

Basophil allergen threshold sensitivity and component-resolved diagnostics improve hazelnut allergy diagnosis

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Clinical & Experimental Allergy

Summary

Background IgE sensitization to hazelnut is common, especially in birch endemic areas. However, its clinical significance often needs to be confirmed by a food challenge.

Objective To evaluate the clinical significance of IgE antibodies to hazelnut components and basophil allergen threshold sensitivity (CD-sens) to hazelnut, in relation to double-blind placebo-controlled food challenge (DBPCFC) in children with a suspected hazelnut allergy.

Methods Forty children underwent a DBPCFC. CD-sens to hazelnut as well as IgE antibodies to hazelnut and its components Cor a 1, Cor a 8, Cor a 9 and Cor a 14 were analysed. Serum tryptase was measured before, during and after DBPCFC.

Results Eight children had a positive DBPCFC, and all of them had a high CD-sens value to hazelnut. Of the 32 children that passed the DBPCFC, 31 were very low or negative in CD-sens. A positive DBPCFC corresponded with significantly higher CD-sens values (median 8.9, range 3.3–281) compared to children negative in challenge (median 0.05, range 0–34.7, $P < 0.0001$). Children positive in challenge also had higher levels of IgE-ab to Cor a 9 and Cor a 14 ($P < 0.01$ and $P < 0.001$, respectively) compared with those with a negative challenge. In relation to the results from DBPCFC, the sensitivity of CD-sens and IgE-ab to Cor a 14 was excellent (100%) and the specificity was very high ($> 97\%$ and $> 94\%$, respectively). Five of the eight patients positive at challenge showed an increase in tryptase $> 20\%$ compared to tryptase baseline levels.

Conclusions and Clinical Relevance CD-sens and component-resolved diagnostics to hazelnut, used separately or in combination, may improve the diagnostic accuracy and safety and reduce overdiagnosis of hazelnut allergy.

Keywords basophils, CD-sens, flow cytometry, IgE antibody, molecular allergology, oral food challenge, tryptase

Submitted 2 September 2014; revised 9 February 2015; accepted 13 February 2015

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Cite this as: J. Brandström, A. Nopp, S. G. O. Johansson, G. Lilja, A.-C. Sundqvist, M. P. Borres, C. Nilsson, *Clinical & Experimental Allergy*, 2015 (45) 1412–1418.

Introduction

Sensitization to hazelnut is common in northern Europe and can be explained by extensive cross-reactivity to birch pollen but also to other tree nuts. Ten to seventeen percent of adults have been reported to be sensitized to hazelnut [1], but no figure is available for children.

Diagnosis of hazelnut allergy has mainly relied on patient history, analysis of IgE antibodies (IgE-ab) and/or skin prick test (SPT) to hazelnut. However, to achieve a more reliable diagnosis, oral food challenges (OFC) can be performed. An OFC is a time-consuming proce-

dure, where the patient is at risk of developing a potentially life-threatening allergic reaction, anaphylaxis. In patients coming to the emergency room, the dominant cause of anaphylaxis is peanuts and tree nuts [2]. Tryptase increases in blood during anaphylaxis in adults and can be used to confirm a suspected anaphylactic reaction when the clinical picture is unclear [3]. However, it is uncertain whether tryptase increases to the same extent in children.

Component-resolved diagnostics (CRD) have made it possible to measure IgE-ab to allergenic proteins in different allergen sources. Cor a 1, a PR-10 protein in

hazelnut, is highly cross-reactive with the main birch protein Bet v 1. Hazelnut also contains lipid transfer protein Cor a 8, 11S globulin Cor a 9 and 2S albumin Cor a 14 which seem to be better markers for systemic allergic reactions [4, 5]. A recent study showed that elevated IgE-ab levels to Cor a 9 and Cor a 14 were found to correlate with objective symptoms upon hazelnut ingestion in children [6].

