

# Modified oral food challenge used with sensitization biomarkers provides more real-life clinical thresholds for peanut allergy

Katharina Blumchen, MD,<sup>a</sup> Alena Beder,<sup>a</sup> John Beschorner, MD,<sup>a</sup> Frank Ahrens, MD,<sup>b</sup> Armin Gruebl, MD,<sup>c</sup> Eckard Hamelmann, MD,<sup>d</sup> Gesine Hansen, MD,<sup>e</sup> Andrea Heinzmann, MD,<sup>f</sup> Katja Nemat, MD,<sup>g</sup> Bodo Niggemann, MD,<sup>a</sup> Ulrich Wahn, MD,<sup>a</sup> and Kirsten Beyer, MD<sup>a</sup> Berlin, Hamburg, Munich, Bochum, Hannover, Freiburg, and Dresden, Germany

**Background:** Threshold levels for peanut allergy determined by using oral challenges are important for the food industry with regard to allergen labeling. Moreover, the utility of biological markers in predicting threshold levels is uncertain.

**Objective:** We sought to use a modified oral food challenge regimen that might determine threshold levels for peanut allergy mimicking a more real-life exposure and to correlate the eliciting dose (ED) and severity of clinical reaction in children with peanut allergy with B-cell, T-cell, and effector cell markers.

**Methods:** A modified food challenge procedure with doses scheduled 2 hours apart was used in 63 children with peanut allergy. All children received a maximum of 8 semi-log

increasing titration steps of roasted peanuts ranging from 3 to 4500 mg of peanut protein until objective allergic reactions occurred. Severity of symptoms was graded from I to V. Biological markers were measured before challenge.

**Results:** Forty-five of 63 patients showed objective symptoms after greater than 30 minutes, with a median latency of clinical reaction of 55 minutes. By using a log-normal dose-distribution model, the ED<sub>5</sub> was calculated to be 1.95 mg of peanut protein. The ED was significantly and inversely correlated with peanut- and Ara h 2-specific IgE levels, skin prick test responses, basophil activation, and T<sub>H</sub>2 cytokine production by PBMCs. Symptom severity did not correlate with any of the markers or the ED.

**Conclusion:** This modified food challenge procedure might better reflect threshold levels for peanut allergy than the standard procedure because most of the patients reacted at a time interval of greater than 30 minutes. By using this model, threshold levels, but not severity, could be correlated with biological markers. (*J Allergy Clin Immunol* 2014;134:390-8.)

**Key words:** Basophil activation, Ara h 2, children, cytokines, peanut allergy, food challenge

From <sup>a</sup>the Department of Pediatric Pneumology and Immunology, Charité-Universitätsmedizin Berlin; <sup>b</sup>Children's Hospital "Altona," Hamburg; <sup>c</sup>the Department of Pediatrics, Technical University Munich; <sup>d</sup>the Department of Pediatrics, Ruhr-University Bochum; <sup>e</sup>the Department of Pediatric Pneumology, Allergology and Neonatology, Hannover Medical School; <sup>f</sup>the Center for Pediatrics and Adolescent Medicine, University of Freiburg; and <sup>g</sup>the Department of Pediatrics, University Hospital Carl Gustav Carus, Technical University of Dresden.

Supported in part by a grant from Charité (Rahel Hirsch grant), the Foundation for the Treatment of Peanut Allergic Patients (bea Stiftung), and the Berlin Sparkassen Foundation for Medicine.

Disclosure of potential conflict of interest: K. Blumchen has received grants from the Foundation for the Treatment of Peanut Allergic Patients (bea Stiftung), the Berlin Sparkassen Foundation for Medicine, and the Nutricia Research Foundation (2012-15); is employed by Charité, Department of Pediatric Immunology and Pneumology; has received payment for lectures from Novartis Pharma, GmbH, Meda Pharma, the Society to Promote Dermatologic Research and Education, and the Society for Education of Chronically Ill Patients. E. Hamelmann is a board member for the National Allergy Society Germany (DGAKI): General Secretary of DGAKI, chair of the Pediatric Section; has consultant arrangements with Boehringer Ingelheim, ALK-Abelló, Novartis, and Bencard; has provided expert testimony on behalf of GLG for respirator medicine and allergology; and has received payment for lectures from ALK-Abelló, and Novartis. G. Hansen has received research support from Deutsche Forschungsgemeinschaft (HA 2799/3-3) and Bundesministerium für Bildung und Forschung (82DZL00201). K. Nemat has received payment for lectures from Thermo Fisher Scientific, Nutricia GmbH, Novartis Pharma AG, HAL Allergy Group, and Bencard Allergie GmbH. K. Beyer has received research support from the Foundation for the Treatment of Peanut Allergic Patients (bea Stiftung), the Berlin Sparkassen Foundation for Medicine, Charité, Deutsche Forschungsgemeinschaft, European Union, Danone, Thermo Fisher, DST Diagnostic Systems, Food Allergy and Anaphylaxis Network, and Pina e. V.; has received consultancy fees from Danone, Novartis, ALK-Abelló, Meda Pharma, and Unilever; and has received lecture fees from Danone, Infectopharma, CSL, Behring, Novartis, UCB, Meda Pharma, Med Update, Allergopharma, and Thermo Fisher. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication March 20, 2013; revised February 2, 2014; accepted for publication March 12, 2014.

Available online May 13, 2014.

Corresponding author: Katharina Blumchen, MD, Department of Pediatric Pneumology and Immunology, Charité-Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany. E-mail: [nina.blumchen@charite.de](mailto:nina.blumchen@charite.de).

0091-6749/\$36.00

© 2014 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2014.03.035>

#### Abbreviations used

BA:	Basophil activation
CDmax:	Maximum number of activated basophils after allergen stimulation of all peanut extract dilutions tested minus crude values after RPMI stimulation
CD-sens:	Basophil sensitivity
DBPCFC:	Double-blind, placebo-controlled food challenge
ED:	Eliciting dose
fMLP:	N-formyl-methionyl-leucyl-phenylalanine
OIT:	Oral immunotherapy
SPT:	Skin prick test

threshold levels within a group of patients with peanut allergy, an estimation of population-based thresholds can be calculated (eg, by using dose-distribution models).<sup>7,8</sup>

Recently, these threshold levels have been proposed for populations with peanut allergy.<sup>9-11</sup> However, all these studies used common oral food challenges with dose increases every 15 to 30 minutes. Because immediate symptoms in food allergy usually occur within 2 hours, the proposed threshold levels might be too high because of cumulative effects. The threshold levels might not closely resemble a real-life situation with single-meal exposures. In our previously published study on oral immunotherapy (OIT) in children with peanut allergy,<sup>12</sup> 23 patients received a double-blind, placebo-controlled food challenge (DBPCFC) with dose increments every 30 minutes (dose steps: 0.03, 0.06, 0.13, 0.25, 0.5, 1, and 2 g and, the next day, 4 g of whole crushed roasted peanut in boiled apple as a matrix). OIT began the following day by using a rush protocol, with dose increments every 2 hours for a maximum of 7 days (starting doses ranged from 0.4 to 24 mg of whole crushed roasted peanut in boiled apple as a matrix, with incremental steps as stated in Blumchen et al<sup>12</sup>). We observed that most patients took more than 30 minutes to have symptoms during rush OIT, with a median time to reaction of 102 minutes (range, 5-275 minutes; see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Although the dosing protocols differed between the DBPCFC and the initial days of rush OIT, 30% of patients had lower threshold reaction levels during rush OIT than during their DBPCFCs (see Table E1). This prompted us to choose a modified challenge protocol with a 2-hour interval between dose steps, which might better reflect the real-life ED for children with peanut allergy.

There are few published studies on dose response or on predictive laboratory markers, which correlate the severity of the reaction and ED during challenge. Most of these studies were conducted retrospectively or with a small number of subjects.<sup>13-20</sup> Moreover, the studies show conflicting results. The aim of this prospective study was first to determine threshold levels by using a modified peanut challenge procedure with 2-hour time intervals between dose steps, which might better reflect a real-life ED response, and to correlate the determined EDs and the severity of the clinical reaction with biological markers in a large number of high-risk children with peanut allergy.

## METHODS

### Study population

The study population consisted of peanut-sensitized children between 3 and 18 years of age who had undergone an oral food challenge test to

participate in a randomized, double-blind, placebo-controlled study on OIT. One hundred eighty-six children with suspected peanut allergy were approached either consecutively in our outpatient clinic or from a list of peanut-sensitized children followed in our department (Pediatric Pneumology and Immunology, Charité, Berlin, Germany) or those of our partners (Children's Hospital "Altona," Hamburg, Germany; Department of Pediatrics, Technical University Munich, Munich, Germany; Department of Pediatrics, Ruhr-University Bochum, Bochum, Germany; Department of Pediatric Pneumology, Allergology and Neonatology, Hannover Medical School, Hannover, Germany; Centre for Pediatrics and Adolescent Medicine, University of Freiburg, Freiburg, Germany; and the Department of Pediatrics, University Hospital Carl Gustav Carus, Technical University of Dresden, Dresden, Germany).

