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Impact of native, heat-processed and encapsulated hazelnuts on the allergic response in hazelnut-allergic patients

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Clinical and Experimental Allergy

Summary

Background Pollen-associated food allergy is common. However, systemic reactions or even life-threatening anaphylaxis are rare.

Objective The aim of this study was to investigate the clinical impact of native, heat-processed and encapsulated hazelnuts (HN) in patients with proven HN allergy.

Methods One hundred and thirty-two patients with a positive history of HN allergy were recruited. Sensitization was confirmed by a skin prick test (SPT) and sIgE against HN. After an HN-free diet, double-blind placebo-controlled challenges were performed with increasing amounts of native and roasted HN. A subset of patients were given HN capsules to circumvent the oral mucosa. Basophil activation was measured by flow cytometry before and after provocation but also *ex vivo* using native and roasted HN extracts.

Results Three groups of HN-allergic patients were identified depending on their clinical reaction pattern. The dosages by which allergic reactions were elicited varied for native HN from 0.01 to 2.0 g, with a median of 0.1 g, for roasted HN from 0.01 to 10.0 g, with a median of 0.23 g, and for encapsulated HN from 0.1 to 3.0 g, with a median of 0.3 g. Accordingly, the SPT was more frequently positive and resulted in greater weal reactions if native HN was used. This finding was confirmed by *ex vivo* basophil activation showing that significantly higher allergen extract concentrations (roasted > native) were necessary to induce 50% basophil activation.

Conclusion Our data show that heat processing of HN reduces its allergenicity. SPT but also the basophil activation test can be used to determine the reactivity of an allergen extract.

Keywords atopic dermatitis, basophil activation, double-blind placebo-controlled food challenge, hazelnut allergy, heat processing, oral allergy syndrome, sIgE, skin prick test, systemic reaction

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Introduction

Adverse reactions to food are common and are caused by various mechanisms. Food allergy affects approximately 2–3% of the general population in industrial countries. In a recent study, a prevalence of 2.5% of IgE-mediated food allergy in Germany was determined [1]. The most frequent food allergens, in infants and children are egg, cow milk, soy and wheat. The development of tolerance, 1–2 years after avoidance of these food allergens, is possible, but seems to depend on the sensitization pattern towards particular epitopes of the corresponding allergen [2].

The most frequent cause of IgE-mediated food allergy in adults is a pre-existing pollen allergy. Owing to similarities between proteins of pollen and plants, cross-reactivity is possible. Birch pollen are one of the most important allergens in Europe and birch pollen-allergic patients may suffer from food allergy to hazelnut (HN), apple, carrot or celery due to cross-reactivity [3, 4]. The estimated prevalence of HN allergy in the European population is 0.1–0.5% [5]. The major allergen of birch pollen is Bet v 1 and that of hazel pollen is Cor a 1. The corresponding allergen in HN is Cor a 1.04, which belongs to the pathogenesis-related protein family 10 [6, 7]. This

protein family shares a high amino acid sequence identity with Bet v 1. In Northern Europe, most cases of birch pollen-related HN allergy are caused by this cross-reactivity. However, other allergens like Cor a 2 (profilin), Cor a 8 (lipid transfer protein = LTP), Cor a 9 (11S globulin), Cor a 11 (vicilin-like protein), 2S albumin and a thaumatin-like protein have also been described previously [8–11]. Food-allergic patients with a history of severe allergic reactions need to avoid the offending food stringently [12]. However, insufficient labelling of food products may be the reason for unexpected severe allergic reactions [13]. The EU-directive 2000/13/EC, last amended by directive 2006/142/EC, lists 14 major food allergens, including HN, which have to be declared on packed food [14–16]. As the amount of an allergen within a food product plays a crucial role in whether sensitized patients may experience a clinical reaction, the relevance of oral threshold levels has become a matter of debate [12, 14, 17]. Moreover, for the estimation of the allergenicity of industrially prepared food products, food processing and its influence on the allergen stability thresholds levels for food-allergic reactions are important. Recent studies indicate that the manner of food treatment may alter the protein and therefore the allergen structure [18]. The roasting process leads to a decrease of allergenicity in HN [19], but e.g. to an increase in the allergenic activity of peanuts. On the other hand, it has been shown that cooking methods like boiling or frying may also reduce the allergenic property of peanuts [20].

