos de estudio.

**Palabras clave:** alergia alimentaria; cantidades mínimas de alérgenos; seguridad alimentaria; etiquetado de los alimentos; test de activación de basófilos.

## Introduction

The incidence of food-induced anaphylaxis is increasing steadily [1]. In the absence of a cure, management of food allergy is based on strict allergen avoidance informed by correct diagnosis. Inadvertent exposure [2] is not uncommon and can result in life-threatening and fatal anaphylaxis [3]. A major reason for accidental exposure is incorrect, incomplete, or even misleading labeling [4]. Therefore, the allergic consumer can benefit from improved labeling, as issued by European [5] and US [6] legislators.

Studies and publications on the detection of trace amounts of food allergens are evolving rapidly. Currently applied techniques such as enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), real-time (RT)-PCR, and mass spectrometry have recently been comprehensively reviewed [7]. Although all these tests provide valuable information, none of them addresses the issue of allergenic potential, ie, the capacity of the detectable food constituents to trigger activation of mast cells and basophils. Theoretically, the allergenicity of food could be disclosed by skin and challenge tests. However, application of these tests is hampered by practical and ethical considerations. Therefore, functional in vitro tests that closely resemble the in vivo pathway leading to symptoms would be more than welcome.

Recently, Vogel et al [8] described an assay based on activation of passively sensitized "humanized" rat

basophilic leukemia cells that makes it possible to quantify the allergenicity of peanut and hazelnut proteins in complex food matrices. However, stable transfection of the human high-affinity receptor for immunoglobulin (Ig) E (FcεRI) is required, stimulation conditions vary widely from person to person, and even sera from patients with severe peanut allergy do not always elicit cell activation.

Flow-assisted analysis of basophils, known as the basophil activation test (BAT), has been adopted for the diagnosis of IgE-mediated hypersensitivity [9-12].

We anticipated that the technique could be adopted to study the allergenic potential of trace amounts of food allergens in complex matrices. Peanut (*Arachis hypogaea*) allergy was selected as a proof-of-concept model, since peanut can elicit systemic reactions in trace amounts [13,14] and is responsible for about 60% of fatal food-induced anaphylaxis [3].

## Patients and Methods

### Patient and Controls

The local ethics committee approved the study.

The study population comprised 5 peanut-allergic patients (3 males/2 females; median age, 12 years [interquartile range (IQR), 9-16]) and 5 peanut-tolerant healthy controls (2 males/3 females; median age, 8 years, [IQR, 4-12]).

Table. Clinical and Biological Features of Peanut-Allergic Patients<sup>a</sup>

Patient	Sex	Age	Clinical Reaction to Peanut	Skin Prick Test (Wheal/Flare), mm	sIgE ImmunoCAP (kU <sub>A</sub> /L)			
					CPE	rAra h 1	rAra h 2	rAra h 3
1	M	16	Anaphylactic shock	25/60	11.9	7.21	5.62	1.03
2	M	12	Anaphylactic shock	ND	>100	41.4	83.7	8.7
3	F	9	Edema of mouth/larynx Gastrointestinal symptoms	ND	>100	71.8	>100	12.8
4	M	9	Anaphylactic shock	5/30	>100	>100	>100	38.1
5	F	12	Edema of mouth/larynx, Gastrointestinal symptoms	ND	>100	26.7	>100	34.6

Abbreviations: CPE, crude peanut extract; slg, specific immunoglobulin; ND, not done.

<sup>a</sup>None of the patients showed slgE or had a positive skin prick test result for cow's milk and wheat.

Peanut allergy was documented as a compelling history of severe allergic reaction, peanut-specific IgE  $\geq 15$  kU<sub>A</sub>/L (ImmunoCAP FEIA, Phadia, Uppsala, Sweden), and/or a peanut skin prick test result  $\geq 8$  mm (HAL Allergy, Haarlem, The Netherlands). As these cutoff values demonstrate a positive predictive value (PPV)  $\geq 95\%$  [15], a hazardous challenge was deemed unethical [16]. Furthermore, all the patients showed concomitant sensitization to the peanut allergens Ara h 1 (vicilin), Ara h 2 (2S albumin), and Ara h 3 (legumin), a combination that is repeatedly observed in patients with more severe disease [17].

