Hazelnut allergy in children and adults:

diagnosis and underlying mechanisms

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Lay-out kaft: Eefke Brandsma
Lay out and printed by: Optima Grafische Communicatie, Rotterdam, The Netherlands

ISBN: 978-94-6169-463-8

HAZELNUT ALLERGY IN CHILDREN AND ADULTS: DIAGNOSIS AND UNDERLYING MECHANISMS

Hazelnootallergie bij kinderen en volwassenen: diagnostiek en onderliggende mechanismen (met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op vrijdag 20 december 2013 des middags te 4.15 uur

door

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geboren op 2 januari 1983 te Sittard

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Het drukken van dit proefschrift werd mede mogelijk gemaakt met financiële steun van Thermo Fisher Scientific\IDD, AbbVie, ABN-AMRO, ALK-Abéllo BV, Fagron BV, Galderma Benelux B.V., Infection & Immunity Center Utrecht, LeoPharma

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Chapter 1

General introduction

FOOD ALLERGY: PREVALENCE AND IMPACT

Allergies are among the ten most common diseases and health issues. However, in the case of food allergy, the perceived prevalence largely exceeds the accurately confirmed prevalence. The self-reported prevalence of food allergy is around 10-20%[1-3]. Accurate diagnostic procedures have shown that food allergic disorders affect 6-8% of children in their first 3 years of life[4;5] and the prevalence decreases over the first decade. It is estimated that about 2-4% of the adult population is affected with food allergies[3;6]. In contrast to food allergies like cow's milk and hen's egg allergies, which in 80-85% of the cases will be outgrown in the first 5-10 years of life, only about 20% of children with peanut allergy[7;8], and 9% of children with tree nut allergy will outgrow their allergy[9]. For most individuals, a tree nut or peanut allergy will persist throughout life. Hazelnut allergy is fairly common in Europe and the United States, with a prevalence of around 0.2% in children and 4.5% in adults[10-14]. In Norway, Sweden and Germany hazelnut is even the most often reported trigger of food allergic symptoms, as is peanut in the USA[1].

The reported clinical symptoms vary among different food allergies. Cow's milk allergy in infants is predominantly associated with mild and moderate symptoms. Hazelnut and peanut allergy are more divergent symptomatically, ranging from mild, oral symptoms to severe symptoms like urticaria, asthma attack or even anaphylaxis[10;11;15]. Our own previous studies showed that most children with a hazelnut allergy have severe symptoms[16], while most adults have symptoms limited to mild and local reactions[17-19]. More than 80% of fatalities due to food allergy are provoked by hazelnut, other tree nuts or peanut, in both children and adults[20].

To prevent severe allergic reactions, children and adults with hazelnut, other tree nut and/or peanut allergy are advised to eliminate (tree) nuts and peanut from their diet. At the present time there is no curative treatment for tree nut and peanut allergic patients. Therefore, allergic individuals must rely on a strict elimination diet, in combination with rescue medication. Adherence to elimination diets is difficult nowadays. In recent decades, the diversity of foods has greatly increased, and a larger proportion is industrially prepared with a more complex composition of ingredients. This increases the risk of (unexpected) allergic reactions. To prevent this, an increasing number of food products are labeled as "may contain". This strongly reduces the product choices of food allergic individuals, and stimulates disregard for warning labels, which will further increase the risk of adverse reactions. Elimination diets also have several disadvantages, including the risk of nutritional deficiencies, growth retardation, eating disorders and impaired psychosocial wellbeing, including high levels of anxiety in children, their parents and adult patients[15], as well as continuous awareness of the danger of a potentially severe reaction[21].

DIAGNOSIS OF HAZELNUT ALLERGY

The diagnosis of a hazelnut, other tree nut and/or peanut allergy significantly influences the daily lives of allergic individuals and their environment. Therefore it is important to verify a suspected food allergy with a careful diagnostic work-up, and to advise appropriate, though not excessive, elimination diets.

The diagnostic work-up of a suspected hazelnut allergy consists of a detailed clinical history, determination of the (level of) sensitization, by measuring the presence of specific IgE and/or positive skin prick test, and oral food challenges, either open or – ideally - double-blind placebo-controlled (DBPCFC). The clinical history in children is often unknown or unreliable, probably because many children are not yet able to report particular subjective symptoms. Moreover symptoms are often derived via heteroanamnesis from their parents. Children with a sensitization to hazelnut are often advised to eliminate hazelnut (and other nuts) from their diet as a preventive measure. Another factor complicating the history is that hazelnut is notorious as a hidden ingredient in a number of food products, and therefore accidental ingestion may not always be acknowledged[22;23]. Sensitization to hazelnut may be false-positive, as has been shown for several other allergens like peanut and egg[5]. For hazelnut, sensitization is associated with clinical disease in less than 50% of the cases [24]. Because of the low predictive value of sensitization, DBPCFC is currently the 'gold standard' to prove clinical reactivity[25]. However, this is an elaborate and expensive test, which is not available at all hospitals and is generally performed above the age of 4 to 5 years for hazelnut. Therefore, the diagnosis of hazelnut allergy is still frequently based on the patient's history and (level of) sensitization alone. Because sensitization is often clinically irrelevant, and history in children is often unreliable, this is likely to result in unnecessary elimination diets in a significant number of sensitized children and adults[16]. Improvement of current diagnostic testing is strongly desired to reduce the number of burdensome DBPCFCs and unnecessary elimination diets.

HAZELNUT ALLERGENS

To recommend appropriate but not excessively strict dietary restrictions and rescue medication to allergic individuals, information about the potential severity of the allergic reaction is required. Current diagnostic test results, including the level of allergen specific IgE and the size of SPTs, are related to the probability that an individual will develop allergic symptoms, but not to the severity of symptoms[24;26]. Previous studies have tried to correlate clinical reaction patterns of mild and severe symptoms with sensitizations to major hazelnut allergens. Several allergens have been identified in hazelnut

(Table 1). The birch pollen-related allergens are Cor a 1 and Cor a 2. Sensitization to Cor a 1 has mainly been associated with mild and local symptoms[27-29]. The clinical relevance of allergens from the profilin family, such as Bet v 2 and related Cor a 2 allergen, is less clear[30].

Sensitization to other, non pollen-related hazelnut allergens has been linked to more severe symptoms. In general, sensitization to allergens from the family of lipid transfer proteins (LTPs), Cor a 8 in hazelnut, has been associated with systemic symptoms in individuals from the Mediterranean area[31]. However, our previous studies also showed that Cor a 8 may be an important allergen in severe hazelnut allergy in children[32] in the Netherlands, whereas this was much less clear in adults[33]. Sensitization to the 11S globulin Cor a 9 was shown in 86% of individuals with a systemic hazelnut allergy from the USA[34]. Other molecules that have been identified in hazelnut and may be involved in severe symptoms are the 7S globulin Cor a 11[35], the 2S albumin Cor a 14[36] and hazelnut oleosins; Cor a 12 and Cor a 13[37]. However, the clinical relevance of these allergens is still uncertain. Cor a 11 is a minor allergen, which is glycosylated, and its glycan (carbohydrate) structure does not contribute to its allergenicity[35]. In general, sensitization to specific carbohydrate structures, so-called cross-reactive carbohydrate determinants (CCDs), does not seem to be clinically important[35;38].

Table 1. Structural relationship between major allergens in hazelnut, peanut and birch pollen

Allergen family	Hazelnut	Peanut	Birch pollen
PR-10	Cor a 1	Ara h 8	Bet v 1
Profilins	Cor a 2	Ara h 5	Bet v 2
Lipid transfer proteins	Cor a 8	Ara h 9	
11S Globulins	Cor a 9	Ara h 3	
7S Globulins	Cor a 11	Ara h 1	
Oleosins	Cor a 12, 13	Ara h 10, 11	
2S Albumins	Cor a 14	Ara h 2, 6	

ROUTE OF SENSITIZATION

Previous exposure to the culprit allergen is needed before sensitization occurs. However, several studies have indicated that young children can already be sensitized to hazelnut, other tree nuts and/or peanut without a previously known ingestion[39-41]. This suggests that other routes of sensitization may be involved.

In patients with a hazelnut and birch pollen allergy, sensitization to hazelnut is generally considered to occur through inhalation or ingestion of pollen allergens. Hazelnut-induced allergic symptoms are caused by cross-reactivity between IgE raised against

pollen allergens and homologous proteins in hazelnut[28]. It is estimated that around 70% of birch pollen allergic individuals will develop allergic symptoms to cross-reactive foods, like fruits, legumes, nuts and seeds[42]. Hazelnut sensitization secondary to birch pollen sensitization, which is common in birch abundant areas such as Northern Europe, predominantly induces mild symptoms.

In Mediterranean countries, where birch trees are absent, severe hazelnut allergy is more prevalent[43]. Severe symptoms to hazelnut have been shown in individuals without sensitization to birch pollen, also in birch abundant areas[33]. In patients with a hazelnut allergy without pollen allergy, the sensitization to hazelnut is assumed to have taken place through ingestion.