The aim of this study was to identify HN-allergic patients regarding their clinical reaction pattern and to determine their reactivity levels depending on the processing of HN. The level of basophil activation *in vivo* before and after provocation was determined to evaluate the usefulness of CD203c expression during a provocation test.

Methods

Subjects

One hundred and thirty-two adult patients with a history of birch pollen allergy and of adverse reactions to HN were recruited at the outpatient clinic of dermatology. Each subject underwent a thorough medical history. The reported symptoms included oral allergy syndrome (OAS), systemic reactions (SR) like dyspnoea or urticaria or aggravation of atopic eczema after the consumption of HN. Exclusion criteria for further analysis were a concomitant severe disease, pregnancy, lactation, the use of antihistamines, β -blockers, ACE inhibitors or immunosuppressive drugs. The study was approved by the local ethics committee and all participants gave written informed consent before participation in the study. A group

of age-matched skin healthy, non-allergic volunteers ($n = 13$) served as a control group for the basophil activation analysis.

Preparation of the hazelnut extracts

Allergen extracts were prepared from native and roasted (144 °C) HN as described previously [18]. HN were crushed and then ground to a fine powder. After extraction with ammonium carbonate, the crude extract was filtered and stored until use at –20 °C.

Skin prick test and immunoglobulin E determination

Participants received skin prick tests (SPT) with native HN and roasted HN (prick to prick), with prepared HN extracts at different dilutions (1 : 2, 1 : 10 and 1 : 100) and also with a commercial extract (ALK Scherax, Hamburg, Germany). Histamine (10 mg/mL) and sodium chloride (0.9% NaCl, ALK Scherax) served as positive and negative controls, respectively. The reactions were documented after 15 min. SPT were considered positive if the diameter of the weal was ≥ 3 mm [21].

To confirm the specific sensitization, a blood sample was taken for the determination of total IgE and specific IgE (birch pollen, HN) using the Phadia CAP System (Uppsala, Sweden). The determination of specific IgE for HN was performed with extracted as well as with spiked material.

Double-blind placebo-controlled food challenge

After an HN-free diet, for at least 1 week, a double-blind placebo-controlled food challenge (DBPCFC) was performed in each patient according to the recommendations of the EAACI position paper [20]. The ingredients for the provocation test were the following: 100 mL water, 15 g bitter cocoa powder (Krüger GmbH, Bergisch Gladbach, Germany), 10 g sugar, 25 g rice flakes (Demeter, Bauckhof, Rosche, Germany), 15 g SINLAC[®], a nut-, soy-, milk- and gluten-free infant formula (Nestlé, Frankfurt am Main, Germany) and 40 mL peppermint syrup (Monin[®], Birmensdorf, Switzerland). For the verum provocation, the following dose steps of finely ground native HN were given: 0.01–0.02–0.03–0.05–0.1–0.2–0.4–1.0–2.5–5.0–10.0 g. Instead of HN, ground rice flakes were added for the placebo provocation. The DBPCFCs were titrated with a time interval of 15 min between each provocation dose to determine the individual oral threshold dose for HN in each patient.

Because in daily life an exposure to roasted rather than native HN is more frequent, a DBPCFC with roasted, finely ground HN was performed additionally in 20 patients using the recipe described above. Finally, we exposed a subgroup of patients ($n = 30$) to capsules containing HN

flour to circumvent the oral mucosa. In both cases, the threshold determined in the first DBPCFC was decisive for the first challenge dose.

Before and after each single challenge, vital parameters like blood pressure, heart frequency and the forced expiratory volume in 1 s (FEV₁) were measured.

Basophil activation in vivo and ex vivo

For the measurement of *in vivo* basophil activation, 5 mL of heparinized blood was obtained from 51 patients before and after the provocation procedure. In the control group, the blood was obtained before and 60 min after the ingestion of 10 g native HN. Analysis was performed as described below.