Clinical and serological sensitization to milk and wheat in both groups of individuals and against peanut in controls was ruled out.

Table 1 displays the features of the peanut-allergic patients.

### Biscuit Preparation

The procedure for preparing blank and peanut-spiked biscuit (Institute of Reference Materials and Measurements [IRMM, Geel, Belgium]) has been described elsewhere [18]. Briefly a fine powder obtained as a mixture from equal aliquots of 5 heat-treated peanut varieties (IRMM-481f standard test material) was used to spike milk- and wheat-containing dough. Blank dough was baked at 180°C for 16 minutes (standard time); peanut-spiked dough was baked at 180°C for 16, 11, 21, and 26 minutes. Blank and peanut-spiked biscuits were ground and sieved. For protein extraction, 1 g of ground biscuits was solubilized in 10 mL of 60°C phosphate-buffered saline (PBS) (10 mM, pH 7.4) for 15 minutes and centrifuged (1900g for 10 minutes) at 4°C. The supernatant was filtered and stored at -20°C until use. For the BAT, biscuit extracts were further diluted to match their peanut protein concentration with the challenge conditions applied in this test.

### Chocolate Preparation

Blank and peanut-spiked chocolate were prepared for the BAT. Nut-free milk chocolate (28% cocoa content), provided by Barry-Callebaut (Lebbeke-Wieze, Belgium), was ground with an Ultra-turrax (IKA, dispersing tool 25N, Staufen, Germany) in liquid nitrogen and spiked with a fine powder obtained from the IRMM-481f standard test material (IRMM). Next, 970 g of fine milk chocolate powder was mixed with 20 g of cocoa butter (Barry-Callebaut) containing 10 g peanut powder. The prewarmed cocoa butter and peanut mixture was added to melted chocolate (80°C for 15 minutes). The chocolate mixture (10 g/kg peanut content) was cooled at room temperature and ground with an Ultra-turrax (IKA, dispersing tool 25N).

Aliquots of 450 g of peanut-free (blank) chocolate powder were added to 50 g of spiked chocolate to progressively obtain samples with 10-fold lower peanut concentrations.

The chocolate extracts applied in the BAT were prepared and further diluted as described for biscuits.

### BAT

The BAT is detailed elsewhere [9-12]. Aliquots of endotoxin-free heparinized whole blood were stimulated with dilution buffer as a negative control, anti-IgE (10 µg/mL; Pharmingen, BD Biosciences, San Jose, California, USA) as a positive control, "pure" peanut (IRMM-481f standard material) extract (serial dilutions 10, 1, and 0.1 ng/mL), and blank and peanut-spiked (0.1, 0.01, and 0.001 ppm corresponding to 10, 1, and 0.1 ng/mL of peanut protein, respectively) biscuits/chocolate extracts. Cells were stained with 10 µL of a mixture of CD63-FITC/CD123-PE/AntiHLADR-PerCP (BD Biosciences, catalogue number 341068). Activated basophils were gated as CD123+/HLADR-/CD63+.

### Statistical Analysis

All results were expressed as median (IQR).

Statistical analysis was conducted using SPSS 16.0 (SPSS Inc, Chicago, Illinois, USA). The Kruskal-Wallis and Wilcoxon tests were used where appropriate. A *P* value <.05 was considered significant.