In IgE-mediated allergy, mast cells and basophils are sensitized with IgE-ab. Mast cells are found in the tissue, while basophils are easily accessible in the blood. By triggering basophils *in vitro* with decreasing concentrations of allergen, the lowest triggering dose, the basophil allergen threshold sensitivity, CD-sens, can be determined [7]. Basophil stimulation, as a diagnostic test, has been introduced earlier in the diagnosis of food allergy [8–11].

In this study, we aimed to evaluate the clinical significance of presence of IgE-ab to hazelnut and to Cor a 1, Cor a 8, Cor a 9 and Cor a 14, as well as CD-sens to hazelnut when diagnosing hazelnut allergy in children using double-blind, placebo-controlled food challenge (DBPCFC) as method of reference. Given the structure of this study, we also wanted to evaluate the usefulness of repeated tryptase measurements during the challenges.

Methods

Study population

Forty children, aged 6–18 years, who were referred to Sachs's Children and Youth Hospital, Stockholm, Sweden, for oral hazelnut challenge, were invited to participate (Table 1). The inclusion criteria were as follows: the presence of IgE antibodies to hazelnut > 0.35 kU_A/L or SPT > 3 mm, a hazelnut-free diet and a referral for hazelnut challenge. Exclusion criteria were ingestion of antihistamines or steroids within 4 days prior to the challenge or history of severe anaphylaxis to hazelnut confirmed in medical records. Written consent was provided by the parents of the children, or if age > 18 years, by the patient. This study was approved by the regional ethics committee in Stockholm, Sweden (approval number 2012/990-31/3).

Hazelnut challenge

All DBPCFC were performed by experienced personnel, in a hospital setting. To achieve blinding, a dietitian made chocolate balls based on a modified, previously validated recipe for DBPCFC to peanut [12], with 11% raw hazelnut and fat content 14%. The children came to the clinic twice for challenges, one day they received placebo only and the other day active substance only,

of which the order was randomized. A minimum of 4 days separated the two challenges. Dosage started at 1 mg of hazelnut (if active challenge) and was gradually increased every 30 min (10 mg, 100 mg, 1 g, 5 g). A total of five doses were given, and the accumulative dose of raw hazelnut was 6.11 g equivalent to 800 mg of hazelnut protein. Patients were observed an additional 2 h after the final dose. A positive challenge was defined by the presence of objective symptoms scored according to Astier et al. (Table 2) [13] because subjective oral symptoms are often absent or hardly recognized by children [14]. A negative test was defined as no objective allergic symptoms at the challenge and during the following observational time. For safety reasons, and to obtain blood samples, all children had a peripheral venous catheter during challenge.

Blood sampling

Venous blood samples for CD-sens and IgE-ab analyses were taken before the first challenge was started. Whole blood for CD-sens analyses was analysed within 24 h, and serum was separated and stored in -20°C pending IgE analyses. Blood samples for analyses of tryptase were collected before and after the challenge. In the event of a positive challenge, another blood sample was taken 30–60 min after the allergic reaction.

Preparation of hazelnut extract

The hazelnut extract used for CD-sens analyses was prepared from the same raw hazelnut batch used for the chocolate balls. Ground hazelnuts were mixed with phosphate buffer pH 7.4 and incubated for 2 h in $+4^{\circ}\text{C}$. Extraction was followed by centrifugation (3100 g for 15 min), filtration and measurement of protein content.

Basophil analyses

Basophils were stimulated [7, 15] with decreasing concentrations of crude hazelnut (protein concentration range 2–200 000 ng/mL). The hazelnuts used in basophil stimulation came from the same batch as the hazelnuts used in all oral challenges. Anti-Fc ϵ RI (Bühlmann Laboratories AG, Schönenbuch, Switzerland) was used as positive control and RPMI (a cell culture media developed at Roswell Park Memorial Institute) as negative control. Leucocytes were stained for CD203c for basophil identification and CD63 for basophil activation (Immunotech, Marseille, France) and counted in a Navios flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA). The cut-off determining a positive test was set to 5% of CD63-positive basophils.