HAZELNUT SENSITIZATION AND ALLERGY IN RELATION TO OTHER TREE NUTS AND PEANUT

Hazelnut allergic individuals are frequently allergic to multiple tree nuts and peanut[41]. This was also observed in our own patient population[16]. Children with a severe hazelnut allergy are more prone to develop multiple sensitizations than children with a mild hazelnut allergy[16]. The multiple sensitizations in the severe phenotype are already observed early in life[39-41], which suggests cross-reactivity between seed storage proteins (globulins and albumins) in hazelnut, other tree nuts and peanut. Cross-reactivity between peanut, hazelnut, and other tree nuts in patients with severe peanut allergy has been demonstrated by De Leon et al[44;45]. They showed inhibition of IgE binding to peanut by almond, Brazil nut and hazelnut. Data in patients with a mild peanut allergy were not available. In a case report of a patient with an anaphylactic reaction to walnut and additional sensitizations to hazelnut and peanut, IgE reactivity to walnut could not be inhibited by hazelnut or peanut, whereas IgE reactivity to hazelnut, but not to peanut, was strongly inhibited by walnut[46]. This shows that the reactivity to hazelnut was due to cross-reactivity to walnut, and not the other way around. Knowledge about the primary sensitizing allergen may add to the understanding of the difference in severity of clinical symptoms. On the basis of their structural relationship, the hazelnut allergens Cor a 9, Cor a 11 and Cor a 14 were suggested to be potential cross-reactive allergens between hazelnut and the major peanut allergens Ara h 3, Ara h 1, Ara h 2 and Ara h 6 respectively[47;48]. This has not been confirmed yet at the level of IgE binding or cross-inhibition. Cross-reactivity at the IgE level may be due to the mere binding of IgE to one food allergen, to similar IgE-binding epitopes present in homologous allergens in other foods. However, cross-reactivity may also be induced at the level of T cells. In a Th2-skewed environment, which is associated with allergy, cross-reactive T cells that recognize similar epitopes present in different allergens may induce IgE to these related

allergens. This was previously shown for cross-reactivity between birch and apple via Bet v 1 and Mal d 1[49]. Preliminary data in our own lab showed that peanut-specific T cell lines respond well to extracts from hazelnut, indicative of cross-reactivity between hazelnut and peanut at the T cell level. More insight into the level of cross-reactivity (IgE and/or T cell) may explain the observed diversity in IgE responses to hazelnut and peanut, and might further improve diagnosis and dietary advice in cases of a suspected hazelnut and/or peanut allergy.

OUTLINE OF THIS THESIS

Previous studies in our department evaluated clinical aspects of hazelnut allergy in children and adults[16;18;19;33]. In adults, hazelnut allergy was often mild, and associated with birch pollen sensitization[18;19], whereas in children, hazelnut allergy was often severe and associated with sensitization to other tree nuts and peanut[16]. PR-10 cross-reactivity between Bet v 1 and Cor a 1 seemed predominant in mild hazelnut allergy and Cor a 8 was suggested to be important in severe hazelnut allergy[32]. As a follow up to these studies, this thesis further explores the clinical and mechanistic aspects of mild and severe hazelnut allergy, and studies the differences in characteristics between children and adults. We further evaluated the presence of peanut allergy in relation to the severity of hazelnut allergy and aimed to elucidate the potentially underlying mechanism. Cross-reactivity between hazelnut and peanut is studied at IgE and T cell levels. Potential routes of sensitization other than the oral route are considered.

In Chapters 2 and 3, the value of current diagnostic tests for hazelnut allergy in children and adults is evaluated. Differences in the diagnostic work-up and clinical presentation of a hazelnut allergy between children and adults are addressed.

Chapter 4 analysed the value of new diagnostic tests for hazelnut allergy. IgE to specific hazelnut allergens is evaluated in relation to the severity of a hazelnut allergy in children and adults.

Chapter 5 illustrated the eliciting dose for hazelnut in children and adults with a hazelnut allergy. The influence of several patient characteristics on the threshold distribution is studied.

In Chapter 6 evaluated the presence and severity of peanut allergy among hazelnut sensitized children and adults. The molecular basis of the frequently observed sensitization to both hazelnut and peanut is studied by ImmunoCAP inhibitions between seed storage proteins.

Chapter 7, the potency of hazelnut and peanut allergens is studied at IgE level in the indirect basophil activation test and at T cell level in allergen-specific T cell lines. Cross-reactivity between hazelnut and peanut is studied at T cell level.

Chapter 8 is the result of a systematic review of the current literature with regard to the influence of thermal processing on the allergenicity of tree nuts. Implications for source materials used for IgE testing and food challenges and dietary advice are formulated.

Finally, the implications of the different chapters are discussed in Chapter 9.

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Chapter 2

Diagnostic value of hazelnut allergy tests including rCor a 1 spiking in double-blind challenged children

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Allergy 2012;67(4):521-527

ABSTRACT

Background: The diagnostic value of hazelnut allergy tests in double-blind challenged children is largely unknown. The aim of this study was to analyse the performance of current diagnostic tests for hazelnut allergy in children and the effect of spiking.

Methods: Data of 151 children who underwent a DBPCFC for hazelnut were analyzed. The positive and negative predictive value (PPV/NPV) of level of specific IgE (sIgE) for hazelnut, the influence of rCor a 1 spiking of the ImmunoCAP, and size of the skin prick test (SPT) for hazelnut was determined, also in relation to the severity of the hazelnut allergy. Reported accidental ingestion leading to an allergic reaction to hazelnut was also analyzed in relation to hazelnut allergy.

Results: sIgE ≥0.35 kU_A/L for hazelnut was a moderate predictor for hazelnut allergy. The spiking decreased the PPV from 41% to 38% and increased the NPV from 91% to 100% for sIgE ≥0.35 kU_A/L. The maximum reached PPV was 73% for sIgE cutoff of 26 kU_A/L. Level of sIgE before spiking was significantly different between different grades of severity and was lost after spiking. SPT was a better predictor for hazelnut allergy and severity than the level of sIgE. A history of accidental ingestion leading to an allergic reaction to hazelnut had a predictive value of 59% for hazelnut allergy.

Conclusions: This study showed a good NPV of diagnostic tests for hazelnut allergy in children which further improved by rCor a 1 spiking. However, the PPVs are moderate and decreased by spiking.

INTRODUCTION

Hazelnut allergy is often associated with other manifestations of the atopy syndrome[1-3] and accounts together with other tree nuts and peanut for >80% of fatalities due to food allergy[4]. Patients with a hazelnut allergy are advised to follow an elimination diet. This has a great impact on the daily life of children and their parents[5-7]. This stresses the need for reliable diagnostic tools. The diagnostics of hazelnut allergy consist of a careful taken history and determination of the (level of) sensitization, followed by a food challenge, ideally the double-blind placebo-controlled food challenge (DBPCFC). If a challenge is not available, the diagnosis is often based on sensitization only. Several studies have determined the role of the level of sIgE and/or the size of the SPT in relation to the risk of an allergic reaction with varying results. An IgE level ≥15 kU_A/L was reported to give a 95% chance of having a suggestive history of tree nut allergy[8]. However, this could not be confirmed in a population exclusively suspected of hazelnut allergy[9]. An SPT wheal size ≥8 mm diameter has been shown to predict a positive (mainly open) hazelnut challenge in 95% of the children[10].

To improve the performance of the ImmunoCAP to detect IgE to hazelnut extract (in birch pollen related hazelnut allergy), the ImmunoCAP for hazelnut was enriched with recombinant Cor a 1 (rCor a 1) in 2007 (further referred to as spiking). However, two studies have shown that rCor a 1 spiking can lead to problems in distinguishing between sensitization to hazelnut and birch pollen[11;12]. Furthermore, a DBPCFC is performed sporadically in previous diagnostic studies for hazelnut allergy and a distinction between hazelnut allergy in children and adults is often not made, although children more frequently suffer from severe symptoms [2] whereas adults often suffer from mild symptoms[13].

The aim of this retrospective study was to determine the diagnostic value of the level of sIgE, the influence of rCor a 1 spiking and the size of the SPT to diagnose DBPCFC confirmed hazelnut allergy in children. These analyses were also related to the severity of the hazelnut allergy. Other determinants such as accidental ingestion leading to an allergic reaction to hazelnut, the presence of asthma, rhinoconjunctivitis and atopic dermatitis were analyzed in relation to a hazelnut allergy.

METHODS

Patient selection, sIgE and SPT

Children who underwent a DBPCFC for hazelnut in the Center for Paediatric Allergology in Utrecht, (the Netherlands,) were selected for this study. The indication for a DBPCFC for hazelnut was based on a history suggestive for hazelnut allergy, or a positive

sensitization for hazelnut by sIgE and/or SPT while previous ingestion of hazelnut was unknown. sIgE for hazelnut, and birch pollen, was determined by ImmunoCAP (Phadia, Uppsala, Sweden). A value ≥0.35 kU_A/L was considered positive. SPT was performed with commercial hazelnut extract supplied by ALK-ABELLO (Nieuwegein, The Netherlands). Hazelnut extract, histamine 1% as positive control and saline as negative control were pricked into the skin of the upper back or volar aspect of the forearm. The maximum diameter of the weal size was determined and considered positive if ≥ 3 mm in the context of a positive histamine control and a negative reaction to the negative control[14].

Data with respect to accidental ingestions leading to an allergic reaction to hazelnut and the presence of other atopic features were collected from the clinical treatment chart.

Double-blind placebo-controlled food challenge

The diagnosis hazelnut allergy was based on the DBPCFC. The DBPCFC was performed in a standardized way[2]. The challenge was discontinued if objective symptoms occurred (Table 1). Suggestive subjective symptoms resulted in a positive assessment of the challenge. In three children the DBPCFC was prematurely discontinued after the occurrence of three times oral allergy symptoms. The severity of the hazelnut allergy was based on the outcome of the DBPCFC. The severity was classified in three ways: subjective and objective symptoms (Table 1), per organ system (Mueller)[15] and severity per organ system (Sampson)[16].

Data analysis

sIgE levels were compared before and after spiking, and in patients with a positive and negative DBPCFC using the Mann Whitney U test. The diagnostic value of the sIgE and the SPT for the presence of hazelnut allergy was determined by calculating the area under the curve (AUC) of the receiver operating characteristic (ROC) curve for all patients, and before and after spiking. The positive and negative predictive value (PPV and NPV) were determined for different cutoff points of level of sIgE and size of the SPT. Levels of sIgE and sizes of SPT were compared between patients with a hazelnut allergy with subjective and objective symptoms (using the Mann Whitney U test), and between different grades according to Sampson using the Kruskall Wallis test. The relation between a history of accidental ingestion leading to an allergic reaction to hazelnut and presence of hazelnut allergy was assessed by Chi-square. The presence of other atopic features in relation to a hazelnut allergy and sIgE levels was assessed by Chi-square and Mann Whitney U tests, respectively. All analyses were performed with SPSS (version 15).