Seventy-one children meeting the inclusion criteria of being peanut sensitized (peanut-specific IgE, >0.35 kU/L) but not undergoing other forms of immunotherapy and not having uncontrolled asthma were recruited to participate in the study. Between the first screening visit and the in-patient, open oral peanut challenge to confirm diagnosis, 4 patients dropped out, citing anxiety before oral challenge or lack of time for the following OIT. Sixty-seven patients underwent oral provocation testing on our children's ward. Four of these patients had no allergic reactions on provocation. These were considered tolerant toward peanuts and excluded from the analysis of this study. Sixty-three children with IgE-mediated challenge-proved peanut allergy and a median age of 6.5 years (range, 3.2-17.8 years) were finally enrolled in the study (Table 1): 62% were male, and 54% had asthma or increased airway reactivity. Fifty-six children had a history of at least 1 allergic reaction to peanut after accidental ingestion, as reported by the parents, with a median severity of grade IV, as determined by using a modified version of the grading system for food-induced anaphylaxis.<sup>15,21</sup> As recommended in the PRACTALL consensus report,<sup>22</sup> before challenge, patients were considered to be stable concerning their other atopic conditions: none of the patients were receiving treatment with antihistamines or systemic steroids. Of the 34 patients with asthma/increased airway reactivity, 24 received daily medications (inhaled steroids, montelukast, or long-acting  $\beta_2$ -agonists), which they did not stop during challenge. The local ethics committees approved the study, and informed consent was obtained from the subjects and their families. The study was registered with the German Clinical Trials Register (DRKS00004553).

### Study design

During the baseline visit, medical histories were obtained, and patients were physically examined and screened for peanut sensitization. After approximately 8 weeks, children were admitted to our ward for an open oral peanut challenge. Before challenge, patients were assessed for stability of other atopic conditions (history, SCORAD score, and lung function). A physical examination and measurement of vital signs and rectal temperature was carried out clinically, ruling out any infection. Each patient received a skin prick test (SPT) and an intravenous line from which blood samples were taken and analyzed within 2 hours for B-cell, T-cell, and effector cell markers.

### Open oral peanut challenges

Patients received whole crushed roasted peanuts in boiled apple as a matrix. A new challenge protocol was chosen with an increased time interval between dose steps to maximize the accuracy of the ED under challenge. Instead of administering dose steps every 30 minutes, as in the standard protocol, patients were given the peanut doses at intervals of 120 minutes over 2 days. A maximum of 7 semi-log titration steps (0.012 g, 0.04 g, 0.12 g, 0.4 g, 1.2 g, 4 g, 12 g of whole crushed roasted peanut, corresponding to 0.003 to 3 g of peanut protein)<sup>23</sup> were given every 120 minutes (0.012 g, 0.04 g, 0.12 g, 0.4 g on the first day and 0.4 g, 1.2 g, 4 g, 12 g on the second day). If no reaction occurred, a single open dose of 18 g of whole peanut (ie, 4.5 g of peanut protein) was administered on a third day. The whole challenge was conducted as an inpatient procedure, with patients staying continuously on the ward for 3 days. The procedure was stopped if objective clinical symptoms were observed. The whole challenge procedure and also the decision to stop the

**TABLE I.** Baseline characteristics of study participants (n = 63)

Clinical characteristics		Laboratory characteristics	
Age (y)	6.5 (3.2-17.8)	Peanut-specific IgE (kU <sub>A</sub> /L)	74 (0.57-624)
Male sex (%)	62	Ara h 2-specific IgE (kU <sub>A</sub> /L)	45 (0.04-256)
Positive family history for atopy (%)	89	Peanut-specific SPT response (mm)	8 (3-14.5)
Asthma/increased airway reactivity (%)	54	IL-4 production (pg/mL)	17 (0-80)
Atopic dermatitis (%)	67	IL-5 production (pg/mL)	333 (0-2006)
Allergic rhinitis (%)	51	IL-10 production (pg/mL)	24 (0-229)
Further food allergies (%)	38	IL-2 production (pg/mL)	171 (0.5-1131)
Accidental ingestion of peanut before study (%)	89	IFN- $\gamma$ production (pg/mL)	10 (0-476)
No. of accidental ingestions	1 (0-6)	TNF- $\alpha$ production (pg/mL)	7 (0-163)
Severity of worst reaction after accidental ingestion (grade of severity)	IV (I-V)	BA RPMI stimulation (%)	0.2 (0-13)
ED at oral challenge (mg whole peanut)	400 (12-18,000)	BA anti-Fc $\epsilon$ RI mAb (%)	71 (4-95)
Severity of reaction at oral challenge (grade of severity)	III (I-IV)	BA fMLP (%)	13 (0-85)
Lower respiratory symptoms at oral challenge (grade III or IV [%])	35	BA 5 $\times$ 10 <sup>-4</sup> $\mu$ g/mL peanut extract stimulation (%)	0.4 (0-65)
Time to onset of objective symptoms at oral challenge (min)	55 (5-210)	BA 5 $\times$ 10 <sup>-2</sup> $\mu$ g/mL peanut extract stimulation (%)	34 (0-74)
		BA 5 $\mu$ g/mL peanut extract (%)	17 (0-61)
		BA 5 $\times$ 10 <sup>2</sup> $\mu$ g/mL peanut extract (%)	51 (1-93)
		CDmax (%)	47 (14-87)
		CD-sens (%)	3995 (0.4-4 $\times$ 10 <sup>5</sup> )

Values are presented as medians (ranges), where shown.

challenge were conducted by a single study physician with application of strict, well-defined stopping criteria.<sup>22</sup>

The clinical reaction was characterized by both (1) the ED, which was defined as the actual single dose at which patients showed their first objective allergic symptoms, and (2) the severity of objective symptoms displayed during challenge. Symptom severity was graded on a scale of I to V by using a modified version of the grading system for food-induced anaphylaxis (see also the [Methods](#) section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).<sup>15,21</sup> The most severe objective symptom determined the grade. In the current study objective symptoms leading to a stop in challenge included prominent angioedema of the lips or eyes (grade I or grade II, if very prominent), generalized hives (defined as  $\geq 3$  noncontact hives [grade II]), single vomiting if the time between dose ingestion and reaction was more than 10 minutes (grade II), repeated diarrhea or vomiting (grade III), continuous cough (grade III), wheeze (grade IV), shortness of breath (grade IV) with wheeze/cough/decrease in peak flow or generalized hives being present simultaneously, and stridor with a simultaneous decrease in peak flow (grade IV). Grade V reactions (not observed) would have been respiratory arrest, bradycardia, arterial hypotension, cardiac arrest, or loss of consciousness. Symptoms, such as flush, perioral hives, vomiting directly after ingestion of peanut caused by disgust, and mild rhinoconjunctivitis, were not considered stopping criteria as such. Also, subjective symptoms (see also [Results](#)) did not lead to stopping the challenge.

A detailed description of which patients were considered to need medical treatment (systemic antihistamines, steroids, or both; inhaled  $\beta_2$ -agonists; and intramuscular adrenalin) is presented in the [Methods](#) section in this article's Online Repository.

### SPTs

SPTs for peanut were performed as prick-to-prick tests with natural, intact, roasted whole peanut. Saline solution was used as a negative control. Histamine dihydrochloride (10 mg/mL; ALK-Abelló, Hørsholm, Denmark) was used as the positive control. SPTs were read after 15 minutes, and the mean diameter of the elicited wheal was recorded for all patients.

### Measurement of serum allergen-specific IgE levels

Blood samples were analyzed for peanut- or Ara h 2-specific IgE by using the CAP System FEIA (Thermo Fisher, Uppsala, Sweden), according to the manufacturer's instructions. In cases in which peanut- or Ara h 2-specific IgE

levels exceeded 100 kU<sub>A</sub>/L, further dilutions of sera were performed to obtain exact values.

### Differential blood counts

Differential blood cell counts were performed from EDTA blood on an automated Coulter counter for analysis of absolute numbers of eosinophils and basophils in peripheral blood (Sysmex XE-2100; Sysmex Corp, Kobe, Japan).

### In vitro stimulation of PBMCs and analysis of cytokines in supernatants

As previously described,<sup>12</sup> PBMCs were isolated for each patient before challenge and cultured *in vitro* for 48 hours with (1) LPS-free crude peanut extract (50  $\mu$ g/mL),<sup>24</sup> (2) medium alone (RPMI 1640 containing 10% autologous serum), and (3) PHA (20  $\mu$ g/mL) in single measurements. Supernatants were collected and analyzed for the presence of IL-2, IL-4, IL-5, IL-10, IFN- $\gamma$ , and TNF- $\alpha$ .

### Basophil activation test

Basophil activation (BA) was measured, as described previously (for a detailed description, see the [Methods](#) section in this article's Online Repository).<sup>25</sup> Whole venous blood was incubated without adding human IL-3 for 20 minutes in a warm water bath with (1) RPMI as a negative control, (2) anti-Fc $\epsilon$ RI mAb as a positive control, (3) N-formyl-methionyl-leucyl-phenylalanine (fMLP) as an IgE-independent positive control, and (4) 4 increasing dilutions of crude peanut extract (0.0005  $\mu$ g/mL, 0.05  $\mu$ g/mL, 5  $\mu$ g/mL, 500  $\mu$ g/mL final concentration of peanut extract).<sup>24</sup> Basophil degranulation was stopped with an ice bath for 5 minutes. Cells were then stained with surface antibodies for 25 minutes, erythrocytes were lysed, and BA was assessed by using flow cytometry on a FACSCanto II (BD Biosciences, San Jose, Calif). Basophils were identified as CD203c<sup>+</sup>CD123<sup>+</sup>HLA-DR<sup>-</sup>CD45<sup>+</sup>, and at least 300 of these cells (ie, events) were measured for each stimulation. Activated basophils were characterized as CD63<sup>+</sup>CD203c<sup>+</sup>CD123<sup>+</sup>HLA-DR<sup>-</sup>CD45<sup>+</sup>.