Individuals with basophils, which after stimulation with the positive control anti-Fc ϵ RI, responded with

Table 1. Patient characteristics at the food challenge

Pat ID	Age	Sex	IgE kU/L	IgE-ab Birch kU _A /L	IgE-ab Hazelnut kU _A /L	Hazelnut IgE/IgE (%)	Challenge +/- and Astier score (eliciting dose, g)
1	16	Female	907	14.2	4.6	0.5	—
2	18	Female	> 5000	455	279	5	—
3	17	Male	407	30.7	11.5	2.8	—
4	13	Female	422	27.3	8.1	1.9	—
5	10	Female	1955	35.2	35.1	1.8	—
6	8	Male	305	8.47	1.6	0.5	—
7	6	Male	1276	171	105	8.2	—
8	18	Male	284	93.1	34.7	12.2	—
9	16	Female	279	0.69	0.7	0.3	—
10	10	Male	181	54.2	10.1	5.6	—
11	6	Male	817	105	37.3	4.6	—
12	18	Female	125	12.2	8.3	6.6	—
13	15	Female	656	167	94.5	14.4	—
14	18	Male	266	3.74	4.3	1.6	—
15	7	Female	118	12.8	9.4	7.9	+ Astier 1 (1 g)
16	10	Male	961	1.02	102	10.6	+ Astier 3 (0.1 g)
17	18	Male	315	78.4	46.6	14.8	—
18	11	Male	337	52.9	28.7	8.5	—
19	17	Male	2895	73.3	29.6	1	—
20	6	Male	449	137	87.9	19.6	—
21	12	Male	109	11.5	3.8	3.5	—
22	17	Male	2368	43.3	57.6	1.8	+ Astier 2 (5 g)
23	18	Female	121	39.6	19.1	15.8	—
24	11	Male	9.13	2.61	2.4	26.3	—
25	18	Female	848	3.63	0.9	0.1	—
26	18	Female	1166	1.25	0.5	< 0.1	—
27	10	Female	308	1.93	1.3	0.4	—
28	16	Female	1030	0.89	14.6	1.4	—
29	6	Female	830	0.52	0.62	< 0.1	—
30	9	Male	2083	8.82	5.63	0.3	—
31	18	Male	401	2.31	7.23	1.8	+ Astier 3 (1 g)
32	10	Male	748	12.9	3.66	0.5	—
33	8	Female	424	0.62	13.4	3.2	+ Astier 2 (5 g)
34	8	Male	630	8.79	5.14	0.8	—
35	18	Female	1677	< 0.1	310	18.5	+ Astier 3 (5 g)
36	9	Male	12.1	< 0.1	< 0.1	< 0.1	—
37	6	Male	125	< 0.1	5.8	4.6	+ Astier 4 (5 g)
38	11	Male	2004	0.21	39.6	2	—
39	10	Female	11	< 0.1	0.63	5.7	+ Astier 1 (5 g)
40	8	Male	194	11.7	12.0	6.2	—

0–5% CD63 up-regulation, were regarded as non-responders. For individuals with a response between 5% and 16% (low responders), the results should be interpreted with caution. The cut-off of 16% was calculated (mean 76% – 3 SD) from the positive controls of an in-house reference material of 264 allergic children and adults [8].

Definition of CD-sens

To determine the basophil allergen threshold sensitivity, CD-sens, the eliciting allergen concentration giving

50% (EC50) of maximum CD63% up-regulation of the dose–response curve is first determined. CD-sens is defined as the inverted value for EC50 multiplied by 100 and is used to describe the patient's allergen sensitivity [7].