Table 1. Grading of the severity of the hazelnut allergy according to the symptoms occurred during the double-blind placebo-controlled food challenge

Subjective symptoms	Objective symptoms
Oral allergy	Urticaria generalized
Nausea	Angioedema
Abdominal discomfort	Emesis
Throat tightness	Diarrhea
	Rhinoconjunctivitis
	Hoarseness
	Stridor
	Wheezing

RESULTS

Patients

A total of 172 children underwent a DBPCFC for hazelnut between June 2004 and July 2010. One child was excluded due to an inconclusive DBPCFC and 20 children were excluded, because it was unclear whether the level of sIgE was determined before or after spiking. Therefore, data of 151 children were analyzed. The DBPCFC confirmed a hazelnut allergy in 51 children (34%): 19 with subjective and 32 with objective symptoms. Table 2 presents characteristics of the patients. SPT for hazelnut and sIgE for birch pollen was not performed in all children (Table 2). Patient characteristics in these subgroups did not differ from the total patient group (not shown).

Level of sIgE for hazelnut and influence of rCor a 1 spiking

The spiking increased the level of sIgE for hazelnut significantly (median 3.3 kU_A/L versus 8.5 kU_A/L, p=0.019). Level of sIgE for hazelnut was significantly higher in the children with a hazelnut allergy than in children without a hazelnut allergy (median 26.7 kU_A/L versus 2.6 kU_A/L, p<0.001). The area under the curve (AUC) of the ROC-curve was 0.73 (95% CI, 0.65 to 0.81) when considering all 151 children. Before spiking the performance of sIgE was lower with an AUC of 0.70 (95% CI, 0.57 to 0.84) compared to after spiking, with an AUC of 0.75 (95% CI, 0.65 to 0.85). The PPV and NPV for IgE cutoff values are shown in Table 3. The maximum reached PPV before rCor a 1 spiking was 73% for a cutoff level of 26 kU_A/L; after spiking the maximum PPV was 64% for a cutoff level of 31 kU_A/L (Figure 1A). The spiking increased the NPV from 91% to 100% for a cutoff level of 0.35 kU_A/L (Table 3B, Figure 1B). This NPV of 100% accounted for 12% of the children suspected of hazelnut allergy (Table 3B).

Table 2. Patient characteristics

	All		Before spiking		After spiking	
Groups	**n**	**%**	**n**	**%**	**n**	**%**
Number of patients	151		60		91	
Gender, male	104	69	40	67	64	70
Age, median (IQR)	6 (5-9)		6 (5-10)		6 (5-8)	
Asthma	79	52	34	57	45	50
Allergic rhinoconjunctivitis	55	37	20	34	35	39
Atopic dermatitis	131	87	54	90	77	85
Accidental ingestion	39	26	10	17	29	32
sIgE birch pollen >0.35 kU_A/L*	87	58	34	57	53	58
sIgE hazelnut >0.35 kU_A/L	129	85	49	82	80	88
SPT positive ≥3 mm §	43	74	5	83	38	73
DBPCFC positive	51	34	21	35	30	33

IQR, Interquartile range; sIgE, specific IgE; SPT, skin prick test.
*sIgE birch pollen determined in 113 children, 46 before and 67 after spiking.
§SPT performed in 58 children, 6 before and 52 after spiking.

Predictive value of the size of skin prick test (SPT)

SPT, performed in 52 children, was a better predictor for hazelnut allergy than the level of sIgE, with an AUC of 0.87 (95% CI, 0.76-0.98), compared to an AUC of 0.77 (95% CI, 0.65-0.90) for level of sIgE in this same population of 52 children. The PPV and NPV for different SPT cutoff sizes are shown in Table 3A, B.

Effect of combination of level of sIgE with size of the SPT on predictive value

By combining level of sIgE after spiking with size of the SPT, the PPV reached 100% if level of sIgE for hazelnut was ≥0.35 kU_A/L and size of the SPT ≥16 mm or if sIgE for hazelnut was ≥5 kU_A/L and size of the SPT ≥13mm. However, these combinations of sIgE and SPT accounted respectively for 10% and 13% of the children suspected of hazelnut allergy. The PPV of (combinations of) cutoff levels of sIgE and SPT described in literature are shown in Table 3A[8;10].

Severity of hazelnut allergy

For clinical practice it is useful to predict which children will experience severe symptoms after ingestion of hazelnut. Level of sIgE before spiking showed a trend in discrimination between subjective and objective symptoms due to hazelnut allergy (p=0.06, Table 4A). In addition, level of sIgE before spiking showed a significant difference between different grades of severity according to Sampson (p=0.03, Table 4B). However, both disappeared after spiking. Size of the SPT showed a significant difference between children with subjective and objective symptoms (p=0.02, Table 4A) and between different grades of

severity according to Sampson (p=0.02, Table 4B). Level of sIgE and size of the SPT did not show a significant relation with the severity of the hazelnut allergy according to the Mueller classification (data not shown).

Other predictors: history and other atopic disease

26% Of the children suspected of hazelnut allergy reported an accidental ingestion with allergic reaction to hazelnut in the history and resulted in a PPV of 59% for hazelnut allergy (p<0.001). The prevalence of hazelnut allergy was 25% in the children that never ingested hazelnut or in which hazelnut ingestion was unclear. Asthma and rhinoconjunctivitis in the history were not predictive for a hazelnut allergy. Atopic dermatitis in the history showed a trend (p=0.075) to occur more often in the children with hazelnut

Table 3. The positive predictive value (PPV) (A) and negative predictive value (NPV) (B) for different cutoff levels of sIgE and/or SPT and percentage of children suspected of hazelnut allergy above this cutoff level in predicting hazelnut allergy

A

		PPV (%)	% of suspected children	PPV (%)	% of suspected children	PPV (%)	% of suspected children
sIgE (kU_A/L) ≥	SPT (mm)≥	All		Before spiking		After spiking	
0.35		39	85	41	82	38	88
15		57	32	69	22	53	40
	3					40	81
	8					74	37
	17					100	10
0.35	3					45	73
0.35	8					74	37
0.35	17					100	10
15	3					58	23
15	8					75	15
15	17					100	2

B

		NPV (%)	% of suspected children	NPV (%)	% of suspected children	NPV (%)	% of suspected children
sIgE (kU_A/L) <	SPT (mm) <	All		Before spiking		After spiking	
0.35		95	14	91	17	100	12
15		77	52	74	58	80	48
	3					100	19
	8					91	58
	17					74	67

sIgE, specific IgE; SPT, skin prick test.

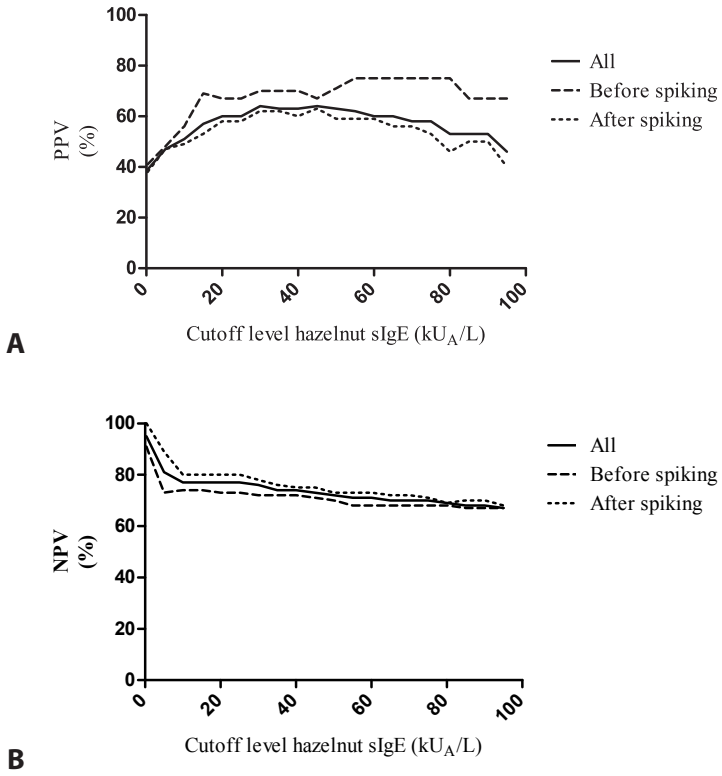

Figure 1. The PPV(A) and NPV(B) for different cutoff levels of hazelnut sIgE. Results are shown for all patients (n=151), and separately for the patients before (n=60) and after (n=91) spiking.

allergy. Children with atopic dermatitis in the history had a significantly higher level of sIgE for hazelnut compared to children without atopic dermatitis in the history (median 6.8 kU$_A$/L versus 1.35 kU$_A$/L, p=0.007).

DISCUSSION

This study showed a good NPV of diagnostic tests for hazelnut allergy in children that further improved by rCor a 1 spiking. However, the PPVs are moderate and decreased by spiking. The size of SPT was a better tool to diagnose hazelnut allergy also in combination with the level of sIgE. A history of accidental ingestion leading to allergic reaction attributed to the diagnostic work-up of hazelnut allergy in children.

The function of a sIgE test is to detect serological reactivity to a specific allergen, whereas the diagnosis food allergy is based on clinical criteria. However, sIgE for ha-

Table 4. Median of the sIgE and SPT for different grades of severity of hazelnut allergy: subjective symptoms vs objective symptoms (A) and grading according to Sampson (B)

A

Severity hazelnut allergy		Subjective		Objective		P value
		Median	n	Median	n	
sIgE (kU_A/L)	All	12	19	29	32	0.33
	Before spiking	3	9	36	12	0.06
	After spiking	56	10	25	20	0.42
SPT (mm)	After spiking	8	6	15	11	0.02

B

Sampson grade		1		2		3		4		5	P value
		Median	n	Median	n	Median	n	Median	n		
sIgE (kU_A/L)	All	10	17	38	21	22	6	7	7	ξ	0.23
	Before spiking	1	9	47	10	ξ		14	2	ξ	0.03
	After spiking	56	8	33	11	22	6	7	5	ξ	0.84
SPT (mm)	After spiking	7	5	10	5	16	4	23	3	ξ	0.02

sIgE, specific IgE; SPT, skin prick test.