Results of BA are presented for each subject in a number of ways: (1) as crude values (percentage of CD63<sup>+</sup>CD203c<sup>+</sup>CD123<sup>+</sup>HLA-DR<sup>-</sup>CD45<sup>+</sup> cells of CD203c<sup>+</sup>CD123<sup>+</sup>HLA-DR<sup>-</sup>CD45<sup>+</sup> cells) for RPMI stimulation (ie, negative control); (2) as a percentage of activated basophils (CD63<sup>+</sup>CD203c<sup>+</sup>CD123<sup>+</sup>HLA-DR<sup>-</sup>CD45<sup>+</sup>) of all basophils after

stimulation with anti-FcεRI mAb, (3) fMLP, or (4)  $5 \times 10^{-4}$  μg/mL, (5)  $5 \times 10^{-2}$  μg/mL, (6) 5 μg/mL, or (7)  $5 \times 10^2$  μg/mL of peanut extract minus crude values after RPMI stimulation; (8) as the maximum number of activated basophils after allergen stimulation of all peanut extract dilutions tested minus crude values after RPMI stimulation (CDmax)<sup>26</sup>; or (9) as basophil sensitivity (CD-sens; defined as 1/Calculated allergen concentration used for stimulation of 50% of maximal BA × 100 [= 1/50% of CDmax × 100]) (adapted from Nopp et al<sup>26</sup>). The higher the values for CD-sens, the higher the basophil sensitivity.

### Statistical analysis

Statistical analyses were performed with SPSS software (version 18.0 for Windows; SPSS, Chicago, Ill). We calculated medians and ranges for continuous variables. The clinical allergic reaction (ED and severity) was correlated to B-cell, T-cell, and effector cell markers of peanut allergy. Also, all laboratory markers were correlated to each other. For strength of correlation, the Spearman correlation coefficient was computed (*r<sub>s</sub>*). A correlation coefficient of 0.8 to 1 was considered very good correlation, 0.6 to 0.8 was considered good correlation, 0.4 to 0.6 was considered moderate correlation, 0.2 to 0.4 was considered mild correlation, and 0.0 to 0.2 resembled no correlation. Differences between the group of patients who needed treatment during challenge and the group who did not need treatment were tested by using the Mann-Whitney *U* test for nonparametric distribution and the Fisher exact test for categorical variables. Statistical significance was defined by a 2-sided  $\alpha$  value of .05.

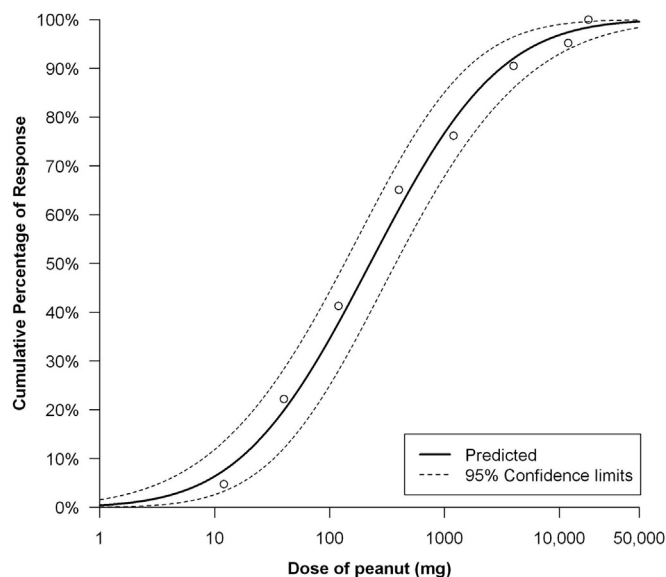
To calculate a population threshold distribution, an interval-censoring survival analysis approach was used, analyzing individual no observed adverse effect levels (ie, the highest dose that will not produce any objective symptom) and lowest observed adverse effect levels, as described by Taylor et al,<sup>8</sup> using SAS version 9.3 software (SAS Institute, Cary, NC). Children reacting to the lowest dose at challenge (12 mg of whole peanut) were left censored. A log-normal dose-distribution model was used to estimate the ED5, ED10, and ED50 values, which are the predicted threshold doses to elicit objective symptoms in 5%, 10%, and 50% of the population with peanut allergy.

## RESULTS

### Threshold levels for peanut allergy using a modified oral challenge procedure

By using a modified challenge protocol with dose increments every 2 hours, 3 (5%) of the 63 patients already had objective allergic symptoms at the first dose tested (12 mg of whole peanut = 3 mg of peanut protein). For all patients, the median ED for objective symptoms was 400 mg of whole peanut (100 mg of peanut protein, Table I). Fig 1 shows the estimated probability distribution of thresholds calculated by using a log-normal dose-distribution model. The ED5 was calculated as 7.81 mg of whole peanut (ie, 1.95 mg of peanut protein; 95% CI, 3.5-17.6 mg of whole peanut), ED10 was calculated as 16.41 mg of whole peanut (95% CI, 8-33.5 mg of whole peanut), and ED50 was calculated as 225.37 mg of whole peanut (95% CI, 134.9-376.4 mg of whole peanut).

Median time to onset of the first objective immediate-type symptoms was 55 minutes (range, 5-210 minutes; Table I), underlining the importance of a modified model to determine threshold levels, which better reflects a real-life exposure. Although 1 patient showed objective symptoms (flush, rhinorrhea, conjunctivitis, and vomiting) 5 minutes after ingesting 400 mg of peanuts, 45 of 63 patients had their first objective symptoms after more than 30 minutes. Although the time interval for increasing dose steps was 120 minutes, 3 patients had objective symptoms, even after more than 120 minutes.



**FIG 1.** Log-normal probability distribution model of individual peanut thresholds (in milligrams of whole peanut) for patients with peanut allergy tested in this study. Distribution is based on lowest observed adverse effect levels and no observed adverse effect levels for objective symptoms only.

One patient delayed eating the next dose out of disgust and reacted after 210 minutes with generalized hives. In 2 patients the allergic reaction occurred between days 1 and 2 of the challenge: of these, 1 patient experienced continuous dry cough 160 minutes after ingestion of 400 mg of peanut, which improved after inhalation of salbutamol. One patient had abdominal pain and vomiting 180 minutes after ingesting the last peanut dose on the first challenge day.

### Clinical reaction at oral peanut challenge

**Objective symptoms.** Except for fatal or near-fatal symptoms (grade V), patients experienced the whole spectrum of allergic symptoms: 14% had only skin symptoms, with hives, angioedema, or both; 19% showed only gastrointestinal tract symptoms, such as vomiting or diarrhea; and 6% showed only respiratory symptoms, such as continuous cough (*n* = 1) and wheeze (*n* = 3). Thirty-five percent showed any lower respiratory tract symptoms (Table I), such as dry cough (13%), wheeze (8%), or shortness of breath (14%), mostly associated with other objective symptoms. In general, 51% of the study population had objective symptoms in more than 1 system.

Of the 15 of 63 patients reacting with grade IV severity (wheeze, shortness of breath, and stridor with decrease in peak flow), only 4 experienced any objective symptom within 30 minutes of ingesting the individual ED. Eight of these patients had no objective symptoms beforehand but reacted with symptoms of pulmonary obstruction (mostly associated with other simultaneous objective symptoms) after 50 to 110 minutes.

Overall, 29 patients needed treatment when reacting. Patients who did not need treatment included both of the 2 patients with severity grade I; 17 of 24 patients with mild symptoms of severity grade II; 14 of 22 patients with severity grade III who showed only gastrointestinal symptoms, moderate rhinoconjunctivitis, or dry cough; and 1 of 15 patients with severity grade IV

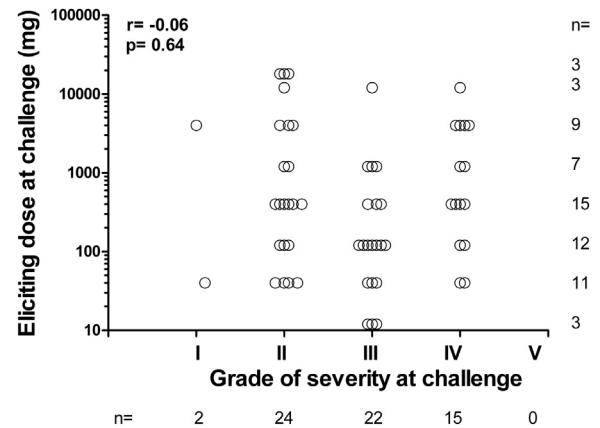
who experienced mild transient stridor and a decrease in peak flow, which rapidly improved again spontaneously. Twelve patients were treated with systemic antihistamines and corticosteroids, 15 patients inhaled a  $\beta_2$ -agonist with or without additional treatment with antihistamines or corticosteroids, and 2 patients were treated with intramuscular adrenalin. When comparing patients who needed treatment during challenge ( $n = 29$ , median grade of severity of most severe objective reactions at challenge = III [range, II-IV]) with those who did not need treatment ( $n = 34$ , median grade of severity = II [range, I-IV]) the only difference other than the severity of the reaction at challenge was seen in sex: there were significantly more boys (16/29 patients) in the group who needed treatment than in the group without treatment (15/34,  $P = .04$ ). There was no difference in markers for sensitization or other clinical parameters tested when these 2 groups were compared (data not shown).