Serological analyses

IgE ('total IgE') and IgE-ab ('specific IgE') to birch, hazelnut and the allergen components Cor a 1, 8, 9 and 14 were analysed in serum with ImmunoCAP® (Thermo Fisher Scientific, Uppsala, Sweden), according to the

Table 2. Symptom score according to Astier to evaluate clinical reactions in DBPCFC

Symptom score	Symptoms
0	No symptoms
1	Abdominal pain that resolved without medical treatment, rhinoconjunctivitis or urticaria, ≥ 10 papules, rash
2	One organ involved, *abdominal pain requiring treatment, *generalized urticaria, *non-laryngeal angioedema, *mild asthma (cough, fall of peak expiratory flow 20%)
3	Two organs involved (of symptoms mentioned under 2)
4	Three organs involved (of symptoms mentioned under 2) or asthma requiring treatment or laryngeal oedema or hypotension
5	Cardiac and respiratory symptoms requiring hospitalization in the intensive care unit

DBPCFC, double-blind placebo-controlled food challenge.

instructions of the manufacturer. The ImmunoCAP IgE-ab hazelnut (f17) is based on an optimized hazelnut extract and is spiked with Cor a 1.

Tryptase was analysed with ImmunoCAP Tryptase (Thermo Fisher Scientific) according to the instructions of the manufacturer.

Statistics

IgE-ab levels and CD-sens values are presented as median and range. ROC curves were calculated, generating the cut-off levels used for specificity and sensibility. Differences between patients with positive and negative challenge were tested with Wilcoxon's rank-sum test. Statistical significance was considered at a P -value of < 0.05 . No adjustment for multiple testing has been performed. Thus, significant results should be regarded as descriptive and explorative. Statistical analyses were carried out using GraphPad Prism (version 5.01) (GraphPad Software Inc, La Jolla, California, USA).

Results

Patient characteristics

Forty children and adolescents (median age 11 years; range 6–18 years) sensitized to hazelnut at the inclusion (IgE-ab to hazelnut > 0.35 kU_A/L and/or SPT > 3 mm), 17 girls and 23 boys, went through a DBPCFC to hazelnut (Table 1).

DBPCFC to hazelnut. Eight children (20%) were positive, that is had objective symptom at the challenge, and 32 children (80%) were negative at the DBPCFC to hazelnut (Table 3). Two children required hospitalization after the food challenge and were admitted to a general paediatric ward. No child had an allergic reaction on placebo.

CD-sens to hazelnut. None of the eight children who had a positive DBPCFC were negative in CD-sens. Of the 32 children that passed the oral challenge, 24 had very low CD-sens values and seven were CD-sens

Table 3. Severity score at the challenge

	Positive DBPCFC	Negative DBPCFC
Total; n (%)	8 (20)	32 (80)
Severity score		
Grade 0	0	32 (80)
Grade 1	2 (25)	
Grade 2	2 (25)	
Grade 3	3 (38)	
Grade 4	1 (12)	
Grade 5	0 (0)	

DBPCFC, double-blind placebo-controlled food challenge.

negative (Fig. 1). The eight children with a positive DBPCFC had significantly higher levels of hazelnut CD-sens, 8.9 (range 3.3–281) compared to children negative in hazelnut challenge, 0.05 (range 0–34.7, $P < 0.0001$). If the CD-sens value was > 1.7 , the sensitivity was 100% and the specificity 97% according to ROC calculations (Fig. 2). One hazelnut-tolerant adolescent had a high CD-sens value, and one child was a low responder and did not allow further evaluation.

IgE-ab to hazelnut and hazelnut components

The median IgE-ab level to hazelnut in the whole group at the time of challenge was 9.7 kU_A/L (range < 0.10 –310). IgE-ab levels to hazelnut and Cor a 1 both correlated to the levels of IgE-ab to birch ($r = 0.79$, $P < 0.0001$ and $r = 0.98$, $P < 0.0001$, respectively). IgE-ab levels to hazelnut did not differ with statistically significance between those positive in food challenge and those with a negative food challenge (Fig. 3). However, IgE-ab levels to Cor a 9 and Cor a 14 were significantly higher in children positive, compared to children negative, at challenge. Thus, median IgE-ab levels to Cor a 9 in children positive at the challenge were 4.5 kU_A/L (range 0.7–97.5) and in children negative at challenge 0.1 kU_A/L (range < 0.10 –36.2, $P < 0.01$). The corresponding IgE-ab levels to Cor a 14 in children positive at the challenge were 5.6 kU_A/L (range 0.9–78.7) compared to children negative at challenge, 0.04 kU_A/L (range < 0.10 –13.9, $P < 0.001$ (Fig. 3). If the level of