ξ Zero children experienced these symptoms according to Sampson during the DBPCFC for hazelnut.

zelnut is often used as a diagnostic test for hazelnut allergy. Cutoff levels for hazelnut sIgE with a 95% PPV could not be determined in this study nor in a previous study on hazelnut allergy[9]. Cutoff levels with a lower PPV than 90-95% have limited decisive importance[17]. sIgE cutoff levels with 95% PPV could be established for other allergens as milk, egg, peanut and walnut[18-20]. The inability of hazelnut sIgE to reach 95% PPV might be caused by the measurement of birch pollen sIgE due to serological cross-reactivity. The effect of this serological cross-reactivity might be different between children and adults, because hazelnut allergy in children, even in a birch endemic area, may be partly pollen independent[2], in contrast to adults[12;13;21]. The spiking of the ImmunoCAP for hazelnut with rCor a 1 was aimed to increase the sensitivity of the test in birch pollen-related hazelnut allergy[22] this was shown to be at the expense of a decreased specificity[12]. We evaluated the effect of spiking in two consecutive groups of children, with similar clinical characteristics (Table 2), selected before and after 2007. The spiking increased the level of sIgE for hazelnut significantly. This has already been shown before with consecutive measurements of the sIgE levels within one group of children in the USA[11]. In agreement with this previous paper we detected a strong correlation (Spearman) between level of hazelnut and birch pollen specific IgE ($r = 0.66$, $p < 0.001$, data not shown) in children without a hazelnut allergy, most prominently after spiking. In general, the spiking improved the performance of the sIgE as shown by the AUC. After spiking, the sIgE cutoff value of <0.35 kU_A/L is a reliable tool in the exclusion of hazelnut allergy and accounts for 12% of the children suspected of hazelnut allergy.

However, the spiking resulted in a higher false positive rate for the sIgE cutoff value of <0.35 kU_A/L (62% versus 59%), both higher than the 40% published for tree nuts in previous literature[8]. Earlier studies showed a false negative rate of 22% for the sIgE[8;9], compared to 9% before spiking in our study. These differences might be explained by a different prevalence of hazelnut allergy or the more heterogeneous patient populations in these previous studies. Both children and adults were included[8], hazelnut allergy was not confirmed by a DBPCFC[2;4;23] and patients with a tree nut allergy were included[2].

In agreement with literature[8], our study showed that SPT was more reliable in diagnosing hazelnut allergy compared to the level of sIgE. The clinical used SPT cutoff value (<3mm) was able to rule out hazelnut allergy in 19% of the children. The PPV was 100% for the SPT cutoff value ≥ 17 mm however, accounting for 10% of the children. This suggests that the SPT might be a useful tool for a significant proportion of the children to diagnose or exclude hazelnut allergy, especially in settings in which the DBPCFC is not or limited available. In contrast with the previously published >95% PPV for the SPT cutoff value ≥8 mm[4], the PPV for this cutoff value was 74% in our study. A different commercial SPT extract was used in this previous study. The composition of commercially available reagents shows variability between different batches and companies[24], hampering SPT standardization. The PPV for hazelnut allergy could be increased by combining the level of the sIgE and the size of the SPT. However, only a minority of the children suspected of hazelnut allergy will reach these cutoff levels with a high PPV.

Our study was performed in a third line referral population. It is important to realize that the prevalence of a disease influences the PPV and the NPV. Therefore, our data may represent an overestimation of the PPV and an underestimation of the NPV as compared to other settings (primary and secondary care).

sIgE levels showed a trend in discriminating between severity of hazelnut allergy on group level. The spiking resulted in a loss of this discrimination. Previous studies could not show a predictive role for level of sIgE for (hazel)nuts on the severity of the clinical reaction based on history[8;25]. Size of the SPT was a good predictor for severity of the hazelnut allergy in children (on group level), comparable to a previous study[8].

Our study showed a predictive value of 59% for reported accidental ingestion of hazelnut. In peanut allergy, a history of accidental ingestion of peanut was shown to predict peanut allergy in children independent of sensitization. The severity of the previous reaction was the strongest predictor for peanut allergy in that model[26]. However, the severity of the symptoms after accidental ingestion was not a predictor for hazelnut allergy in our study (data not shown). Additionally, atopic dermatitis had a higher prevalence in children with hazelnut allergy. (Early onset) eczema has been shown to be a risk factor for peanut allergy in children[27;28].

In conclusion, this study showed that the NPV of diagnostic tests for hazelnut allergy in children is good and further improved by rCor a 1 spiking. However, the PPVs are

moderate and decreased by spiking. The SPT might reduce the amount of necessary DBPCFCs. This shows that until better tools have been developed, such as component-resolved-diagnosis, the DBPCFC remains the mainstay of the diagnostics of hazelnut allergy in children, to correctly diagnose hazelnut allergy and prevent unnecessary elimination diets.

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Chapter 3

Hazelnut allergy differs between children and adults in frequency of severity and relevance of diagnostic parameters

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Submitted

ABSTRACT

Background: In adults hazelnut allergy is often birch pollen-related, whereas in children non-pollen-related hazelnut allergy is more frequent.

Objective: To compare the diagnostic workup of hazelnut allergy, routinely available data in adults were evaluated and compared to previously published data in children.

Methods: Adults (n=101) who underwent a double-blind placebo-controlled food challenge (DBPCFC) for hazelnut were selected and compared to 151 hazelnut-challenged children from a previous study.

Univariate and multivariate logistic regression analyses were performed to build a prediction model. The area under the curve (AUC) of the ROC curve was determined for level of hazelnut-specific IgE, skin prick test (SPT) and the prediction model.

Results: Hazelnut allergy was confirmed by DBPCFC in 80/101 (79%) adults, 66% had only subjective and 33% objective symptoms, whereas in children 63% had objective symptoms to hazelnut. Birch pollinosis, oral symptoms and symptoms specifically attributed to hazelnut were included in the prediction model for adults, AUC of 0.83 (0.65 in children). In children, SPT for hazelnut alone was the strongest predictor for a hazelnut allergy, AUC of 0.87 (0.58 in adults). History of accidental ingestion was specifically attributed to hazelnut in 35% of allergic adults with a positive predictive value of 93%. Most children (74%) had never ingested nuts and a reliable history was therefore difficult to obtain. In adults, the severity of the symptoms after accidental ingestion was predictive for the severity during challenge.

Conclusions: Hazelnut allergy differs between children and adults with respect to frequency of severity and relevance of diagnostic parameters. Therefore, age has to be taken into account in the diagnostic work-up of a hazelnut allergy.

INTRODUCTION

In Northern Europe (Norway, Sweden and Germany), hazelnut is the most often reported cause of food allergic symptoms in adults[1], with an estimated prevalence of 0.5-6.6%[1-4]. Adults with a hazelnut allergy have often also birch pollinosis. Via cross-reactivity, sensitization to birch pollen can lead to a birch pollen-related hazelnut allergy[5;6], which is part of the so-called pollen-food syndrome. A primary or non-pollen related hazelnut allergy can occur in adults from a birch-endemic area[7], but is reported more frequently in patients from the Mediterranean area[8;9] and in children[10;11]. This suggests that the nature of the hazelnut allergy and allergens involved may be different between geographical regions[8] and between children and adults[7;11]. This might further determine the appropriate choice of diagnostic tools. Therefore, it is important to evaluate the value of diagnostic tests separately in adults and children. In birch-endemic areas, IgE to hazelnut extract or the PR-10 protein rCor 1.04 appeared unable to discriminate between patients with or without hazelnut allergy[8;12]. IgE to (non-pollen related) hazelnut storage proteins, nCor a 9 and rCor a 14, which is highly specific for an objective hazelnut allergy, was only detected in a minority of the hazelnut allergic adults[13]. Therefore, this study focused on the predictive value of routinely available diagnostic tools. In a recent study we observed that in children the history of allergic symptoms to hazelnut is often unknown, and, if known, is confirmed by food challenge in only 59%[11]. So to date, the diagnosis of hazelnut allergy in children strongly relies on sensitization and double-blind placebo-controlled food challenge (DCPCFC). Similar data in adults are not available. Therefore, this study evaluated the routinely available data in the diagnosis of hazelnut allergy in adults suspected of hazelnut allergy and compared these data to children from the same geographical area.

METHODS

Patient selection, sIgE and SPT

Adults (n=101), who underwent a DBPCFC with hazelnut between 2000 and 2011 in the University Medical Center Utrecht, the Netherlands, were retrospectively selected for this study. Approval by a local ethical review board was not required, because of the retrospective character of this study. These adults were compared to 151 hazelnut-challenged children from the same geographical area, already described in a previous study[11]. The indication for a DBPCFC was based on a history suggestive for hazelnut allergy, or sensitization to hazelnut by IgE and/or SPT. The level of IgE was determined by ImmunoCAP to hazelnut extract (spiked with rCor a 1 since 2007) (Thermofisher scientific, Uppsala, Sweden). In sixty patients, the level of IgE to hazelnut extract was de-

termined in the same sera before and after spiking with rCor a 1. A value ≥0.35 kU_A/L was considered positive. SPT was performed with commercial hazelnut extract supplied by ALK-ABELLO (Nieuwegein, The Netherlands). Hazelnut extract, histamine 1% and saline were pricked into the skin of the volar aspect of the forearm. The maximum diameter of the weal size was determined and considered positive if ≥ 3 mm[14].

The history of hazelnut allergy and atopic features were collected from the patient records. The history with regard to the ingestion of hazelnut was classified into the following five categories: ingestion specifically attributed to hazelnut (hazelnut); exact culprit nut not specified in patient record (nuts); ingestion attributed to nut mixture including hazelnut (nut mixture); never ingested hazelnut; missing data, unknown or unclear (unknown). Presence of asthma, atopic dermatitis and birch pollinosis was based on doctor's diagnosis.

Double-blind placebo-controlled food challenge

The diagnosis of hazelnut allergy was based on the DBPCFC[11]. The challenge was discontinued if objective symptoms occurred or after subjective symptoms on three consecutive dosages. The severity of the hazelnut allergy was defined as 1) subjective or objective symptoms or 2) according to organ system (Mueller)[15] or 3) severity per organ system (Sampson)[16]. In patients with birch pollinosis, the DBPCFC was performed outside the pollen season.