**Subjective symptoms.** Forty-two (67%) of 63 patients experienced subjective symptoms, such as abdominal pain, nausea, oral allergy symptoms, throat tightness, itching, headache, and mild vertigo, at a peanut dose lower than their individual objective threshold dose. Of the remaining 21 patients, 12 showed subjective symptoms and then objective symptoms at their threshold dose, whereas 9 patients showed no subjective symptoms before their objective reaction at threshold. The median threshold for the occurrence of subjective symptoms in 54 patients experiencing subjective symptoms was 40 mg of whole peanut (range, 12-4000 mg).

There was no correlation between the ED and the clinical severity of the patient's symptoms at oral challenge (Fig 2). There was also no correlation between the ED or the severity of symptoms at challenge and either the time interval to onset of symptoms or age (Table II). However, there was an inverse correlation between the ED at challenge and the number of accidental reactions and also the severity of the worst accidental reaction in the patient's history (Table II).

### Biological markers

The median peanut-specific IgE level of the study population was 74 kU<sub>A</sub>/L (0.57-624 kU<sub>A</sub>/L, Table I), with 60 (95%) of 63 patients being sensitized against Ara h 2. After 48 hours of stimulation of PBMCs with peanut extract, IL-2 (median IL-2 production, 171 pg/mL [0.5-1131 pg/mL]) and IL-5 (median IL-5 production, 333 pg/mL [0-2006 pg/mL]) could be primarily detected in the supernatants of all tested patients (Table I). Also, a low but significant increase in peanut-specific IL-4 and IL-10 production was detected when compared with that seen after stimulation with medium (median IL-4 production after RPMI stimulation, 2.2 pg/mL [0-32 pg/mL]; median IL-4 production after peanut extract stimulation, 17 pg/mL [0-80 pg/mL]; median IL-10 production after RPMI stimulation, 2.7 pg/mL [0-60 pg/mL]; median IL-10 production after peanut extract stimulation, 24 pg/mL [0-229 pg/mL]; Table I). Peanut-specific IFN- $\gamma$  and TNF- $\alpha$  production was similar to production after RPMI stimulation (median IFN- $\gamma$  production after RPMI stimulation, 6.8 pg/mL [0-289 pg/mL]; median IFN- $\gamma$  production after peanut extract stimulation, 10 pg/mL [0-476 pg/mL]; median TNF- $\alpha$  production after RPMI stimulation, 5.2 pg/mL [0-160 pg/mL]; median TNF- $\alpha$  production after peanut extract stimulation, 7 pg/mL [0-163 pg/mL]; Table I). Cross-linking the



**FIG 2.** Correlation of the severity of allergic reactions (grade I-V) with the ED (12-18,000 mg of whole peanut) at oral challenge in children with peanut allergy. There is no correlation between the ED at oral peanut challenge and the severity of reactions at challenge. *n*, Number of patients reacting to an individual ED/experiencing an individual grade of severity at challenge.

Fc $\epsilon$ RI receptor as a positive control showed an increase in BA in almost all patients. Three (5%) patients were characterized as nonresponders (see the **Methods** section in this article's Online Repository). Bacterial peptide stimulation (fMLP) did not enhance BA as much as seen in the children with peanut allergy as did Fc $\epsilon$ RI cross-linking (Table I). In most patients *in vitro* peanut-specific stimulation resulted in a dose-dependent BA (Table I).

### Correlation of eliciting peanut dose during challenge with biological markers

There was a significant mild-to-moderate inverse correlation of the markers for sensitization (peanut-specific IgE, Ara h 2-specific IgE, and wheal size of peanut SPTs) with the ED at challenge (Fig 3, A-C, and Table II). Also, peanut-specific markers of BA could be inversely correlated to the ED: the percentage of CD63<sup>+</sup> activated basophils stimulated *in vitro* with different low concentrations of peanut extract (Fig 3, D, and Table II), basophil sensitivity (Fig 3, E, and Table II), and also the number of activated peripheral basophils not stimulated *in vitro* with peanut extract (BA RPMI stimulation, Table II) were inversely correlated to the ED. In addition, peanut-specific T<sub>H</sub>2 and IL-2 cytokine production by PBMCs stimulated *in vitro* with peanut extract was also inversely correlated to the ED (Fig 3, F, and Table II), whereas peanut-specific T<sub>H</sub>1 cytokine production (IFN- $\gamma$  and TNF- $\alpha$ ) could not be correlated (Table II).

### Correlation of severity of symptoms during challenge with biological markers

There was no correlation between the biological markers tested and the grade of severity of the most severe objective allergic reaction at challenge (Table II and see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). With regard to Ara h 2, neither the quantitative value as such could be correlated to the grade of severity of reaction at challenge nor did the qualitative evaluation of Ara h 2 give a significant correlation. Only 3 patients within this study population showed an Ara h

**TABLE II.** Correlation of severity/ED of the allergic reaction at oral challenge versus clinical and all laboratory data analyzed

	Severity of reaction		ED of reaction	
	Spearman correlation coefficient ( $r_s$ )	P value	Spearman correlation coefficient ( $r_s$ )	P value
Age (y)	0.04	.8	-0.14	.28
No. of accidental ingestions	0.16	.21	<b>-0.36</b>	<b>.004</b>
Severity of worst reaction after accidental ingestion (grade of severity)	-0.25	.86	<b>-0.28</b>	<b>.04</b>
ED at oral challenge (mg)	-0.06	.64	1	NA
Cumulative ED at oral challenge (mg)	-0.05	.7	0.99	NA
Severity of reaction at oral challenge (grade of severity)	1	NA	-0.06	.64
Time to onset of objective symptoms at oral challenge (min)	0.18	.17	-0.03	.8
Peanut-specific IgE (kU <sub>A</sub> /L)	0.013	.92	<b>-0.36</b>	<b>.004</b>
Ara h 2-specific IgE (kU <sub>A</sub> /L)	0.05	.71	<b>-0.38</b>	<b>.002</b>
Peanut-specific SPT response (mm)	0.09	.46	<b>-0.27</b>	<b>.04</b>
Total IgE (kU <sub>A</sub> /L)	-0.13	.33	-0.12	.34
IL-4 production (pg/mL)	0.01	.9	<b>-0.36</b>	<b>.004</b>
IL-5 production (pg/mL)	0.12	.36	<b>-0.42</b>	<b>.001</b>
IL-10 production (pg/mL)	-0.07	.6	<b>-0.37</b>	<b>.003</b>
IL-2 production (pg/mL)	0.13	.3	<b>-0.35</b>	<b>.006</b>
IFN- $\gamma$ production (pg/mL)	0.09	.5	-0.02	.88
TNF- $\alpha$ production (pg/mL)	-0.06	.66	-0.18	.18
BA at RPMI stimulation (%)	0.03	.8	<b>-0.27</b>	<b>.03</b>
BA at anti-Fc $\epsilon$ RI mAb stimulation (%)	-0.2	.13	-0.05	.68
BA at fMLP stimulation (%)	0.21	.1	-0.21	.11
BA at $5 \times 10^{-4}$ $\mu$ g/mL peanut extract stimulation (%)	-0.13	.35	<b>-0.41</b>	<b>.001</b>
BA at $5 \times 10^{-2}$ $\mu$ g/mL peanut extract stimulation (%)	0	1	<b>-0.32</b>	<b>.02</b>
BA at 5 $\mu$ g/mL peanut extract stimulation (%)	-0.21	.12	-0.14	.32
BA at $5 \times 10^2$ $\mu$ g/mL peanut extract stimulation (%)	-0.19	.16	0.01	.94
CDmax (%)	-0.24	.08	-0.03	.82
CD-sens (%)	-0.12	.41	<b>-0.36</b>	<b>.008</b>
Eosinophil count (/nL)	0.04	.8	<b>-0.27</b>	<b>.03</b>
Basophil count (/nL)	0.13	.32	<b>-0.3</b>	<b>.02</b>

Significant ( $P < .05$ ) values are shown in boldface.

2-specific IgE level of 0.35 kU/L or less, and their grade of severity ranged from II to III.

In 5 patients there was a progression in severity from the first objective symptom noticed (ie, stopping criteria for challenge) to more severe ones later. This occurred in 2 of these patients, although they received systemic steroids and antihistamines at the first objective symptom noticed. The other 3 patients had only mild symptoms, such as vomiting, diarrhea, or allergic rhinoconjunctivitis, for which they did not receive any treatment but progressed to more severe symptoms later. However, there was still no correlation found between the severity of the first objective symptom and the biological markers tested (data not shown).

Because the severity of the reaction might be reflected by the need for medical treatment during challenge, patients were grouped into those who needed treatment and those who did not need treatment. However, there was also no difference found in any biological markers tested or the ED when these 2 groups were compared (data not shown).