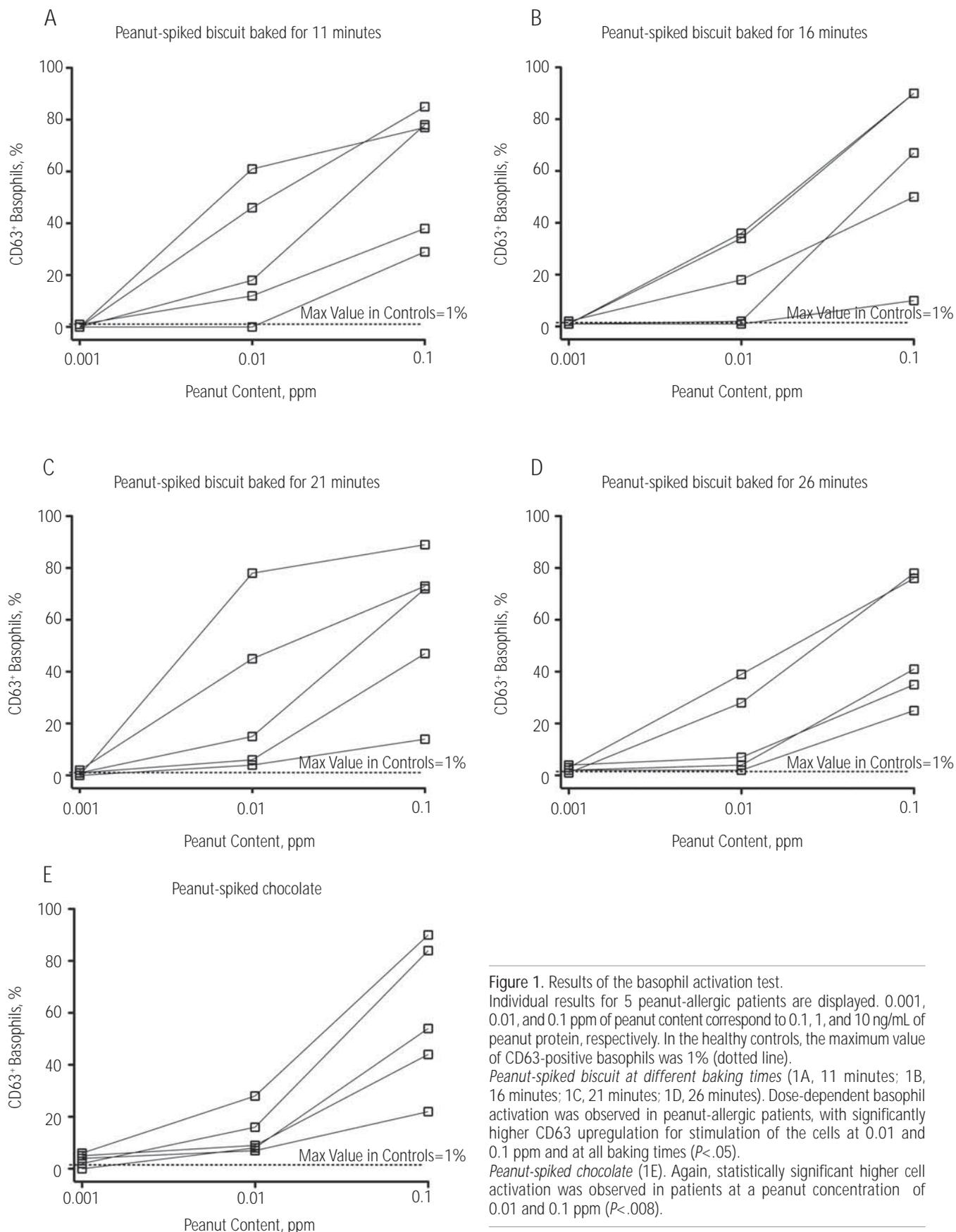
## Results

Peanut-allergic patients and healthy controls showed comparable spontaneous and anti-IgE-induced expression of CD63 (data not shown).