Data analysis

Patient characteristics were compared between hazelnut allergic and tolerant adults and between different severity grades of hazelnut allergy. Mann-Whitney U-test and Kruskall-Wallis test was used for age, IgE to hazelnut extract and birch pollen and SPT to hazelnut extract. Chi-square was used for gender distribution, presence of asthma, atopic dermatitis, birch pollinosis, reported culprit nut and allergic symptoms after accidental ingestion. Odds ratios were calculated for association between asthma, atopic dermatitis and birch pollinosis and hazelnut allergy. The diagnostic value of IgE and SPT to hazelnut extract was determined by calculating the area under the curve (AUC) of the receiver operating characteristic (ROC) curve. A multivariate logistic regression analysis was performed to build a prediction model. The IgE levels to hazelnut (before and after spiking) were compared between children and adults with Mann-Whitney U-test. All analyses were performed with SPSS version 20.0.1 (SPSS Inc., Chicago, IL, USA).

RESULTS

Clinical characteristics in adults

A total of 121 adults underwent a DBPCFC for hazelnut between 2000 and 2011, of which 101 were selected for this study. The other twenty patients were excluded from further analyses, due to missing values for IgE levels to hazelnut extract (after spiking). DBPCFC confirmed hazelnut allergy in 79% (80/101) of patients, with subjective symptoms in 53 (66%), and additional objective symptoms in 26 (33%). Reported symptoms were missing in one patient. No allergic symptoms during the DBPCFC were reported by 21 patients of the 101 and were considered tolerant. Patient characteristics are shown in Table 1. Birch pollinosis was more common in hazelnut allergic than tolerant adults ($p<0.01$). Age was inversely related to severity of the hazelnut allergy: adults with subjective symptoms had higher median age (33 years) than adults with objective symptoms (24 years, $p=0.03$). Other classification systems for severity of hazelnut allergy (Mueller $p<0.01$ and Sampson $p=0.01$) confirmed the inverse relation between age and severity of hazelnut allergy.

Table 1. Patient characteristics and reported culprit nut of accidental ingestion in adults with a suspicion of hazelnut allergy who underwent a DBPCFC for hazelnut

	No hazelnut allergy n=21		**Hazelnut allergy n=80**		**p value**
	Median	IQRξ	Median	IQR	
Age (y)	26	22-38	29	32-41	0.48
IgE hazelnut (kU_A/L)	7.9	0.5-26	17	5.8-51	0.04
IgE birch pollen (kU_A/L)#	2.0	0.4-52	35	9.7-75	0.06
SPT hazelnut (mm)~	5	4-6	6	4-10	0.48
	n	%	n	%	p value
Gender (male)	10	48	25	31	0.20
Asthma	12	57	39	49	0.63
Atopic dermatitis	13	62	46^	58^	0.84
Birch pollinosis	11	52	69	86	<0.01
Accidental ingestion	n	%	n	%	p value
Hazelnut	2	10	28	35	0.03
Nuts	2	10	27	34	0.03
Nut mixture	4	19	6	8	0.21
Never ingested hazelnut	3	14	7	9	0.43
Unknown	10	48	12	15	<0.01

ξIQR, Interquartile range.

#in 9 adults without hazelnut allergy and 18 adults with hazelnut allergy sIgE to birch pollen was not determined.

~in 13 adults without hazelnut allergy and 23 adults with hazelnut allergy SPT was not determined.

^in one patient presence of atopic dermatitis was unknown.

History of accidental ingestion in adults

The allergic symptoms after an accidental ingestion were specified by 80 adults.

Adults reporting only oral symptoms had more often a hazelnut allergy (89%) than those reporting more or other symptoms than oral symptoms (70%, p=0.04). If they also reported birch pollinosis, the risk of a positive hazelnut challenge increased further to 93% and 83%, respectively. Most adults with only oral symptoms had also a milder hazelnut allergy during challenge (74% only subjective symptoms) than adults with more or other symptoms than oral symptoms (50% objective symptoms, p=0.10), also confirmed by severity according to Mueller (p=0.02) and Sampson (p=0.08). Respiratory symptoms in history were reported more frequently by adults with objective (30%) than subjective symptoms (9%, p=0.04) during DBPCFC, and in adults with higher grade of severity according to Mueller (p=0.02) and Sampson (p=0.03). This did not account for other allergic symptoms after accidental ingestion.

A history of accidental ingestion specifically attributed to hazelnut was known in 30% of adults, more often in allergic (35%) than tolerant adults (10%, p=0.03), resulting in a positive predictive value (PPV) of 93%. The majority of adults could not attribute their allergic reaction specifically to hazelnut (Table 1).

Sensitization in adults

The level of IgE to hazelnut was determined in all adults, SPT to hazelnut extract in 65 (64%) and IgE to birch pollen in 74 (73%). The prevalence of hazelnut allergy was comparable among those subgroups (data not shown).

The level of IgE to hazelnut extract was higher in allergic than tolerant adults (Table 1), with an AUC of 0.65 (95% CI, 0.51-0.79, Table 2). IgE to hazelnut extract was performed before and after rCor a 1 spiking in a subpopulation of 60 patients. Although the level of IgE to hazelnut after spiking (median 17.6 kU_A/L) was significantly higher than before spiking (median 0.62 kU_A/L, p<0.01), the AUC was comparable (Table 2).

The level of IgE to hazelnut extract (before and after spiking) was not predictive for the severity of the hazelnut allergy.

Most adults (89%, 66/74) were sensitized to birch pollen. The level of IgE to birch pollen showed a trend to be higher in hazelnut allergic than tolerant adults (Table 1).

The size of the SPT was not significantly different between allergic and tolerant adults (Table 1), and had an AUC of 0.58 (95% CI, 0.39-0.77). SPT responses ≥3 mm were common (89%) and not discriminative between allergic and tolerant adults, while SPT response ≥15 mm were hardly observed (3%), but highly specific for a hazelnut allergy (specificity 100%). The size of the SPT was not predictive for the severity of the hazelnut allergic response during DBPCFC.

Table 2. Differences between children and adults with a hazelnut allergy and relevance of diagnostic parameters, best predictor is shown in bold

	Children	Adults
Severity hazelnut allergy		
Objective symptoms DBPCFC	63%	33%
Median age objective vs subjective symptoms	6 vs 10 years (p=0.001)	24 vs 33 years (p=0.03)
Predisposing factor OR (95% CI)		
Asthma	0.8 (0.4-1.6)	0.7 (0.3-1.9)
Atopic dermatitis	3.3 (0.9-11.8)	0.9 (0.3-2.3)
Birch pollinosis	1.2 (0.6-2.4)	5.7 (2.0-16.7)
History after accidental ingestion (%)		
Never ingested nuts	74	10
Symptoms attributed to hazelnut	15	30
Positive predictive value symptoms attributed to hazelnut	61	93
Prediction model or sensitization AUC (95% CI)		
Prediction model: birch pollinosis, oral symptoms, symptoms attributed to hazelnut	0.65 (0.56-0.75)	**0.83 (0.72-0.95)**
IgE after spiking (whole population	0.75 (0.65-0.85)	0.65 (0.51-0.79)
IgE before spiking	0.70 (0.57-0.84)	0.75 (0.59-0.92)
IgE after spiking (in same population)		0.74 (0.49-0.98)
SPT	**0.87 (0.76-0.98)**	0.58 (0.39-0.77)

Prediction model hazelnut allergy in adults

A prediction model was built, by entering the following predictors of the univariate analyses: age, gender, asthma, atopic dermatitis, birch pollinosis, symptoms specifically attributed to hazelnut, oral symptoms, IgE to hazelnut extract. SPT to hazelnut extract was excluded for the model, because it was not discriminative between hazelnut allergic and tolerant adults (univariate analysis) and had many missing data. This resulted in a final model consisting of three variables to predict the outcome of the DBPCFC: birch pollinosis, oral symptoms and symptoms specifically attributed to hazelnut. The AUC of the prediction model was 0.83 (95% CI, 0.72-0.95).

Hazelnut allergy in children and adults

Most children (63%) had a hazelnut allergy with objective symptoms during DBPCFC[11], in contrast to 33% in adults (p<0.01). Within the group of children and adults, severity of the hazelnut allergy was inversely related to age (Table 2). Birch pollinosis was only associated with a hazelnut allergy in adults with an OR 5.7 (95% CI 2.0-16.7).

Most children had never ingested (hazel)nuts (74%) compared to 10% of the adults (p<0.01). In both children and adults, a minority (15-30%) could specifically attribute the accidental ingestion to hazelnut. However, in those who identified the culprit nut

as hazelnut, this was more predictive for hazelnut allergy in adults (PPV 93%) than in children (PPV 61%, Table 2). Respiratory symptoms (dyspnoea) after accidental ingestion of hazelnut were only predictive for a severe hazelnut allergy in adults.

Sensitization to hazelnut and prediction model in children and adults

The level of IgE (before and after spiking) had a similar diagnostic value and was not predictive for severity of hazelnut allergy in both children and adults (Table 2). Spiking increased the IgE level to hazelnut in both children ($p=0.02$) and adults ($p<0.01$). The level of IgE to hazelnut before spiking (not after spiking) was significantly higher in children (median level 3.3 kU_A/L) than in adults (median level 0.6 kU_A/L) ($p<0.01$). SPT was a only good predictor of hazelnut allergy in children. The prediction model showed that from the selected variables SPT was the best predictor in children, whereas history of oral symptoms, accidental ingestion specifically attributed to hazelnut and birch pollinosis were the best predictors in adults (Table 2).

DISCUSSION

In this study, routinely available data during diagnostic work-up of hazelnut allergy were evaluated and compared between adults and children. The majority of adults (66%) had hazelnut allergy with only subjective symptoms, while 33% had additional objective symptoms during DBPCFC. A strong association with birch pollen was shown by presence of birch pollinosis in 86% and IgE to birch pollen in 89% of adults. A history of accidental ingestion specifically attributed to hazelnut was known in 35% of allergic adults, with a high PPV of 93%. Most adults were unable to identify the exact culprit nut, because they were exposed to a nut mixture or hidden nuts or could not recognize the tree nuts[17].