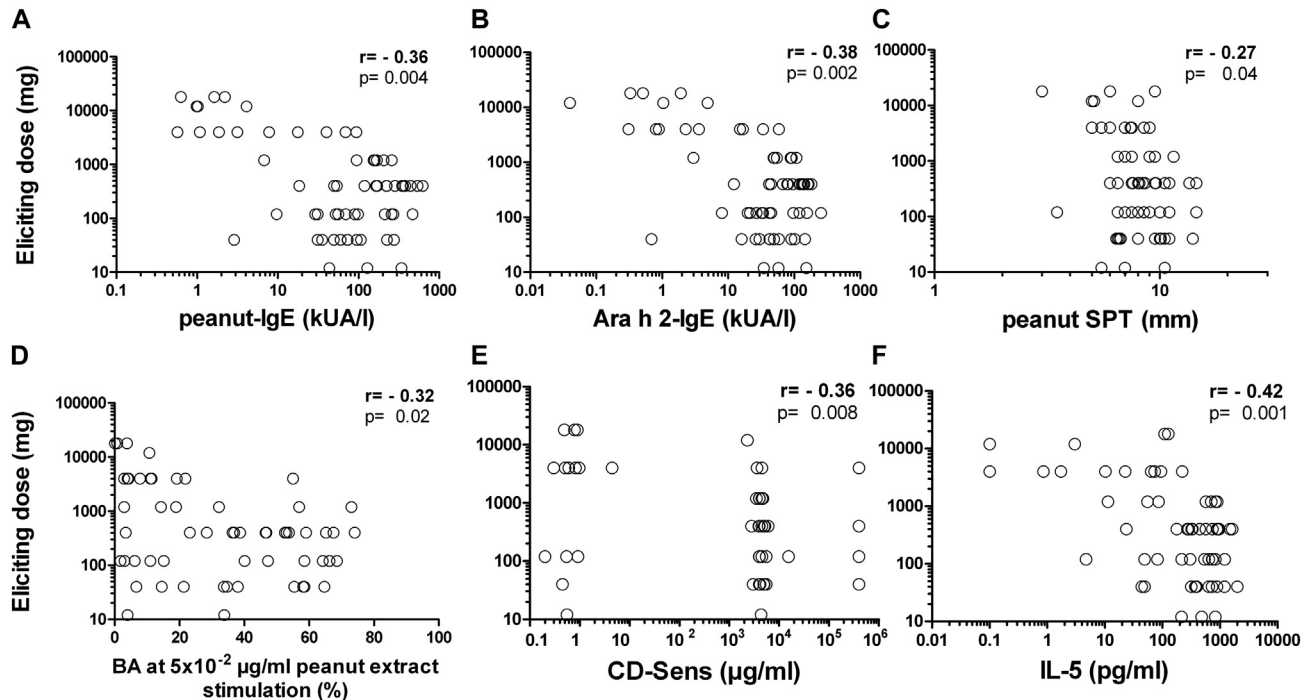
## DISCUSSION

In this prospective study, for the first time, we were able to identify threshold levels in a high-risk population with peanut allergy by using a modified procedure for oral food challenges with dose increments every 2 hours. We show that the ED during oral peanut challenge could be correlated not only to peanut-specific biomarkers for sensitization but also to markers

for T<sub>H</sub>2 cell activation and BA. However, the severity of symptoms during oral challenge could not be correlated to any of these markers.

By using a modified challenge protocol with a 2-hour interval between dose steps, the majority of patients in this study reacted with objective immediate-type symptoms after more than 30 minutes. Of 15 patients experiencing symptoms of grade IV severity during challenge, 8 had these symptoms 55 to 110 minutes after the ingestion of the peanut dose that elicited the symptoms. This highlights the point that intervals of 2 hours between peanut doses during oral food challenge might increase the specificity of the ED to estimate the threshold and better predict symptom severity during challenge.

Because thresholds are usually estimated based on the cumulative dose of allergen immediately before symptom development, shorter time intervals during challenge (eg, 30 vs 120 minutes) might result in a more rapid accumulation of doses, leading to estimation of higher final threshold levels. Previous challenge protocols, including all published studies on population thresholds in children with peanut allergy,<sup>8-11</sup> have used dose increments every 15 to 30 minutes, which might result in thresholds that are too high. To compare threshold levels within different study populations, it was postulated to use the ED5 (ED at which 5% of the population had objective symptoms), which is derived from population thresholds by using an interval-censored dose-distribution model. In the current study the estimated ED5 was 1.95 mg of peanut protein (ie, 7.81 mg of whole peanut). This ED5 is much lower than previously



**FIG 3.** Correlation of laboratory markers for sensitization, BA, and T-cell activation with the ED of the allergic reaction at oral challenge. There is a correlation between the individual eliciting dose of the allergic reaction at oral challenge (12-18,000 mg of whole peanut) and markers for peanut-specific sensitization (ED [in milligrams] vs peanut-specific IgE [in kilounits of allergen per liter, **A**]; ED [in milligrams] vs Ara h 2-specific IgE [in kilounits of allergen per liter, **B**]; and ED [in milligrams] vs peanut-specific wheal size in SPTs [in millimeters, **C**]). There is a correlation between the individual ED of the allergic reaction at oral challenge (in milligrams) and BA after *in vitro* stimulation with  $5 \times 10^{-2}$   $\mu\text{g}/\text{mL}$  peanut extract (**D**) and basophil sensitivity (**E**) and IL-5 production (**F**; in picograms per milliliter) by PBMCs after *in vitro* stimulation with peanut extract.

described by Eller et al,<sup>11</sup> who report an ED<sub>5</sub> of 77 mg of whole peanut for a Danish population of 175 children and adults with peanut allergy. Some of these Danish patients received a high starting dose at challenge (85 mg of whole peanut), which might have influenced the ED<sub>5</sub>. However, a similar threshold level as in the current study was identified by a Dutch study, which analyzed data from 135 children with positive oral peanut challenge results.<sup>9</sup> By using a “standard” oral food challenge test with dose increments every 30 minutes, an ED<sub>5</sub> of 1.6 mg of peanut protein equals 6.4 mg of whole peanut was determined. Similar results were also obtained by Taylor et al,<sup>10</sup> studying a French population of 286 children and adults with peanut allergy who had been challenged with dose increments every 15 minutes, showing an ED<sub>5</sub> of 7.3 mg of whole peanut for this population. However, given that the majority of our population reacted later than 30 minutes, the ED<sub>5</sub> determined by Taylor et al<sup>10</sup> or Blom et al<sup>9</sup> might be even lower within their population if their patients had been challenged with the modified protocol.

The population recruited for this study does not resemble a normally distributed population of children with peanut allergy because recruitment was for an interventional study on OIT. This was a high-risk group with high anxiety about experiencing severe allergic reactions at accidental ingestion of peanut. The majority of these children (90%) had at least 1 allergic reaction after accidental ingestion of peanut before the study, with half of them having an allergic reaction of severity grade IV or V (Table I). Fifty-four percent of the study population was

considered high risk because they had asthma or increased airway reactivity. Furthermore, peanut-specific sensitization was the highest for all 3 parameters tested (peanut-specific IgE levels, Ara h 2-specific IgE levels, and peanut-specific SPT responses) when compared with other published studies on challenged patients with peanut allergy.<sup>10,16-20,27-29</sup> To our knowledge, this is one of the largest studied groups of children with peanut allergy at high risk for serious or fatal food-induced allergic reactions when undergoing oral challenges. However, whether these patients were also at high risk for severe reactions to low allergen doses is not known.

One drawback of this study is that a nonblind but open food challenge protocol was used. However, the definition of the allergic reaction under challenge was very strict because only clear-cut objective symptoms counted as stopping criteria and all challenges were conducted by the same study physician throughout the study. Moreover, patients were free of any signs of infection or instability of their other atopic conditions before and after challenge. Furthermore, an indoor environment throughout the peanut challenge was controlled because of the hospitalization.

In the current study there was no correlation of the severity of clinical reactions during challenge with any of the laboratory markers tested or with the ED. This differs from other published studies, showing a correlation between markers for food sensitization and the severity of reactions at challenge.<sup>14,15,18,19,29,30</sup> There is no standardized scoring system for the severity of clinical

reactions at oral challenge. Similar to other published studies,<sup>11,14,15</sup> we used a modified version of the grading system for food-induced anaphylaxis published by Sampson,<sup>21</sup> which has the limitation of not specifically accounting for a multiorgan reaction. Applying different scoring systems, such as the adapted “Mueller” scoring used by Wensing et al<sup>19</sup> or Flinterman et al,<sup>17</sup> or scoring systems better reflecting multiorgan reactions, as used by Taylor et al (published in Astier et al<sup>31</sup>) or van der Zee et al<sup>27</sup> for grading accidental ingestions, we still could not identify any correlation between reaction severity at challenge and either biological markers tested or the ED (data not shown). Thus the differences in results seem not to be due to the different scoring systems used. The differences might be explained by the fact that in some of these studies, tolerant children with no clinical reactions during oral food challenge were included in the data analysis.<sup>14,15,29</sup> Other studies have focused only on adults,<sup>11,19</sup> some used mainly subjective symptoms for stopping the oral food challenge,<sup>11,14,19</sup> some were conducted in children with food allergies other than peanut,<sup>14,15,30</sup> and 1 used a non-high-risk study population (median peanut-specific IgE level, 5.65 kU/L), with a scoring system combining reaction severity and the ED.<sup>18</sup> Calculating the ratio between specific activation in response to peanut extract and the nonspecific activation of basophils mediated by FcεRI cross-linking (percentage of CD63<sup>+</sup>/CD203c<sup>+</sup>/CD123<sup>+</sup>/HLA-DR<sup>-</sup>/CD45<sup>+</sup> cell peanut stimulation divided by percentage of CD63<sup>+</sup>/CD203c<sup>+</sup>/CD123<sup>+</sup>/HLA-DR<sup>-</sup>/CD45<sup>+</sup> cell anti-FcεRI mAb stimulation), as was suggested by Rubio et al,<sup>30</sup> also did not enhance correlation to the severity of reactions at challenge in our study (data not shown). However, similar to the current study, 2 other published studies on children with peanut allergy could not show any correlation between markers for sensitization and severity of reactions under challenge.<sup>17,20</sup>

Recently, a study by Eller and Bindslev-Jensen<sup>32</sup> on 205 patients indicated a correlation between the severity of clinical reactions at challenge and Ara h 2-specific IgE levels, whereas other studies with smaller numbers of patients<sup>17,33</sup> could not show this correlation. However, using the same scoring system as Eller and Bindslev-Jensen (Sampson scoring), the current study could not show this correlation. In the study by Eller and Bindslev-Jensen, tolerant children (symptom score of 0) and children only experiencing subjective symptoms seem to be included within the data analysis, which might explain the different results compared with the current study.