For the measurement of *ex vivo* basophil activation, blood from six patients with a clinical history of birch pollen and HN allergy was collected. Cells were stimulated *ex vivo* with native and roasted HN extract. In each experiment, different allergen doses were used (56 ng, 560 ng, 5.6 µg, 56 µg, 560 µg and 2.8 mg) in comparison with unstimulated cells as negative control. Cell stimulations with anti-IgE (anti-IgE HP 6029/HP 6061, Calbiochem, San Diego, CA, USA; 50 µg/mL) and buffer (NH₄⁺H⁺CO₃²⁻) served as positive and negative controls, respectively. Cells were stained for 45 min with anti-CD203c-PE (Beckman Coulter, CA, USA) with 2 µL Beriglobin, (Aventis Behring, Marburg, Germany). Activated basophils were analysed by flow cytometric analysis and data were analysed using the 'FACS Cell Quest' programme.

Scoring of atopic dermatitis

Pollen-related food allergens may induce flares in patients with atopic dermatitis (AD) [22]. Therefore, the skin status of HN-allergic patients suffering from AD was assessed using the severity scoring of atopic dermatitis (SCORAD) before and 48 h after each provocation by the same

dermatologist. A clinically relevant improvement or aggravation of skin symptoms was defined by a change of ≥ 10 SCORAD points, respectively.

Statistical analysis

The data from the basophil activation were evaluated by applying the Wilcoxon tests and the Mann-Whitney *U* test, respectively. Differences in thresholds between the groups were evaluated by the Mann-Whitney *U* test and the Kruskal-Wallis test, respectively. The threshold levels dependent on the processing of the HN were evaluated by the Wilcoxon test. *P*-values ≥ 0.05 were considered statistically significant. Calculations were performed with SPSS 11.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinical profile of hazelnut-allergic subjects

One hundred and thirty-two patients (61 female, age: 21–65 years) with a history of allergic reactions to HN and a positive SPT and/or elevated HN-specific IgE were included in the study. Forty-two patients were withdrawn due to incompletion or negative SPT results. After the DBPCFC with HN, three groups were identified: the first group had an OAS (*n* = 58), the second group suffered from AD (*n* = 18) and the third group had SR, such as urticaria or dyspnoea (SR, *n* = 14) (Table 1).

The total IgE levels were higher in the AD group, with a median of 697 kU/L and a range from 3 to 2772 kU/L. By contrast, the total IgE levels were significantly lower in the OAS and the SR group (median 117 and 86 kU/L, respectively; *P* = 0.042, Kruskal-Wallis test). Specific IgE for HN with extracted material was low in all three patient groups (median ≥ 1.5 kU/L) but varied between ≤ 0.35 and 18.5 kU/L. Thirty-four patients had sIgE ≤ 0.35 kU/L (23 OAS, five AD and six SR). No significant differences were present among the three groups (*P* > 0.05, Kruskal-

Table 1. Age and sensitization pattern of the patient and control group (median, min and max are shown)

Patient groups (<i>n</i>)	Age (years)	Total IgE (kU/L)	sIgE birch (kU/L)	sIgE HN (extracted) (kU/L)	sIgE HN (spiked) (kU/L)
OAS (58)	41 (21–60)	117 (5.4–614)	21 (1.55–100)	0.44 (< 0.35 –6.5)	(<i>n</i> = 47) 10.5 (0.99–87.0)
AD (18)	34 (22–56)	697 (3–2772.2)	22 (< 0.35 –100)	1.44 (< 0.35 –18.5)	(<i>n</i> = 9) 18.3 (0.01–101.0)
SR (14)	44 (27–65)	86 (21–577)	14 (< 0.35 –100)	0.47 (< 0.35 –1.76)	(<i>n</i> = 11) 5.04 (0.01–66.6)
Controls (13)	33 (23–46)	27 (2–175)	0 –	0 –	0 –

AD, atopic dermatitis; HN, hazelnuts; OAS, oral allergy syndrome; SR, systemic reactions.

Wallis test). The median of specific IgE in an assay using spiked material, i.e. selected HN allergens was 11.1 kU/L and significantly higher than the specific IgE reactive with extracted material. As with extracted material, these sIgE values were not significantly different from among the three clinical groups ($P > 0.05$, Kruskal–Wallis test).

The specific IgE values (median) for birch were comparable within the three groups and were 21 kU/L in the OAS, 22 kU/L in the AD and 14 kU/L in the SR patients ($P > 0.05$, Kruskal–Wallis test) (Table 1).