The main symptoms reported by allergic adults (97%) were oral symptoms, which are helpful for tracing the culprit allergen and also a warning signal, because they occur almost immediately after exposure of the oral and pharyngeal mucosa to allergens[18]. A combination of birch pollinosis, oral symptoms and symptoms specifically attributed to hazelnut were shown to form the best prediction model, with an AUC of 0.83. The level of IgE to hazelnut extract or the size of the SPT did not add significant diagnostic value to the model. The AUC of IgE to hazelnut extract with an AUC of 0.65 was slightly higher than the previously published 0.55 in unselected pollen-sensitized adults[12]. The spiking of the hazelnut extract with rCor a 1 increased the level of IgE to hazelnut extract significantly, but did not alter the diagnostic value[11;19].

The SPT with hazelnut extract was not discriminative between adults with and without a hazelnut allergy. This could be caused by cross-reactivity between Cor a 1 and Bet v 1 from birch pollen, resulting in difficulties in discrimination between hazelnut and birch

pollen specific SPT responses. Prick-to-prick with fresh nuts (AUC 0.84)[12;20] performed better than SPT with hazelnut extract as used in this study (AUC 0.58).

The degree of the sensitization (specific IgE or SPT for hazelnut) was not predictive for the severity of the hazelnut allergy. Only oral symptoms after accidental ingestion were predictive for a hazelnut allergy with only subjective symptoms during challenge (74%), while more or other symptoms than oral symptoms, such as respiratory symptoms, after accidental ingestion were predictive for a hazelnut allergy with objective symptoms during challenge. IgE to specific hazelnut components, Cor a 9 and Cor a 14, was highly specific for an objective hazelnut allergy in adults as well as in children[13].

A comparison was made with recently published data from our pediatric population[11]. In adults, only 33% had objective symptoms during DBPCFC in contrast to 63% of the children. Children or adults with objective symptoms had a significantly lower age than children or adults with only subjective symptoms during DBPCFC. This is in line with the clinical observation that birch pollen-related hazelnut allergy, which is mostly associated with subjective symptoms, develops often after childhood.

Birch pollinosis was more common in hazelnut allergic adults (86%) than children (39%, $p<0.01$), and children with birch pollinosis were significantly older than children without birch pollinosis (median age 8 vs 6 years, $p<0.01$). This fits with our previous findings that older children were more often sensitized to Bet v 1 and the related Cor a 1 than younger children[21]. Development of pollen-related hazelnut allergy with increasing age will result in a lower overall proportion of patients with a (non-pollen related) hazelnut allergy with objective symptoms in older age groups. A study with longitudinal design is needed to confirm these data.

The difference in relevance of diagnostic parameters between children and adults was shown by the different prediction models that were identified. In adults, a combination of birch pollinosis, oral symptoms and symptoms specifically attributed to hazelnut were the best predictors ending up in the prediction model (AUC 0.83). In children, the prediction model eventually consisted of only SPT with hazelnut extract (AUC 0.87). These differences are explained by the observations that birch pollinosis was not associated with a hazelnut allergy in children, and oral symptoms were hardly reported by children, because they were absent or not recognized. Furthermore, most children (74%) never ingested nuts intentionally. Overall, it appeared much more difficult to obtain a reliable history of reactivity to nuts in children. The fact that SPT appeared a good diagnostic tool in children (AUC 0.87), but not adults (AUC 0.58), might be explained by the higher frequency of a hazelnut allergy with objective symptoms in our unselected pediatric than adult population.

In conclusion, hazelnut allergy differs between children and adults with respect to frequency of severity and relevance of diagnostic parameters. Therefore, age should be taken into account in the diagnostic work-up of a hazelnut allergy.

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Chapter 4

Sensitization to Cor a 9 and Cor a 14 is highly specific for a hazelnut allergy with objective symptoms in Dutch children and adults

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J Allergy Clin Immunol 2013;132(2):393-399

ABSTRACT

Background: Component-resolved diagnosis has been shown to improve diagnosis of food allergy.

Objective: To evaluate whether component-resolved diagnosis may help to identify patients at risk of objective allergic reactions to hazelnut.

Method: A total of 161 hazelnut-sensitized patients were included: 40 children and 15 adults with objective symptoms in DBPCFC and 24 adults with a convincing objective history were compared to 41 children and 41 adults with no or subjective symptoms in DBPCFC (grouped together). IgE levels to hazelnut extract and single components were analyzed with ImmunoCAP.

Results: IgE to hazelnut extract was significantly higher in children with objective than with no or subjective symptoms. In 13% of children and 49% of adults with a hazelnut allergy with objective symptoms, only sensitization to rCor a 1.04 was observed and not to other water-soluble allergens. Sensitization to rCor a 8 was rare, in contrast to rCor a 1. Sensitization to nCor a 9 and/or rCor a 14 was strongly associated with a hazelnut allergy with objective symptoms. Using adapted cut-off levels, a diagnostic discrimination between severity groups was obtained. IgE to either nCor a 9 ≥1 kU_A/L or rCor a 14 ≥5 kU_A/L (children) and IgE to either nCor a 9 ≥1 kU_A/L or rCor a 14 ≥1 kU_A/L (adults) had a specificity of >90% and accounted for 83% of children and 44% of adults with a hazelnut allergy with objective symptoms.

Conclusion: Sensitization to Cor a 9 and Cor a 14 is highly specific for patients with objective symptoms in DBPCFC as marker for a more severe hazelnut allergic phenotype.

INTRODUCTION

The clinical presentation of hazelnut allergy is highly variable and ranges from local and mild to systemic and severe allergic reactions[1]. The prevalence and severity of a hazelnut allergy is different between children and adults. The reported prevalence of hazelnut allergy is around 0.2% among children[2] and up to 4.5% among adults from birch-endemic areas[3;4]. In a birch-endemic area, children with hazelnut allergy have often objective symptoms (67%)[5-7], in contrast to mainly subjective oral symptoms in adults (93%)[8;9]. A differential sensitization profile might be responsible for this difference. Sensitization to the cross-reactive lipid transfer protein Cor a 8 has mainly been described in patients from the Mediterranean area[10;11], with a strong association with severe allergic symptoms. Evidence that sensitization to Cor a 9, an 11S globulin, may be associated to a severe hazelnut allergy in children has been reported both from the USA and Europe[5;12]. In birch pollen-related hazelnut allergy[10;13], sensitization to Cor a 1, the Bet v 1 homologue, belonging to the pathogenesis-related protein 10 (PR-10) family, has been implicated as a cause of typically mild and local symptoms, mainly in adults[5;9;10]. The clinical relevance of sensitization to profilin in hazelnut (Cor a 2, another pollen-related allergen) seems limited[14] and sensitization to cross-reactive carbohydrate determinants (CCD) is now well-established as being clinically inert[15;16]. More recently identified allergens in hazelnut are the oleosins: Cor a 12 and Cor a 13[17], and Cor a 14, belonging to the 2S albumins[18]. The clinical relevance of sensitization to the oleosins and Cor a 14 from hazelnut still needs to be evaluated.

Sensitization to hazelnut extract may occur regardless of whether patients react to hazelnut with severe or mild symptoms, or even at all, and is therefore not discriminative[5;7]. Previous studies have suggested that component-resolved-diagnosis may increase the diagnostic specificity and can predict the severity of an allergic reaction to hazelnut[5;10-12]. The number of patients with a hazelnut allergy with objective symptoms was limited in previous studies, especially in adults from birch-endemic areas. The aim of this study was to analyze the sensitization to the currently available components: rCor a 1, rCor a 8, nCor a 9 and rCor a 14 in a population of hazelnut sensitized children and adults from a birch-endemic area. To evaluate whether component-resolved diagnosis can help to identify patients with objective allergic reactions to hazelnut, a large group of children as well as adults responding with objective symptoms in a double-blind placebo-controlled food challenge (DBPCFC) or having a convincing history of a hazelnut allergy with objective symptoms was enrolled. This group was compared to a group of hazelnut-sensitized children and adults with a negative DBPCFC or with only subjective symptoms.

METHODS

Patient selection

Patients sensitized to hazelnut (≥0.35 kU_A/L) were retrospectively recruited. The aim was to enrol equally powered groups of around 40 children and adults that had a more severe phenotype, as judged by objective symptoms in DBPCFC (or if DBPCFC was declined, by a convincing history of a hazelnut allergy with objective symptoms) and similar numbers of sensitized children and adults without allergy or mild phenotype as judged by no symptoms or subjective symptoms in DBPCFC, respectively. To build a prediction model with IgE to four hazelnut components as variables, forty patients per group were necessary. A total of 164 hazelnut sensitized patients were enrolled. Three patients were excluded from further analyses, because they lost their previously determined hazelnut sensitization. The final study population therefore consisted of 161 sensitized patients: 81 children and 80 adults. Based on challenge outcome (performed in all 81 children and 56 adults) or a convincing objective history (24 adults), the population was divided in 79 patients (40 children, 39 adults) with an objective phenotype and 82 (41 children, 41 adults) not having an objective phenotype (DBPCFC negative or subjective symptoms in DBPCFC) (Figure 1). For these reporting adverse reactions to hazelnut in daily life, a detailed clinical history was recorded. In addition, data were collected on asthma, birch pollinosis and atopic dermatitis.

The study was performed at the University Medical Center Utrecht between 2010 and 2012. The study was approved by the local Ethics Committee and all patients provided written informed consent before entering the study.

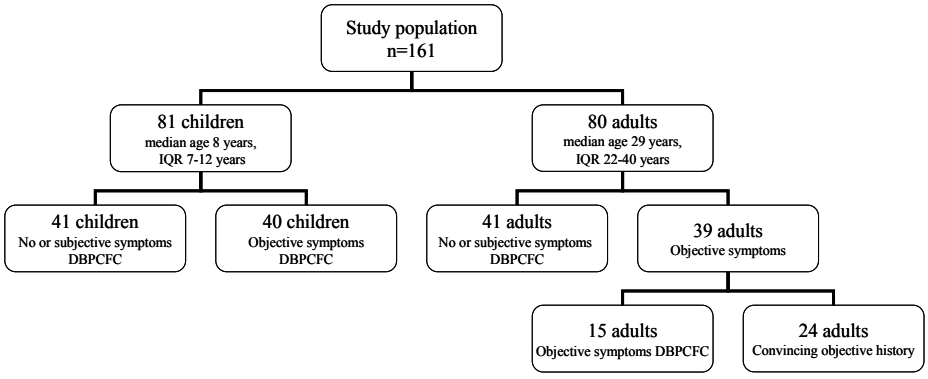

Figure 1. Composition of the study population and different groups with regards to age (children vs adults), severity (hazelnut allergy with no/subjective vs objective symptoms), and judgement of severity (DBPCFC vs convincing history).