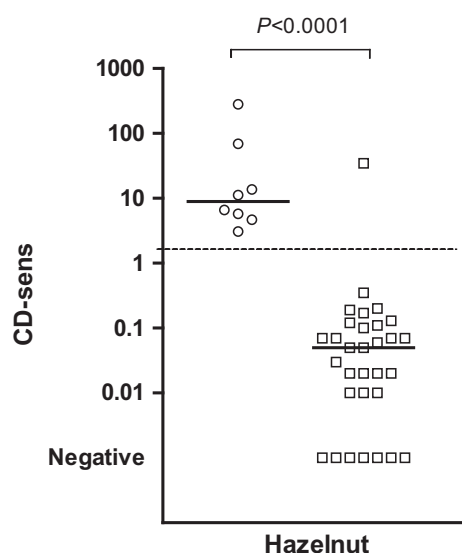


Fig. 1. CD-sens to hazelnut in children with a positive (○) or negative (□) DBPCFC. Horizontal bars, median. The dashed line shows the CD-sens value 1.7 obtained from ROC calculations. DBPCFC, double-blind placebo-controlled food challenge.

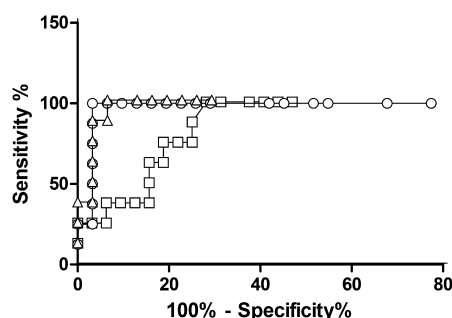


Fig. 2. ROC curves showing specificity for CD-sens > 1.7 to hazelnut (○), IgE-ab to Cor a 14 > 0.65 kU_A/L (△) and IgE-ab to Cor a 9 > 0.65 kU_A/L (□).

IgE-ab to Cor a 14 was > 0.64 kU_A/L, the sensitivity was 100% and the specificity 94%. For Cor a 9, > 0.65 kU_A/L, the corresponding sensitivity was 100% and the specificity 72% according to ROC calculations (Fig. 2). There were no significant differences in levels of IgE-ab to Cor a 1 or Cor a 8 (Fig. 3).

Tryptase in children positive at the hazelnut challenge

All tryptase values were within the reference interval (< 11.4 µg/L) in both children positive and negative at challenge. However, of the eight patients with objective symptoms, five showed an increased serum tryptase value that reached a concentrations > 20% higher than the baseline levels, while having objective symptoms. Two of the 32 hazelnut-tolerant patients had a > 20% increase of tryptase after the food challenge. One of

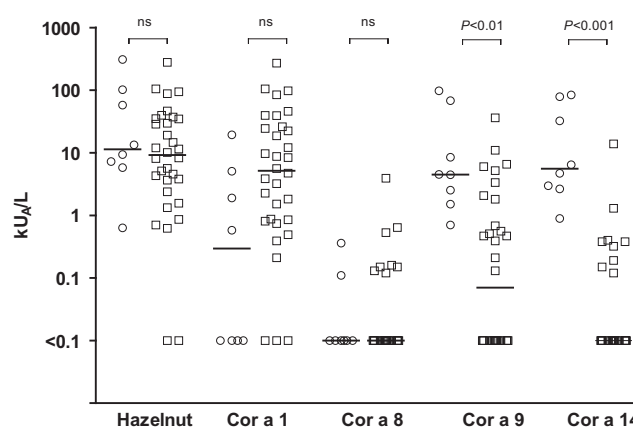


Fig. 3. IgE-ab to hazelnut and hazelnut components in children with a positive (○) or negative (□) DBPCFC. Horizontal bars, median; ns, not significant. DBPCFC, double-blind placebo-controlled food challenge.

them had this increase after placebo challenge without reporting any symptoms. The other patient had a > 20% increase after active challenge and reported mild oral pruritus after the final dose of 5 g hazelnut. The baseline tryptase level for this patient was only 1.8 µg/L, reaching 2.3 µg/L after challenge.