By using a modified challenge protocol, the current study was able to demonstrate an inverse correlation between markers for sensitization or cell activation and the ED similar to another published study on adults with peanut allergy.<sup>16</sup> Recently, a retrospective study on 126 children with peanut allergy with ages similar to those of the current study population was published by van der Zee et al.<sup>27</sup> Using a small range of peanut dose steps from 6 mg to 1.2 g of whole peanut and a regression model, the authors showed that a higher peanut-specific IgE level was a predictor for lower EDs on oral challenge. Because of the smaller number of patients included in the current study, we could not use a regression model. However, we were also able to demonstrate the inverse correlation of ED and sensitization. This stands in contrast to other published studies that did not show any correlation between the ED and food-specific IgE levels<sup>11,15,17</sup> or SPT responses.<sup>20</sup> One explanation might be that the threshold levels determined in these studies could show

cumulative dose responses because of the short time intervals between dose increments at challenge. In addition, many of these studies did not focus on peanut as the allergen.<sup>13-15</sup> Furthermore, one study recruited a non-high-risk population (median peanut-specific IgE, 21 kU/L).<sup>17</sup>

In the current study we have demonstrated an inverse correlation between the ED and the number and severity of accidental reactions in the patients' histories (Table II): patients who reacted to smaller doses of peanut during challenge had experienced more and severe accidental ingestions before the study than patients reacting only to higher doses. This is in contrast to the data published by van der Zee et al<sup>27</sup> and Taylor et al,<sup>10</sup> who did not show an association of the ED and the severity of food reactions by history. As mentioned above, these 2 studies used different scoring systems for the severity of accidental reactions. Applying these scoring systems to the current data on accidental reactions from this study, we were still able to show an inverse correlation of the ED at challenge and the grade of severity at accidental reactions in the patient's history (data not shown). Thus the differences in results are not due to different scoring systems but might be explained because van der Zee et al<sup>27</sup> recruited a lower-risk group of patients (median peanut-specific IgE, 18.6 kU/L) whereas Taylor et al<sup>10</sup> included adults and furthermore because the determined EDs of these studies might be influenced by the short time interval between the dose increments used.

In conclusion, with a modified oral challenge protocol in high-risk children with peanut allergy, a median latency until clinical reaction of 55 minutes and an ED<sub>5</sub> of 1.95 mg of peanut protein have been identified. The ED correlated to all assessed markers of allergen-specific sensitization and immune reactions. However, the severity of the allergic reaction did not correlate with any markers. These results are not only important for future patient care but also for food industry and policymakers in their attempt to establish thresholds for allergen labeling.

We thank Alexander Rohrbach for technical assistance in the laboratory, as well as all nurses and receptionists of the collaborative pediatric departments. Most importantly, we thank all the patients who participated in the study.

#### Key messages

- A modified food challenge procedure with doses scheduled 2 hours apart showed a median latency until clinical reaction of 55 minutes and might be helpful in the determination of more real-life threshold levels for peanut allergy.
- Threshold levels could be correlated to peanut-specific biomarkers for sensitization, T<sub>H</sub>2 cell activation, and BA.
- Severity of symptoms during oral challenge could not be correlated to any of these biomarkers or to the ED.

#### REFERENCES

1. Sicherer SH, Munoz-Furlong A, Sampson HA. Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit telephone survey: a 5-year follow-up study. *J Allergy Clin Immunol* 2003;112:1203-7.
2. Ben-Shoshan M, Kagan RS, Alizadehfard R, Joseph L, Turnbull E, St Pierre Y, et al. Is the prevalence of peanut allergy increasing? A 5-year follow-up study in children in Montreal. *J Allergy Clin Immunol* 2009;123:783-8.



3. Wood RA. The natural history of food allergy. *Pediatrics* 2003;111:1631-7.
4. Pumphrey RS. Lessons for management of anaphylaxis from a study of fatal reactions. *Clin Exp Allergy* 2000;30:1144-50.
5. Yu JW, Kagan R, Verreault N, Nicolas N, Joseph L, St Pierre Y, et al. Accidental ingestions in children with peanut allergy. *J Allergy Clin Immunol* 2006;118:466-72.
6. Hefle SL, Furlong TJ, Niemann L, Lemon-Mule H, Sicherer S, Taylor SL. Consumer attitudes and risks associated with packaged foods having advisory labeling regarding the presence of peanuts. *J Allergy Clin Immunol* 2007;120:171-6.
7. Crevel RW, Briggs D, Hefle SL, Knulst AC, Taylor SL. Hazard characterisation in food allergen risk assessment: the application of statistical approaches and the use of clinical data. *Food Chem Toxicol* 2007;45:691-701.
8. Taylor SL, Crevel RW, Sheffield D, Kabourek J, Baumert J. Threshold dose for peanut: risk characterization based upon published results from challenges of peanut-allergic individuals. *Food Chem Toxicol* 2009;47:1198-204.
9. Blom WM, Vlieg-Boerstra BJ, Kruijzinga AG, van der Heide S, Houben GF, Dubois AE. Threshold dose distributions for 5 major allergenic foods in children. *J Allergy Clin Immunol* 2013;131:172-9.
10. Taylor SL, Moneret-Vautrin DA, Crevel RW, Sheffield D, Morisset M, Dumont P, et al. Threshold dose for peanut: risk characterization based upon diagnostic oral challenge of a series of 286 peanut-allergic individuals. *Food Chem Toxicol* 2010;48:814-9.
11. Eller E, Hansen TK, Bindslev-Jensen C. Clinical thresholds to egg, hazelnut, milk and peanut: results from a single-center study using standardized challenges. *Ann Allergy Asthma Immunol* 2012;108:332-6.
12. Blumchen K, Ulbricht H, Staden U, Dobberstein K, Beschormer J, de Oliveira LC, et al. Oral peanut immunotherapy in children with peanut anaphylaxis. *J Allergy Clin Immunol* 2010;126:83-91.e1.
13. Osterballe M, Bindslev-Jensen C. Threshold levels in food challenge and specific IgE in patients with egg allergy: is there a relationship? *J Allergy Clin Immunol* 2003;112:196-201.
14. Benhamou AH, Zamora SA, Eigenmann PA. Correlation between specific immunoglobulin E levels and the severity of reactions in egg allergic patients. *Pediatr Allergy Immunol* 2008;19:173-9.
15. Rolinck-Werninghaus C, Niggemann B, Grabenhenrich L, Wahn U, Beyer K. Outcome of oral food challenges in children in relation to symptom-eliciting allergen dose and allergen-specific IgE. *Allergy* 2012;67:951-7.
16. Peeters KA, Koppelman SJ, van Hoffen E, van der Tas CW, den Hartog Jager CF, Penninks AH, et al. Does skin prick test reactivity to purified allergens correlate with clinical severity of peanut allergy? *Clin Exp Allergy* 2007;37:108-15.
17. Flinterman AE, van Hoffen E, den Hartog Jager CF, Koppelman S, Pasmans SG, Hoekstra MO, et al. Children with peanut allergy recognize predominantly Ara h2 and Ara h6, which remains stable over time. *Clin Exp Allergy* 2007;37:1221-8.
18. Hourihane JO, Grimshaw KE, Lewis SA, Briggs RA, Trewin JB, King RM, et al. Does severity of low-dose, double-blind, placebo-controlled food challenges reflect severity of allergic reactions to peanut in the community? *Clin Exp Allergy* 2005;35:1227-33.
19. Wensing M, Penninks AH, Hefle SL, Koppelman SJ, Bruijnzeel-Koomen CA, Knulst AC. The distribution of individual threshold doses eliciting allergic reactions in a population with peanut allergy. *J Allergy Clin Immunol* 2002;110:915-20.
20. Sicherer SH, Morrow EH, Sampson HA. Dose-response in double-blind, placebo-controlled oral food challenges in children with atopic dermatitis. *J Allergy Clin Immunol* 2000;105:582-6.
21. Sampson HA. Anaphylaxis and emergency treatment. *Pediatrics* 2003;111:1601-8.
22. Sampson HA, Gerth van Wijk R, Bindslev-Jensen C, Sicherer S, Teuber SS, Burks AW, et al. Standardizing double-blind, placebo-controlled oral food challenges. American Academy of Allergy, Asthma & Immunology–European Academy of Allergy and Clinical Immunology PRACTALL consensus report. *J Allergy Clin Immunol* 2012;130:1260-74.
23. Souci F, Kraut. Food composition and nutrition tables. 7th ed. Stuttgart: Medpharm Scientific Publishers; 2008.
24. Burks AW, Williams LW, Connaughton C, Cockrell G, O'Brien TJ, Helm RM. Identification and characterization of a second major peanut allergen, Ara h II, with use of the sera of patients with atopic dermatitis and positive peanut challenge. *J Allergy Clin Immunol* 1992;90:962-9.
25. Wanich N, Nowak-Wegrzyn A, Sampson HA, Shreffler WG. Allergen-specific basophil suppression associated with clinical tolerance in patients with milk allergy. *J Allergy Clin Immunol* 2009;123:789-94.e20.
26. Nopp A, Johansson SG, Ankerst J, Bylin G, Cardell LO, Gronneberg R, et al. Basophil allergen threshold sensitivity: a useful approach to anti-IgE treatment efficacy evaluation. *Allergy* 2006;61:298-302.
27. van der Zee T, Dubois A, Kerkhof M, van der Heide S, Vlieg-Boerstra B. The eliciting dose of peanut in double-blind, placebo-controlled food challenges decreases with increasing age and specific IgE level in children and young adults. *J Allergy Clin Immunol* 2011;128:1031-6.
28. Ahrens B, Niggemann B, Wahn U, Beyer K. Organ-specific symptoms during oral food challenge in children with food allergy. *J Allergy Clin Immunol* 2012;130:549-51.
29. Wainstein BK, Studdert J, Ziegler M, Ziegler JB. Prediction of anaphylaxis during peanut food challenge: usefulness of the peanut skin prick test (SPT) and specific IgE level. *Pediatr Allergy Immunol* 2010;21:603-11.
30. Rubio A, Vivinus-Nebot M, Bourrier T, Saggio B, Albertini M, Bernard A. Benefit of the basophil activation test in deciding when to reintroduce cow's milk in allergic children. *Allergy* 2011;66:92-100.
31. Astier C, Morisset M, Roitel O, Codreanu F, Jacquenet S, Franck P, et al. Predictive value of skin prick tests using recombinant allergens for diagnosis of peanut allergy. *J Allergy Clin Immunol* 2006;118:250-6.
32. Eller E, Bindslev-Jensen C. Clinical value of component-resolved diagnostics in peanut-allergic patients. *Allergy* 2013;68:190-4.
33. Dang TD, Tang M, Choo S, Licciardi PV, Koplin JJ, Martin PE, et al. Increasing the accuracy of peanut allergy diagnosis by using Ara h 2. *J Allergy Clin Immunol* 2012;129:1056-63.