DBPCFC

DBPCFC with hazelnut was performed during diagnostic work-up as previously described[6] and was discontinued if objective symptoms occurred. Suggestive oral symptoms appearing at the last open dose resulted in a positive assessment of the challenge in five patients. In eight patients the DBPCFC was prematurely discontinued after subjective symptoms on three consecutive dosages. In patients with birch pollinosis, the DBPCFC was performed outside the pollen season. A total of 24 adults declined undergoing a DBPCFC. All had a convincing history of an objective phenotype, with generalized urticaria, dyspnoea or wheezing.

Classification of symptoms during DBPCFC

Symptoms recorded during DBPCFC were used to stratify patients into severe and non-severe phenotype. The approach chosen for this stratification was to grade subjective symptoms (oral allergy, nausea, abdominal discomfort and throat tightness) as non-severe and objective symptoms (generalized urticaria or angioedema, emesis, diarrhea, rhinoconjunctivitis, hoarseness, stridor and wheezing) as severe. Although it could be argued that e.g. throat tightness may be seen as severe, and rhinoconjunctivitis as mild, we have chosen for this approach because it is least influenced by patient and observer bias, and overall, the division subjective versus objective has acceptable overlap with the division mild versus severe. Sensitized patients that were negative in the DBPCFC were stratified together with the mild subjective group. To ensure blinding this clinical stratification was finished before serology data were generated or any further analysis was done.

IgE measurements

IgE to hazelnut extract, rCor a 1, rCor a 8, nCor a 9, rCor a 14, birch pollen extract, rBet v 1, rBet v 2 and CCD was determined by ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden). Experimental tests were used for nCor a 9 and rCor a 14. Natural Cor a 9 was purified from defatted hazelnut extract by ion exchange chromatography (IEC) and reversed phase chromatography (RPC). Recombinant Cor a 14[18] was expressed as a hexahistidine tagged protein and purified by immobilized metal ion affinity chromatography (IMAC) and IEC. A hazelnut extract depleted of Cor a 1 was prepared by immunoadsorption using an affinity column carrying a monoclonal antibody directed against Cor a 1. IgE test results ≥0.35 kU_A/L were considered positive. Values <0.35 kU_A/L were analysed statistically as 0.34 kU_A/L and values >100 kU_A/L as 101 kU_A/L.

Data analysis

Prevalence of asthma, atopic dermatitis and birch pollinosis was compared between children and adults and between patients with a hazelnut allergy with objective and no

or subjective symptoms using Chi-square test. The Mann-Whitney U-test (continuous IgE values) and Chi-square test (binary, ≥ 0.35 kU_A/L) were used to compare levels of IgE between patients with a hazelnut allergy with objective and no or subjective symptoms. The diagnostic value of IgE levels (continuous values) for discrimination between a hazelnut allergy with objective and no or subjective symptoms was determined by calculating the area under the curve (AUC) of the receiver operating characteristic (ROC) curve. Sensitivity and specificity in the discrimination between a hazelnut allergy with objective and no or subjective symptoms were calculated for different cut off values of IgE and different combinations of IgE. $P<0.05$ was considered significant. All analyses were performed with SPSS (Version 20.0, SPSS Inc., Chicago, IL, USA).

RESULTS

Patient characteristics

Clinical characteristics of children and adults are shown in Table 1. There was a male predominance in children and a female predominance in adults. Asthma was more common among adults with a hazelnut allergy with objective than no or subjective symptoms (p=0.03) and more common among children with no or subjective than adults with no or subjective symptoms (p=0.04). Atopic dermatitis was more common in children than in adults (p<0.001), while birch pollinosis was less common in children than in adults (p=0.01) and less common in children with objective than no or subjective symptoms (p=0.01). Clinical characteristics were comparable within the group of children and adults with a hazelnut allergy with no or subjective symptoms, while age was significantly higher in children and adults with subjective symptoms compared to none hazelnut allergy (data not shown). Eliciting dose during DBPCFC was lower in the children and adults with a hazelnut allergy with objective symptoms than no or subjective symptoms (p=0.02).

Sensitization to birch pollen

Birch pollen sensitization occurred in the majority of all children and adults. In line with the clinical history, birch pollen sensitization was less common among children with a hazelnut allergy with objective than no or subjective symptoms (p=0.007). All children (100%) and the majority of adults (97%) with a subjective hazelnut allergy were sensitized to birch pollen. The median level of IgE to birch pollen was lower in sera of children with a hazelnut allergy with objective than no or subjective symptoms (median 8.78 kU_A/L versus 18.2 kU_A/L, p=0.10). No such difference was observed among the adults. Similar to birch pollen, sensitization to rBet v 1 was frequently detected in children and adults, but less common among children with a hazelnut allergy with objective than

Table 1. Patient characteristics

	Children					Adults				
	No/subjective n=41		Objective n=40		p value	No/subjective n=41		Objective n=39		p value
	Median	IQR	Median	IQR		Median	IQR	Median	IQR	
Age (y)	9	8-12	7	5-11	0.002	31	24-45	27	22-37	0.19
	n	%	n	%		n	%	n	%	
Gender (male)	28	68	27	68	1.00	15	37	8	21	0.14
Asthma	27	68	21	53	0.11	18	45	29	73	0.03
Atopic dermatitis	41	100	38	95	0.24	30	73	29^	74^	0.80
Pollinosis	33	81	24	60	0.05	37	90	29	74	0.08
Birch pollinosis	30	73	18	45	0.01	35	85	28	72	0.18
Grass pollinosis	22	54	15	38	0.18	27	66	20	51	0.26

IQR, Interquartile range.
^Missing data in one adult.

no or subjective symptoms (p=0.05). Again, no such difference was observed among the adults. The level of sensitization to rBet v 1 was comparable between patients with a hazelnut allergy with objective and no or subjective symptoms, both in children and adults. Sensitization to rBet v 2 and CCD was present in a minority of children and adults and did not significantly differ between a hazelnut allergy with objective and no or subjective symptoms (Table 2, Figure 2).

Table 2. Number of children and adults sensitized (IgE ≥ 0.35kU$_A$/L) to hazelnut extract, rCor a 1, rCor a 8, nCor a 9, rCor a 14, birch pollen extract, rBet v 1, rBet v 2 and CCD determined by using ImmunoCAP

IgE ≥ 0.35kU$_A$/L	Children					Adults				
	No/subjective n=41		Objective n=40		p value	No/subjective n=41		Objective n=39		p value
	n	%	n	%		n	%	n	%	
Hazelnut extract	41	100	40	100		41	100	39	100	
rCor a 1	37	90	28	70	0.03	39	95	35	90	0.43
rCor a 8	2	5	3	8	0.68	1	2	2	5	0.61
nCor a 9	8	20	33	83	<0.001	2	5	14	36	0.001
rCor a 14	10	24	28	70	<0.001	2	5	15	39	<0.001
Birch pollen extract	39	95	29	73	0.007	40	98	35	90	0.20
rBet v 1	37	90	29	73	0.05	39	95	35	90	0.43
rBet v 2	10	24	4	10	0.14	5	12	4	10	1.00
CCD	2	5	2	5	1.00	2	5	6	15	0.15

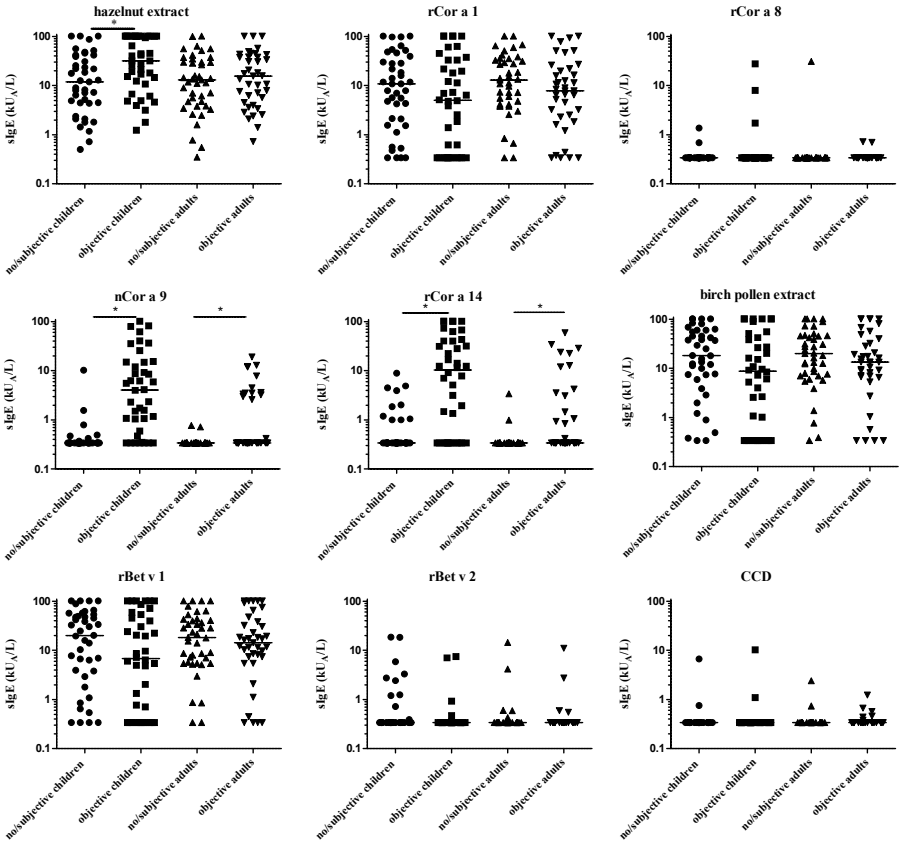

Figure 2. Level of sensitization to hazelnut extract, rCor a 1, rCor a 8, nCor a 9, rCor a 14, birch pollen extract, rBet v 1, rBet v 2 and CCDs in children and adults with a hazelnut allergy with objective and those with no or subjective symptoms. Horizontal bars indicate median levels and IgE values of less than 0.35 kU$_A$/L were set to 0.34 kU$_A$/L. *P <0.05.