Discussion

Children positive at DBPCFC had significantly higher CD-sens values to hazelnut and significantly higher levels of IgE-ab to the hazelnut components Cor a 9 and Cor a 14 compared to those negative at challenge. These tests show good ability to discriminate between IgE sensitization and allergy to hazelnut with objective symptoms. Implementing these tests could reduce the need for OFC.

Standard diagnostic testing for hazelnut allergy, that is SPT or measuring specific IgE-ab to hazelnut, has high sensitivity, but low specificity, mostly due to the allergenic cross-reactivity with birch pollen. In our study, as well as in other studies, many individuals who are IgE-sensitized to foods are negative at challenge [16, 17]. However, previous studies of IgE-ab and food allergy have shown that higher levels of IgE-ab to the allergen of interest correlate with a higher probability of reaction upon exposure [17]. In this study, no significant differences of IgE-ab levels to hazelnut, in relation to the outcome of DBPCFC, were seen, neither could we observe any tendencies in this direction, for instance 4/8 (50%) patients with a positive DBPCFC had IgE-ab to hazelnut of less than 10 kU_A/L. The same percentage was seen in the non-reacting group that is 16 of 32 of the hazelnut-tolerant children had hazelnut IgE-ab levels above 10 kU_A/L. Low specificity may cause overdiagnosis, which can lead to a lower quality

of life and unnecessary diet restrictions. Thus, there is a need of better diagnostic methods.

Since a couple of years, component-resolved diagnostics for hazelnut are available. In our study, sensitization to both Cor a 9 and Cor a 14 was present in all children having objective symptoms at the DBPCFC. The results are very promising showing that IgE-ab to Cor a 14 > 0.64 kU_A/L give a sensitivity of 100% and a specificity of 94%, and for Cor a 9 > 0.65 kU_A/L, the sensitivity was 100% and the specificity 72% according to ROC calculations. However, our results are based on only eight children positive in hazelnut challenge.

In our study, as well as in a retrospective study from the USA, IgE-ab to Cor a 14 seems to outperform IgE-ab to Cor a 9 when it comes to discriminate between children sensitized or allergic to hazelnut [18]. However, this is in contrast to the study from Masthoff and colleagues where IgE-ab to Cor a 9 was the best to discriminate for hazelnut allergy [6]. Another study, from Belgium, draws the conclusion that both Cor a 9 and Cor a 14 are of comparable importance [19]. It should be emphasized that all these studies, including the present study, draw the conclusions that both components are of great importance in hazelnut allergy diagnostics. In the Masthoff study, there was an age-depending variability concerning both IgE-ab to Cor a 9 and to Cor a 14 where allergic adults were less sensitized to these allergen components. The child group in their study was younger than in our study which can be one reason for the differences. The two studies were also performed in different populations, Scandinavia and central Europe.

In the present study, we evaluated the lowest dose of hazelnut allergens triggering the basophils, CD-sens [7, 15]. This method is a well-documented marker for basophil sensitivity and in contrast to methods measuring basophil reactivity [9, 20], CD-sens will give information about the allergen sensitivity, that is how much allergen the basophils will tolerate before they react. CD-sens is an excellent measure of the patient's allergen sensitivity and has been launched as a safe and relevant complement to allergen provocation tests in allergic rhinoconjunctivitis [21], asthma [22] and food allergy [8]. In the present study, one child, negative in DBPCFC, was a 'low responder' in CD-sens, that is the basophils response to anti-FcεRI, which was used as the positive control, was $< 16\%$. For low responders and non-responders, we have to rely on other diagnostic tests. In addition, one patient tolerant in hazelnut challenge had a positive CD-sens. This patient was sensitized to Cor a 14 (1.3 kU_A/L) and had detectable but low levels of IgE to Cor a 9 (0.39 kU_A/L). At a recent clinical follow-up, the patient claimed that he was tolerating hazelnuts. One might speculate that this individual either has been truly allergic to hazelnut or has recently become tolerant and that IgE antibodies to Cor

a 14 values are lagging. One might also speculate about the opposite that he is at risk of developing hazelnut allergy later on.