Healthy individuals without a history of allergic sensitization ($n = 13$, age: 23–46 years) served as a control group. The total IgE was 27 kU/L (median) and the sIgE HN was negative.

Eighty-eight of 90 patients had a positive SPT with native HN or the commercial HN extract. The SPT with roasted HN was negative in $> 50\%$ ($n = 61$) of the HN-allergic patients, suggesting a reduced reactivity of processed HN (Table 2a). Furthermore, the prick-to-prick test with native HN led to stronger reactions than the commercial extract (data not shown). The comparison of the SPT results within the three clinical groups showed that patients with SR had more frequently greater SPT reactions (Table 2b).

Threshold levels in hazelnut-allergic patients

Next we analysed in each patient the amount of native HN eliciting a clinical reaction. The threshold level defined as the single amount of native HN eliciting symptoms ranged from 0.01 to 2.0 g, with a median of 0.1 g ($n = 82$). The

Table 2a. Results of SPT with native and roasted HN, stated as diameter of the weal (mm) and patient number as absolute value and as percentage

SPT (diameter of the weal)	Native HN	Roasted HN
Negative	5 (6%)	61 (68%)
3–4 mm	12 (13%)	17 (19%)
4–6 mm	32 (36%)	10 (11%)
> 6 mm	41 (45%)	2 (2%)

HN, hazelnuts; SPT, skin prick test.

thresholds after the oral provocation test with roasted HN varied between 0.01 and 10.0 g, with a median of 0.23 g ($n = 17$). We also performed provocation tests with capsules to circumvent the oral mucosa. These experiments revealed that the threshold levels after the provocation with HN-filled capsules ($n = 24$) were threefold higher (0.1–3.0 g, median 0.3 g). Note that the lowest eliciting dose of encapsulated material was even increased by 10-fold, as compared with native HN (0.1 vs. 0.01, Fig. 1). Eight of 90 study subjects had no reaction after the intake of up to 10 g HN.

The most frequent clinical outcome of the titrated oral provocation test with native HN of all patients was OAS (78/82; 95% of patients). The provocation with native HN within the AD group showed that 12/18 (67%) had OAS, two (11%) developed skin itching and erythema and one developed rhinoconjunctivitis. None of the AD patients developed an aggravation of eczema, i.e. an increase in the SCORAD by ≥ 10 points after HN challenge (data not shown). In the SR group 2/14 (14%) showed erythema and pruritus, 12/14 (86%) showed SR-like rhinoconjunctivitis (7/12; 58%), flush symptoms (3/12; 25%) and a decline in FEV₁ by $> 20\%$ (2/12; 17%).

Similar clinical patterns were observed when roasted HN or capsules with HN were used. However, in both cases (roasted HN or HN capsules) the frequency of symptoms was decreased and the threshold levels were, in most cases, higher than with native HN.

The threshold levels according to the clinical reaction pattern (three groups) did not differ significantly between these three groups ($P > 0.1$) (data not shown). In the OAS group, the lowest challenge dose was 0.01 g, in AD patients 0.05 g and in the SR group 0.02 g. In the OAS group, statistically significant differences between the thresholds for native, roasted or encapsulated HN were found, respectively ($P < 0.05$) (data not shown).

No significant change in lung function, FEV₁% was measured in the three groups after DBPCFC with native HN ($P > 0.05$). As mentioned above, in two HN-allergic patients of the SR group we observed a decline in FEV₁ $> 20\%$ (data not shown).

Table 2b. Results of SPT with native and roasted HN in the different patient groups, stated as diameter of the weal (mm) and patient number as absolute value and as percentage

SPT (diameter of the weal)	OAS ($n = 58$)		AD ($n = 18$)		SR ($n = 14$)	
	Native HN	Roasted HN	Native HN	Roasted HN	Native HN	Roasted HN
Negative	2 (4%)	39 (67%)	1 (6%)	13 (72%)	2 (14%)	9 (64%)
3–4 mm	8 (14%)	11 (19%)	2 (11%)	2 (11%)	2 (14%)	4 (29%)
4–6 mm	23 (40%)	7 (12%)	7 (39%)	2 (11%)	2 (14%)	1 (7%)
> 6 mm	25 (42%)	1 (2%)	8 (44%)	1 (6%)	8 (58%)	0

HN, hazelnuts; SPT, skin prick test.