Stimulation with pure peanut extract resulted in clear CD63-upregulation (90% [86%-97%]) in patients, whereas no activation of basophils (1% [0%-2%]) was observed in healthy controls (*P*<.05). Blank biscuits and chocolate did not induce basophil activation in patients (1% [1%-2%]) and 1% [0%-2%], respectively) or in healthy controls (1% [0%-1%]) and 0% [0%-1%], respectively).

The results of BAT with extracts from peanut-spiked biscuits are plotted in Figure 1 (A-D). In the healthy controls, the maximum value of CD63-positive basophils was 1% for all the concentrations and at any baking time. In contrast, patients showed clear dose-dependent CD63 upregulation for a stimulation concentration between 0.001 and 0.1 ppm. A comparison between patients and healthy controls showed, for all baking times, significantly higher activation of the peanut-challenged basophils at a stimulation concentration of 0.1 and 0.01 ppm (*P*<.05). Experiments with peanut-spiked chocolate confirmed the data obtained with peanut-spiked biscuits (Figure 1E) with a significantly higher activation of basophils after stimulation with 0.1 and 0.01 ppm of peanut protein (*P*<.008).

As displayed in Figure 2, peanut-spiked biscuits at all baking times and chocolate induced comparable and significantly lower basophil activation than pure peanut extract at all the concentrations tested (*P*=.04).



**Figure 1.** Results of the basophil activation test. Individual results for 5 peanut-allergic patients are displayed. 0.001, 0.01, and 0.1 ppm of peanut content correspond to 0.1, 1, and 10 ng/mL of peanut protein, respectively. In the healthy controls, the maximum value of CD63-positive basophils was 1% (dotted line). *Peanut-spiked biscuit at different baking times* (1A, 11 minutes; 1B, 16 minutes; 1C, 21 minutes; 1D, 26 minutes). Dose-dependent basophil activation was observed in peanut-allergic patients, with significantly higher CD63 upregulation for stimulation of the cells at 0.01 and 0.1 ppm and at all baking times ( $P < .05$ ). *Peanut-spiked chocolate* (1E). Again, statistically significant higher cell activation was observed in patients at a peanut concentration of 0.01 and 0.1 ppm ( $P < .008$ ).

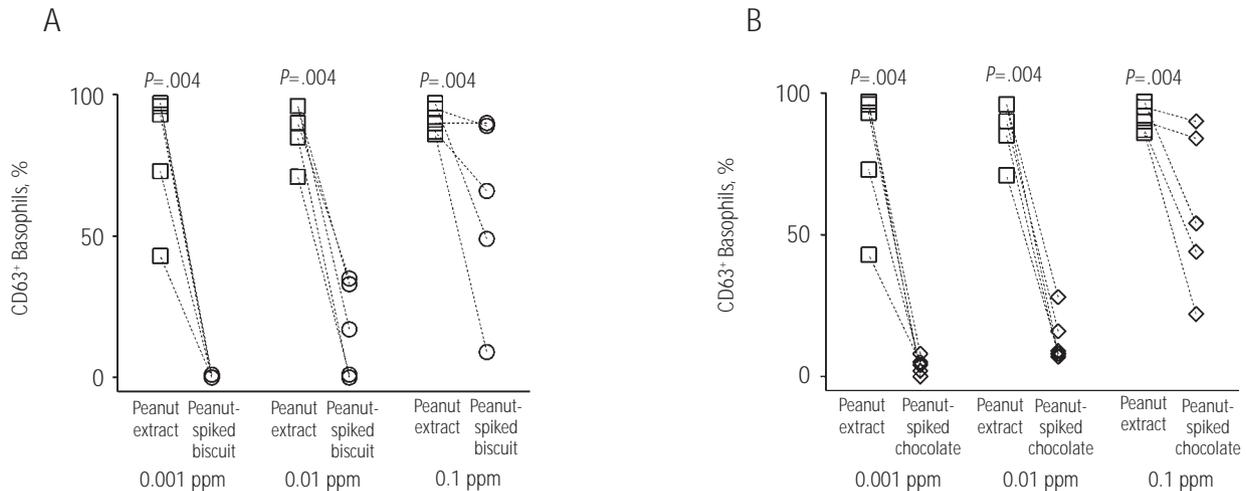


Figure 2. Effect of the matrix.