## METHODS

### Open oral peanut challenges

Symptom severity was graded on a scale of I to V by using a modified version of the grading system for food-induced anaphylaxis.<sup>E1,E2</sup> Only objective symptoms were included. The most severe objective symptom determined the grade. Thus multiple-organ reactions were not considered a higher grade in severity:

- grade I—*skin symptoms*: mild angioedema, perioral urticaria, mild flushing, no respiratory, cardiovascular, or neurologic symptoms;
- grade II—*skin symptoms*: more severe angioedema, generalized urticaria, flushing and/or *gastrointestinal tract symptoms*: single episode of vomiting and/or *respiratory symptoms*: mild nasal congestion, repeated sneezing, no cardiovascular/neurologic symptoms;
- grade III: *skin symptoms*: any of above, and/or *gastrointestinal tract symptoms*: repeated vomiting or diarrhea, and/or *respiratory symptoms*: marked nasal congestion, rhinorrhea, conjunctivitis, throat tightness, dry cough, and/or *cardiovascular symptoms*: tachycardia (increase 15 beats/min);
- grade IV: *skin symptoms*: any of above, *gastrointestinal tract symptoms*: any of the above, *respiratory symptoms*: any of the above and/or hoarseness of voice, dysphagia, barking cough, wheezing, dyspnea, cyanosis, *cardiovascular symptoms*: any of the above and/or dysrhythmia, mild arterial hypotension; and
- grade V: *skin symptoms*: any of above, and/or *gastrointestinal tract symptoms*: any of the above, *respiratory symptoms*: any of the above and/or respiratory arrest, *cardiovascular symptoms*: any of the above and/or severe bradycardia or hypotension or cardiac arrest or loss of consciousness.

Patients were supposed to receive medical treatment (systemic antihistamines and/or steroids, inhaled  $\beta_2$ -agonists, and intramuscular adrenalin) if they experienced allergic objective symptoms of grade V (not observed within this study), IV, or III if a multiple-organ reaction occurred. Some grade III symptoms on their own, such as vomiting, diarrhea, allergic rhinoconjunctivitis, and dry cough, were considered not to require treatment. Except for severe generalized urticaria, all symptoms of grade II and I were considered not to require treatment. Generally, it was the study physician's decision on who to treat.

### *In vitro* stimulation of PBMCs and analysis of cytokines in supernatants

PBMCs were isolated for each patient before challenge by means of density gradient separation (Biocoll; Biochrom AG, Berlin, Germany) of heparinized venous blood. Results are expressed as the amount of cytokine production detected after *in vitro* stimulation with peanut extract or positive control minus the amount of cytokine detected after *in vitro* stimulation with the negative control (medium alone).

### BA test

The following antibodies were used: CD63– fluorescein isothiocyanate (clone H5C6; BD Biosciences, San Jose, Calif), CD203c–phycoerythrin (97A6; Immunotech, Beckmann Coulter, Marseille, France), CD123–phycoerythrin-Cy5 (9F5, BD Biosciences), HLA-DR–phycoerythrin-Cy7 (L243, BD Biosciences), and CD45–allophycocyanin-Cy7 (2D1, BD Biosciences).

Peripheral, heparinized, whole venous blood (100  $\mu$ L) was incubated without adding human IL-3 for 20 minutes in a water bath at 37°C with (1) 100  $\mu$ L of RPMI 1640 as a negative control, (2) 600 ng/mL anti-Fc $\epsilon$ RI mAb (Bühlmann Laboratories AG, Schönenbuch, Switzerland) as a positive control, (3)  $10^{-5}$  mol/L fMLP (Sigma-Aldrich, St Louis, Mo) as an IgE-independent positive control, and (4) 4 increasing dilutions of crude peanut extract (0.0005  $\mu$ g/mL to 0.05  $\mu$ g/mL to 5  $\mu$ g/mL to 500  $\mu$ g/mL final concentration of peanut extract)<sup>E3</sup> covering a suitable stimulation range. Basophil degranulation was stopped by transferring stimulation tubes into an ice bath

for 5 minutes. After that, cells were stained for 25 minutes in a dark room at 4°C with surface antibodies. After lysing of erythrocytes and washing, BA was assessed by using flow cytometry on a FACSCanto II (BD Biosciences). Single-color compensation was prepared by using anti-mouse immunoglobulin beads (anti-mouse Ig,  $\kappa$ /Negative Control [FBS] Compensation Particles Set; BD Biosciences). Fluorescence data were acquired by using FACSDiva software (version 6, BD Bioscience). Basophils were identified as CD203c<sup>+</sup>CD123<sup>+</sup>HLA-DR<sup>-</sup>CD45<sup>+</sup>, and a minimum of 300 of these cells (ie, events) were measured for each stimulation. Activated basophils were characterized as CD63<sup>+</sup>CD203c<sup>+</sup>CD123<sup>+</sup>HLA-DR<sup>-</sup>CD45<sup>+</sup>.

Measurement of BA was not possible in 2 patients because of insufficient amounts of blood. Three patients were characterized as nonresponders, which was defined as having an inadequate reaction (<10% increase from baseline/negative control) after stimulation with anti-Fc $\epsilon$ RI mAb.<sup>E4</sup> These patients were excluded for the analysis of peanut-specific activation. Furthermore, 4 patients had to be excluded for CDmax and CD-sens analysis because BA after peanut stimulation was less than 10%. Thus interpretable BA assays of 56 to 61 patients could be used for further analysis.

Because BA is not widely used as a standardized laboratory test, there is a wide range of how results are summarized and published in the literature.<sup>E5-E8</sup> Certain pitfalls in interpreting BA measurements have to be considered.<sup>E9,E10</sup> There is a broad variability of BA between subjects. This leads to a large range of different measurable interindividual maximum secretory responses (CDmax), which are determined by the subject's intracellular signaling of the basophils and not allergen specific and thus can be, on its own, only used as a positive control measurement when basophils are optimally IgE mediated and stimulated with, for example, an anti-Fc $\epsilon$ RI mAb. CDmax can be seen as a non-allergen-specific marker. However, the maximum secretory response (CDmax) also influences allergen-specific basophil sensitivity (CD-sens), which can be regarded as a marker for allergen-specific BA. CD-sens is measured as follows:

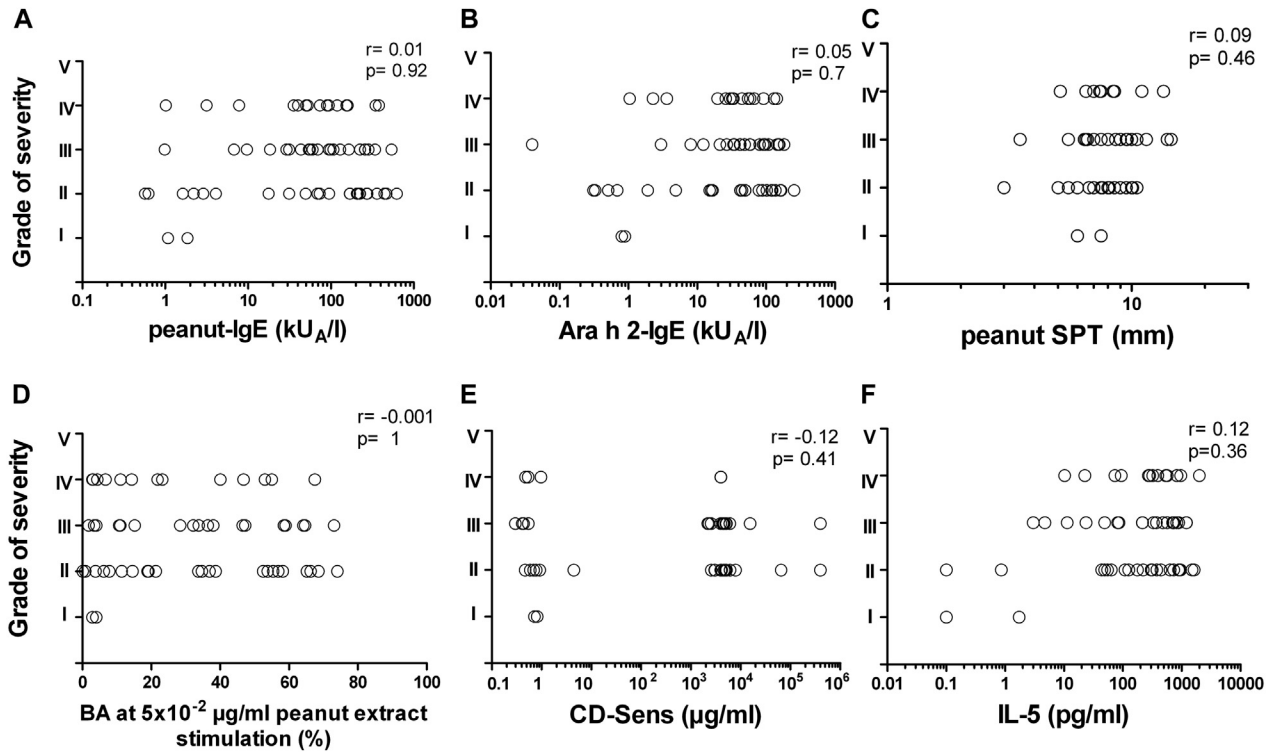
$1/\text{Calculated allergen concentration used for stimulation of 50\% of maximal BA} \times 100 (= 1/\text{Allergen concentration at 50\% of CDmax} \times 100).$

For this calculation, a wide range of BA measurements of different allergen concentrations are needed. This measurement is also highly variable between subjects and is dependent on total basophil IgE receptor cell-surface density; fraction of membrane-bound allergen-specific IgE; intrinsic cellular sensitivity of the basophils, such as the number of IgE molecules required for 50% of the maximal cellular response and cellular reactivity of the basophils (CDmax); and biochemical and structural characteristics of the allergen. Still, CD-sens is considered the best marker for allergen-specific BA measurement. However, none of the results published up to now on peanut-specific BA show CD-sens measurements but publish either only BA at 1 optimal peanut concentration,<sup>E4,E8</sup> crude data for all concentrations used, or population-fitted dose-response curves, which are not suitable for this study purpose. Therefore the authors decided to publish all the measurements used.

## REFERENCES

- E1. Rolinck-Werninghaus C, Niggemann B, Grabenhenrich L, Wahn U, Beyer K. Outcome of oral food challenges in children in relation to symptom-eliciting allergen dose and allergen-specific IgE. *Allergy* 2012;67:951-7.
- E2. Sampson HA. Anaphylaxis and emergency treatment. *Pediatrics* 2003;111:1601-8.
- E3. Burks AW, Williams LW, Connaughton C, Cockrell G, O'Brien TJ, Helm RM. Identification and characterization of a second major peanut allergen, Ara h II, with use of the sera of patients with atopic dermatitis and positive peanut challenge. *J Allergy Clin Immunol* 1992;90:962-9.
- E4. Ocmant A, Mulier S, Hanssens L, Goldman M, Casimir G, Mascart F, et al. Basophil activation tests for the diagnosis of food allergy in children. *Clin Exp Allergy* 2009;39:1234-45.
- E5. Wanich N, Nowak-Wegrzyn A, Sampson HA, Shreffler WG. Allergen-specific basophil suppression associated with clinical tolerance in patients with milk allergy. *J Allergy Clin Immunol* 2009;123:789-94.e20.
- E6. Rubio A, Vivinus-Nebot M, Bourrier T, Saggio B, Albertini M, Bernard A. Benefit of the basophil activation test in deciding when to reintroduce cow's milk in allergic children. *Allergy* 2011;66:92-100.

- E7. Nopp A, Johansson SG, Ankerst J, Bylin G, Cardell LO, Gronneberg R, et al. Basophil allergen threshold sensitivity: a useful approach to anti-IgE treatment efficacy evaluation. *Allergy* 2006;61:298-302.
- E8. Jones SM, Pons L, Roberts JL, Scurlock AM, Perry TT, Kulis M, et al. Clinical efficacy and immune regulation with peanut oral immunotherapy. *J Allergy Clin Immunol* 2009;124:292-300, e1-97.
- E9. de Weck AL, Sanz ML, Gamboa PM, Aberer W, Bienvenu J, Blanca M, et al. Diagnostic tests based on human basophils: more potentials and perspectives than pitfalls. *Int Arch Allergy Immunol* 2008;146:177-89.
- E10. Kleine-Tebbe J, Erdmann S, Knol EF, MacGlashan DW Jr, Poulsen LK, Gibbs BF. Diagnostic tests based on human basophils: potentials, pitfalls and perspectives. *Int Arch Allergy Immunol* 2006;141:79-90.
- E11. Thyagarajan A, Jones SM, Calatroni A, Pons L, Kulis M, Woo CS, et al. Evidence of pathway-specific basophil anergy induced by peanut oral immunotherapy in peanut-allergic children. *Clin Exp Allergy* 2012;42:1197-205.
- E12. Blumchen K, Ulbricht H, Staden U, Dobberstein K, Beschoner J, de Oliveira LC, et al. Oral peanut immunotherapy in children with peanut anaphylaxis. *J Allergy Clin Immunol* 2010;126:83-91.e1.



**FIG E1.** Correlation of laboratory markers for sensitization, BA, and T-cell activation with severity of allergic reactions at oral challenge. There is no correlation of the individual severity of the allergic reaction at oral challenge (grade I-V) with markers for peanut-specific sensitization (peanut-specific IgE [in kilounits of allergen per liter, **A**]; Ara h 2-specific IgE [in kilounits of allergen per liter, **B**]; and peanut-specific wheal size in SPTs [in millimeters, **C**]), markers for BA (BA after *in vitro* stimulation with  $5 \times 10^{-2}$   $\mu g/mL$  peanut extract stimulation [**D**]; basophil sensitivity [**E**]), and markers for  $T_H2$ -cell activation (IL-5 production [in picograms per milliliter [**F**]] after *in vitro* stimulation with peanut extract).

**TABLE E1.** Comparison of peanut EDs at baseline DBPCFCs (30-minute interval for increasing doses) and EDs of objective symptoms during the rush protocol of OIT (2-hour interval for increasing doses) of a formerly published study on OIT<sup>E12</sup>

Patient no.	ED at baseline DBPCFC (mg of peanut)	Objective symptoms at baseline DBPCFC	Starting dose of OIT during rush protocol (mg of peanut)	Dose at first objective adverse event during rush protocol (mg of peanut)	First objective adverse event during rush protocol	Time until first objective adverse event occurred (minutes after last dose eaten)	Tolerated dose after rush protocol (mg of peanut)
4	250	V	3.2	320	V	120	160
7	1000	V, R, C, E, AE	12	320	V, U	160	160
8	250	C, cough, W	3.2	160	Cough, U	35	160
9	250	V	3.2	48	V	5	24
10	250	V	3.2	80	W, D	50	80
11	250	V, U, S	3.2	80	U, S	275	Drop out
12	500	V	6	No objective symptom during rush but repeated abdominal pain			900
13	250	V, R, C, U	3.2	220	R, C, V, S	70	120
14	130	V, E, C	1.6	No objective symptom during rush but repeated abdominal pain			30
15	60	U	0.8	80	V	110	40
16	250	E, S, reduced O <sub>2</sub> -saturation, U, C	3.2	No objective symptom during rush but OAS			140
18	130	V	1.6	140	V, C	45	40
19	1000	Cough, U	12	No objective symptom during rush			1000
20	250	W, S, U, C	3.2	No objective symptom during rush but OAS and repeated abdominal pain			180
21	2000	E, U, W, R	24	No objective symptom during rush but repeated abdominal pain			3000
22	500	Cough, W, S, R, C	6	800	W, cough, U	80	400
23	500	Cough, R, C, U	6	640	W, cough, U	250	400
24	250	Cough, W, U	3.2	80	W, cough	30	50
25	30	AE	0.4	No objective symptom during rush but OAS			140
26	130	V, C	1.6	180	U, S, cough, R, C, AE	105	140
28	1000	Cough, W, E	12	No objective symptom during rush but sneezing			1200
29	500	Flush, U	6	320	Flush, U	260	120
30	2000	Repeated V, R, C	24	No objective symptom during rush but repeated abdominal pain			1000

AE, Angioedema; C, conjunctivitis; D, diarrhea; E, erythema; OAS, oral allergy syndrome; R, rhinitis; S, shortness of breath; U, generalized urticaria; V, vomiting; W, wheeze.