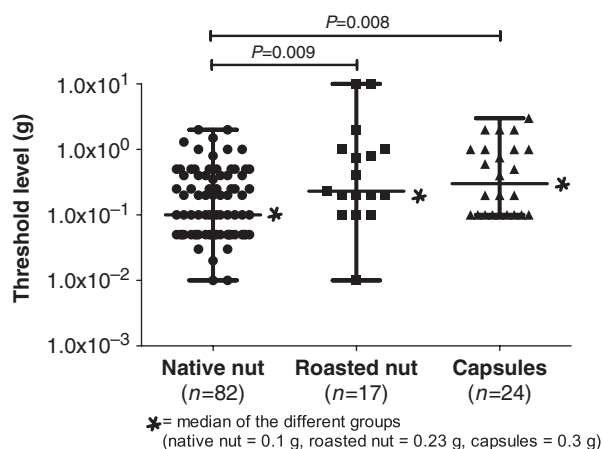


Fig. 1. Oral threshold levels (g) of native and roasted hazelnut (HN) and HN ingested in capsules.

The correlation of threshold levels and SPT results showed a clear trend in which patients with larger wheals in the SPT with native HN also had lower threshold levels ($P = 0.051$) (data not shown).

Basophil activation *in vivo* and *ex vivo*

The expression of the basophil activation marker CD203c was determined in 51 patients to evaluate basophil activation *in vivo* before and after the DBPCFC with native HN. Basophil activation was also measured in the control group before and after the consumption of 10 g native HN (open challenge) (Figs 2a and b). The data show that HN-allergic patients had a low but statistically significant increase of *in vivo* CD203c expression after the oral intake of the threshold amount of native HN (median of 14.7% ranging from 1.6% to 45.5% vs. the median of 17.9% ranging from 1.7% to 57%) ($P < 0.001$, Fig. 3). The difference in the level of basophil activation before provocation between HN-allergic patients and controls was statistically significant ($P = 0.013$). No significant increase in basophil activation after the ingestion of 10 g HN was detected in the control group (median of 4.7% ranging from 1.3% to 30% vs. the median of 5.5% ranging from 1.4% to 37.6%).

To determine the allergenicity of native and roasted HN by *ex vivo* analysis, basophil activation was analysed after stimulation with native and roasted HN extracts. These data reinforce the SPT results because they show that the roasting of HN results in a reduction of reactivity, as 50% basophil activation was reached at significantly lower concentrations of native HN extract compared with roasted HN extract ($AC_{50(\text{native})} = 0.15 \mu\text{g/mL}$; $AC_{50(\text{roasted})} = 8.2 \mu\text{g/mL}$; Fig. 4).

Discussion

An increasing prevalence of food-dependent anaphylaxis is a matter of concern, especially because of the possible

presence of hidden traces of allergenic foods in several food products [13, 14]. In the present study, we performed DBPCFC to determine threshold levels in HN-allergic patients. Native and roasted HN but also capsules filled with ground HN were used for the challenges in order to analyse the role of different application forms of HN in the allergenicity and its impact on the clinical response. The exposure with native HN resulted in positive reactions in 82/90 (91%) patients. Although the remaining eight patients had a positive SPT to native HN and 4/8 also had positive sIgE, they developed no symptoms after ingesting 10 g native HN, indicating that these eight patients are HN tolerant.

Within the OAS group, statistically significant different threshold levels were determined depending on whether HN was given in its native form, roasted or in capsules. In the other two groups, the numbers of patients tested with the different materials were small (two AD and three SR patients) and no further statistical analysis could be performed.

Seventeen of 20 patients developed OAS after the DBPCFC with roasted HN. Although the range (min/max) of threshold levels with roasted HN was similar to native HN, the majority of patients developed symptoms at higher HN doses. Moreover, after the challenge with native HN, the SR patients who developed symptoms like tachycardia, hypotension or urticaria did not develop symptoms after the challenge with roasted HN. This observation demonstrates that the allergenicity of HN is reduced by roasting and indicates its thermolability as reported in previous studies [18, 19, 22]. Our findings support the study results by Skamstrup Hansen et al. [19], who also reported that the allergenicity of HN is reduced by the roasting processes. However, these observations do not indicate that roasted HN are harmless to all HN-allergic consumers [19].