Pure peanut extract and peanut-spiked biscuit (A) and chocolate (B) induced CD63 upregulation in 5 peanut-allergic patients. For peanut-spiked biscuits, 16 minutes of baking time was chosen as a representative example. Significantly lower basophil activation than with pure peanut extract was observed for both peanut-spiked matrices at each concentration and, for biscuits, at each baking time. 0.001, 0.01, and 0.1 ppm of peanut content correspond to 0.1, 1, and 10 ng/mL of peanut protein, respectively.

## Discussion

Correct labeling of foodstuff ingredients is a prerequisite for the safety of allergic consumers. Although, ELISA, PCR, RT-PCR, and mass spectrometry are reliable techniques, they cannot measure the allergenic potential of detectable food constituents. Skin tests and double-blind placebo-controlled food challenges (DBPCFCs) are time-consuming and are hampered by the risk of severe systemic reactions. In fact, patients with a history of life-threatening anaphylaxis are at the highest risk and are excluded a priori from challenges [16]. Therefore, a safe *in vitro* test that closely mirrors the *in vivo* pathway leading to the symptoms of an IgE-mediated reaction and that could disclose traces of biologically active food allergens would be more than welcome.

The most relevant and original finding of this study is that the BAT constitutes a sensitive and specific instrument to detect biologically active trace amounts of peanut allergen in biscuits and chocolate.

Furthermore, our data show that, using basophils from patients with a well-established severe peanut allergy, our technique attains high analytical sensitivity with a limit of detection (LOD) of 0.01-0.1 ppm, irrespective of the matrix and, for biscuit, of baking time. This finding clearly differs from that of Scaravelli et al [19], who recently demonstrated, using the same standardized biscuits, that the LOD of the ELISA and PCR methods was in the range of 1-10 ppm and that increasing baking time had a deleterious effect on the accuracy of both tests.

Although the BAT is a safe and sensitive method that can outperform classic analytical techniques, large-scale use to screen food products is precluded due to the continuous need for fresh basophils from recently exposed patients, as

the analytical sensitivity of the BAT can decrease over time. Nevertheless, the BAT could help assess the reliability of the commonly applied analytical techniques.

In the absence of DBPCFCs, a criticism of our study could be that our approach does not assess the clinical relevance of our *in vitro* findings. At present, 3 controlled studies identifying a no-observed-adverse-effect-level (NOAEL) for peanut have been published and show the NOAEL to be as low as 30-500 µg for peanut protein [13,14,20]. Moreover, it was recommended that a 100-fold uncertainty factor be applied along with these NOAEL in order to account for the small size of the study population and the exclusion of potentially more sensitive patients [21]. Therefore, with a NOAEL of 30 µg for peanut protein, the potential threshold for an allergic reaction would be 0.3 µg of peanut protein. Based on the assumption that our 0.1-ppm peanut-spiked biscuits or chocolate matrices (peanut protein content, 0.01 µg/g) could be ingested at once, an allergic reaction could be expected at a serving size of 30 g. This peanut protein content, clearly below the LOD of commonly used ELISA and PCR methods, was readily detectable with BAT.

Another issue is the assessment of the residual allergenicity of food ingredients that are derivatives of allergenic foods. Directive 2007/68/EC [5] contains a list of allergenic foods that require a mandatory declaration on product labels and names derivatives that are exempt from this requirement. We anticipate that the BAT could be useful to assess the (residual) allergenicity of such derivatives.

Taken together, we provide proof-of-concept that flow-assisted analysis of *in vitro*-activated basophils from severely food-allergic patients is an entirely safe and functional tool to assess the allergenic potential of food. Whether these findings apply to less severe cases remains to be established. Additional DBPCFCs could eventually be performed to strengthen our conclusions.

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