In the present study, the investigation of the basophil allergen threshold sensitivity *in vitro* seems to be a good method for discriminating between hazelnut allergy and IgE sensitization. If the CD-sens value was > 1.7 , the sensitivity was 100% and the specificity 97% according to ROC calculations. Of highest importance is that no false-negative CD-sens tests were seen. Additional basophil studies have shown similar results for other foods, that is peanuts and cow's milk [8, 11]. Using CD-sens, the authors could discriminate between children reacting to cooked vs. raw milk, and they also showed that children with more severe clinical cow's milk reactivity had a higher CD-sens [11]. However, in our study, the patients who were positive at the challenge were too few to allow us to perform calculations concerning severity score.

Of great interest is that our study indicates that CD-sens is a test that provides a positive predictive value (PPV) and specificity far better than serological routine testing. In the present study, this can be exemplified by comparing 'IgE-ab to hazelnut > 10 kU_A/L' which generates a PPV of only 20% for reaction in food challenge, whereas a CD-sens value of > 1.7 results in a PPV of 89%. This noticeable difference can only partially be explained by the fact that the ImmunoCAP IgE-ab hazelnut (f17) is spiked with Cor a 1.

The majority of children, 5 of 8, positive at challenge showed a $> 20\%$ increase in tryptase after the allergic reaction. The interindividual differences at baseline levels were quite large, and a single tryptase level taken in the emergency room might not be enough to prove a recent event of anaphylaxis, but the tendencies of rising tryptase values were seen in more than half of our patients with allergic symptoms at DBPCFC. We encourage future studies that address tryptase levels both in the setting of food challenges and at emergency rooms.

The main limitation of this study is the size of the study population and that only 20% of the patients had a positive DBPCFC. This hampers statistical analysis, when it comes to recommending cut-off values and calculating sensitivity and specificities. However, this illustrates the, far from acceptable, ability to accurately distinguish between hazelnut allergy or hazelnut sensitization in clinical routine. Another obstacle is that hazelnuts contain more fat than, for example, peanuts and this must be taken into account when evaluating a challenge because the fat content may affect the allergen uptake. The more the fat, the longer the time for allergen uptake; therefore, a short interval between doses at the challenge should be avoided. It can be discussed whether a negative DBPCFC should be followed by an open challenge. It is recommended in some papers, but

many studies do not perform this. In our study, we have not followed up the negative DBPCFC with an open one. The strengths of this study are that all children underwent DBPCFC, with a cumulative dose of hazelnut protein of 800 mg, which is in line with previous studies based on blinded hazelnut challenges [6, 23] and that material for CD-sens and IgE-ab tests were obtained the very same day and from the same source. Another strength is that tryptase levels were measured at all food challenges, that is both with ingestion of hazelnut and placebo, thus giving us a more precise idea of the specific individuals' normal tryptase levels.

This study indicates that a large proportion (80%) of children with a doctor's diagnosis of hazelnut allergy

and on a hazelnut-free diet may not react at a hazelnut dose of 6.11 g, thus emphasizing the need for improved diagnostic tests. We conclude that CD-sens to hazelnut and component-resolved diagnostics may improve the diagnostic accuracy when dealing with suspected hazelnut allergy and could reduce the number of OFC. However, more studies are needed to confirm these results.

Conflict of interest

Magnus Borres is employed by Thermo Fisher Scientific, Uppsala Sweden. The other authors declare no conflict of interest.

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