The challenge with capsules was performed in 30/90 patients; 18 OAS, eight AD and four SR patients, respectively. Most interestingly, the majority of patients developed OAS after DBPCFC using capsules although the aim was to avoid this early-phase symptom by circumventing the oral mucosa. This finding suggests that mast cells in the oral mucosa are activated via intestinal allergen absorption and transportation through the blood stream. This finding is of high importance as many authors regard OAS as contact urticaria, postulating that the digested allergens are not capable of inducing SR. This hypothesis is contradicted by our results. The provocation with capsules elicited OAS, suggesting this reaction as a manifestation of a systemic response. Such reactivity patterns are also seen in drug allergy where, upon systemic provocation, some patients react solely with specific organ symptoms e.g. skin rash or bronchial obstruction. Possible explanations are locally increased levels of sIgE or factors influencing the vascular permeability for blood-borne allergens in specific locations and/or organs.

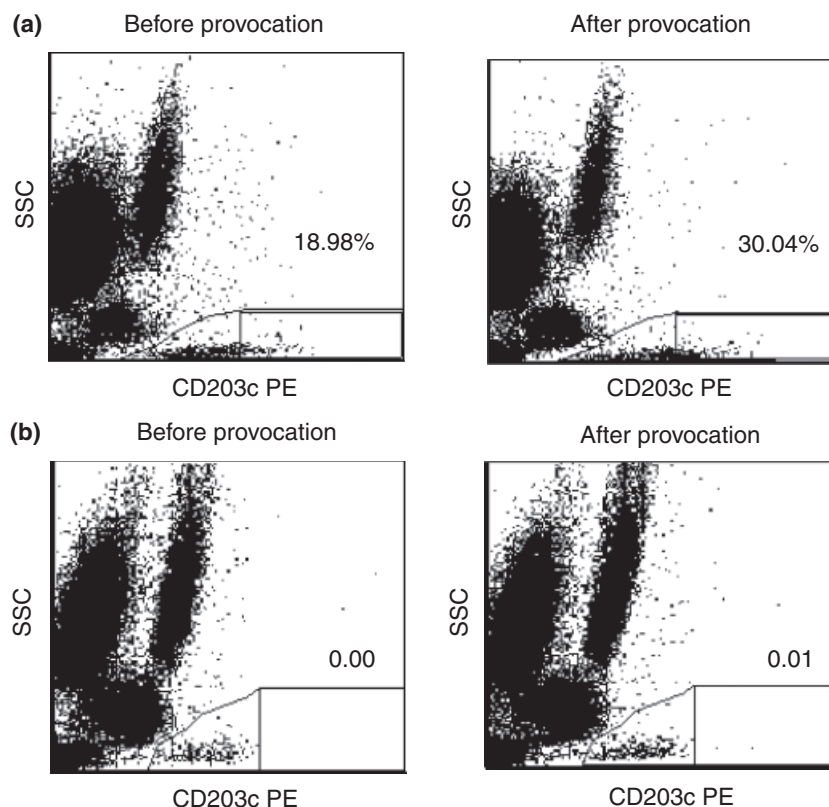


Fig. 2. (a) CD203c expression in one patient before and after hazelnut (HN) provocation. (b) CD203c expression in one control individual before and after HN provocation.

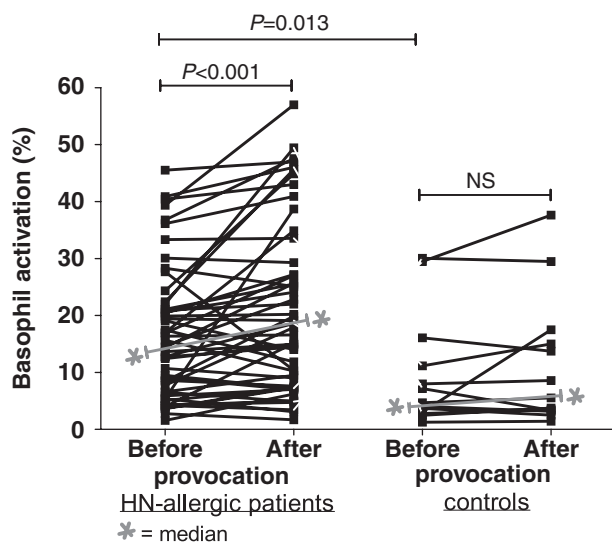


Fig. 3. *In vivo* basophil activation in hazelnut (HN)-allergic patients after native HN provocation in comparison with the control group (after intake of 10 g HN).

Contrary to our expectations, none of the AD patients developed an aggravation of eczema. This might be due to the low doses of HN that might be sufficient to elicit an OAS but no eczema. Other studies have indicated that

aggravation of AD by ingestion of pollen-associated food is possible [23–25].

The attempt to correlate cut-off levels [26] of specific IgE for HN to predict a clinically relevant reaction failed. The data analysis revealed no correlation between HN sIgE and the SPT result or the oral sensitivity to HN. The patients in this study developed only mild symptoms after the challenges. Anyhow, a systemic activation cannot be excluded as the *in vivo* basophil activation seen after the oral HN challenge is highly suggestive of a systemic activation in these patients. A statistically significant increase in basophil activation was observed in the OAS group only, which might be explained by the different threshold doses. In the OAS group, a twofold higher amount of HN (median) was needed to elicit symptoms. This leads to the hypothesis that only higher amounts of HN can induce an SR and concurrently activate basophils in the blood stream.

In recent studies, the determination of CD203c expression was found to be a novel diagnostic tool [27, 28]. It is based on the allergen-mediated up-regulation of CD203c on the cell surface of basophils. Because CD203c is exclusively expressed by basophils in the peripheral blood, no other markers are necessary to prove basophil activation [29]. Up to now, the measurement of CD203c

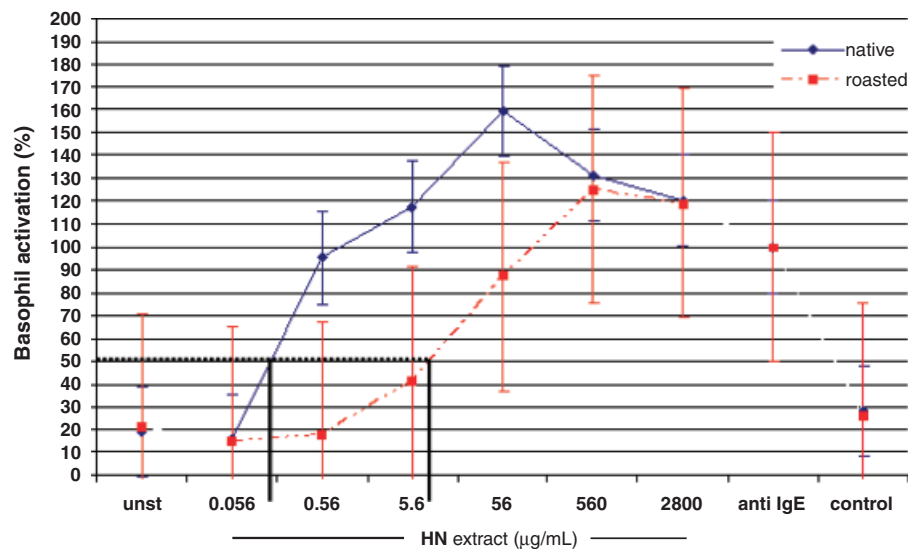


Fig. 4. *Ex vivo* basophil activation using native and roasted hazelnut (HN) extracts at different allergen concentrations (x-axis), in comparison with unstimulated cells and, as positive control, anti-IgE- and buffer-stimulated cells.

expression was used only for *in vitro* analyses. Our data suggest that CD203c expression may also be used as a predictive marker for clinically relevant food allergy. However, this hypothesis will require further investigations.

In summary, oral threshold levels for HN indicate a wide individual range. The low dose of HN inducing OAS might protect affected individuals from the onset of SR by leading to avoidance of the ingestion of higher amounts of HN. More importantly, the patients tolerated roasted HN at higher amounts, suggesting that the ingestion of processed HN may be of lower risk for the majority of HN-allergic patients. Nevertheless, an HN-allergic individual should exercise caution because thresholds are individually different and may vary over time. Also, even roasted HN may lead to severe allergic reactions. In conclusion, our data have an important impact on the risk assessment of HN-containing food for HN-allergic patients.

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