Sensitization to hazelnut extract and components

The level of IgE to hazelnut extract was significantly higher in children with a hazelnut allergy with objective than with no or subjective symptoms (median 31.8 kU$_A$/L versus 11.9 kU$_A$/L, p=0.006), while a corresponding difference was not observed among these phenotypes in adults (Figure 2).

The majority of patients (70-95%) in all four groups were sensitized to rCor a 1 and the levels of IgE strongly correlated to those against rBet v 1 (r=0.96, p value<0.001). Absence of sensitization to rCor a 1 was most frequently observed among children (12/16) and adults (4/6) with a hazelnut allergy with objective symptoms. The level of IgE to rCor a 1 was comparable between patients with a hazelnut allergy with objective and no or subjective symptoms. In 13% (5/40) of the children and 49% (19/39) of the adults with a

hazelnut allergy with objective symptoms, sensitization to no other allergen than rCor a 1 was observed. The level of IgE to rCor a 1 was not significantly different between these 24 patients and other patients with sensitizations to rCor a 1.

Sensitization to rCor a 8 was only detected in eight patients, distributed across all four study groups and mostly at moderate levels.

Sensitization to nCor a 9 and to rCor a 14 was significantly more common in patients with a hazelnut allergy with objective than with no or subjective symptoms (Table 2). The level of IgE to nCor a 9 and to rCor a 14 were significantly higher in children with a hazelnut allergy with objective (median 4.07 and 10.3 kU_A/L, respectively) than with no or subjective symptoms (median <0.35 kU_A/L for both allergens). Also among the adults, patients with a hazelnut allergy with objective symptoms had significantly higher levels of IgE to nCor a 9 and rCor a 14 than those with no or subjective symptoms (Figure 2, Table 2). Both children and adults without birch pollinosis had significantly higher IgE levels to nCor a 9 than those with birch pollinosis. Level of IgE to rCor a 14 was also higher in adults without than with a history of allergic symptoms to birch pollinosis, no such difference was observed for IgE to rCor a 14 among children (data not shown).

Identification of patients with a hazelnut allergy with objective symptoms

In addition, the capacity of these components to identify patients with a hazelnut allergy with objective symptoms was evaluated using ROC curves and the AUC. Hazelnut extract, rCor a 1 and rCor a 8 did not display a useful level of discrimination between

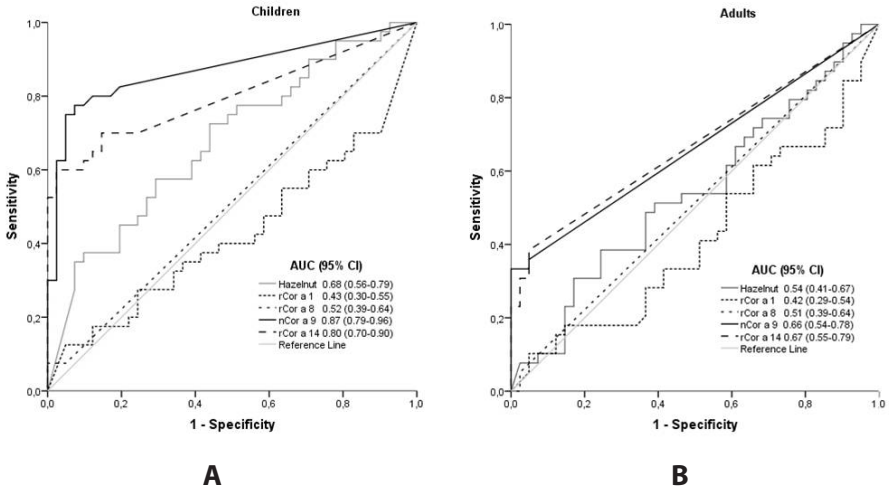

Figure 3. Receiver operating characteristic curves for IgE to hazelnut extract and individual hazelnut components in regard to discrimination between a hazelnut allergy with objective and no or subjective symptoms in children (A) and adults (B) with sensitization to hazelnut.

the severity groups in this study (Figure 1, 2). In contrast, the prevalence and magnitude of sensitization to nCor a 9 and rCor a 14 differed markedly between these groups. The sensitivity and specificity with regard to discrimination between patients with objective symptoms and those with no or subjective symptoms, as a function of different cut-off levels, were calculated, as shown in Tables 3 and 4.

IgE to nCor a 9 had better capacity than rCor a 14 to identify children with objective symptoms in DBPCFC, based on AUC values of 0.87 and 0.80, respectively (Figure 3). This resulted in a sensitivity of 75% and a specificity of 95% for IgE to nCor a 9 ≥1 kU_A/L (Tables 3 and 4). Sensitization to either nCor a 9 ≥1 kU_A/L or rCor a 14 ≥5 kU_A/L had a sensitivity of 83% and a specificity of 93%. In the group with no or subjective symptoms, only 3/41 (7%) of children (two with a negative challenge and one with subjective symptoms) would have been diagnosed incorrectly as potentially objective hazelnut allergic using these cut-off values.

In adults, IgE to either nCor a 9 and rCor a 14 had a similar capacity to identify adults with a hazelnut allergy with objective symptoms, based on the AUC values of 0.66 and 0.67, respectively. The adults who had not undergone DBPCFC did not display higher or more frequent sensitization to nCor a 9 or rCor a 14 than those who had been chal-

Table 3. Sensitivity and specificity for different cutoff levels of IgE to nCor a 9 and rCor a 14 to identify children and adults with a hazelnut allergy with objective symptoms among our selected population

	Children		Adults	
	Sensitivity	**Specificity**	**Sensitivity**	**Specificity**
IgE to nCor a 9 (kU_A/L) ≥				
0.35	83	80	36	95
1	75	95	33	100
5	48	98	13	100
IgE to rCor a 14 (kU_A/L) ≥				
0.35	70	76	38	95
1	70	76	31	98
5	60	98	18	100

Table 4. Sensitivity and specificity for the combination of different cutoff levels of IgE to nCor a 9 and rCor a 14 to identify children and adults with a hazelnut allergy with objective symptoms among our selected population

			Children		Adults	
IgE nCor a 9 (kU_A/L) ≥	**And/ or**	**IgE rCor a 14 (kU_A/L) ≥**	**Sensitivity**	**Specificity**	**Sensitivity**	**Specificity**
0.35	And	0.35	65	98	23	100
0.35	Or	0.35	88	59	51	90
1	Or	1	88	71	44	98
1	Or	5	83	93	36	100

lenged and developed objective symptoms. In the total adult population, sensitization to nCor a 9 ≥1 kU_A/L was very specific (100%) for objective symptoms, but accounted for only 33% of the adults with a hazelnut allergy with objective symptoms. Sensitization to rCor a 14 was specific (95%) for objective symptoms at a cut-off value ≥0.35 kU_A/L, and accounted for 38% of adults with objective symptoms. The combination of IgE to either nCor a 9 ≥1 kU_A/L or rCor a 14 ≥1 kU_A/L in adults accounted for 44% of the adults with a hazelnut allergy with objective symptoms with a specificity of 98%. One of the 41 adults (2%, with a hazelnut allergy with subjective symptoms) would have been diagnosed incorrectly as potentially objective hazelnut allergic using these cut-off values. For both children and adults, logistic regression analyses showed no significant additional value by combining IgE to the different components in the prediction of a hazelnut allergy with objective symptoms, indicating that a prediction model was not necessary (data not shown).

DISCUSSION

Routine diagnostics tools are no good predictors of a severe hazelnut allergy[7;19]. The present study has demonstrated that sensitization to nCor a 9 and/or rCor a 14 is highly specific for a hazelnut allergy with objective symptoms among the Dutch hazelnut sensitized children and adults. Sensitization to nCor a 9 or rCor a 14 was demonstrated in majority of children and almost half of the adults with a hazelnut allergy with objective symptoms. In children, sensitization to either nCor a 9 ≥1 kU_A/L or rCor a 14 ≥5 kU_A/L had a specificity of 93% and accounted for 83% of children with a hazelnut allergy with objective symptoms. In adults, the combination of IgE to either nCor a 9 ≥1 kU_A/L or rCor a 14 ≥1 kU_A/L had a specificity of 98% and accounted for 44% of the adults with a hazelnut allergy with objective symptoms. For patients negative for nCor a 9 or rCor a 14, or with IgE levels below these cut-off values, a DBPCFC would still be required to exclude or demonstrate a hazelnut allergy with objective symptoms. The outcome of this study has relevance to real-life hazelnut exposure, since there was a significant correlation between symptoms during DBPCFC and reported real-life symptoms ($r=0.412$, $p<0.001$ in children and $r=0.533$, $p<0.001$ in adults).

Previous studies already showed recognition of Cor a 9 on western blot in sera of 86% of patients with a severe hazelnut allergy in the USA[12]. Also in Belgium, sensitization to Cor a 9 was detected in a high proportion of children with a severe hazelnut allergy. Furthermore, the prevalence of sensitization to Cor a 9 was found to differ between age groups: 65% of preschool, 50% of school children and 17% of adults with a severe reaction to hazelnut recognized nCor a 9[5]. This age-related effect was also observed in our study: 83% of the children and 36% of the adults with a hazelnut allergy with

objective symptoms were sensitized to nCor a 9. Sensitization to rCor a 14 also showed an age-related prevalence: 70% of children and 39% of adults with a hazelnut allergy with objective symptoms were rCor a 14 sensitized. An explanation might be that birch pollen-related hazelnut sensitization becomes a more frequent cause of hazelnut allergy through adolescence and early adulthood, resulting in a lower proportion of individuals with a primary sensitization to hazelnut (hallmarked by sensitization to abundant storage proteins). Sensitization to rCor a 14 has previously been described in a pilot study of eleven Dutch patients, of which eight reported a history suggestive of hazelnut allergy, including five with reported severe symptoms[18].

Cor a 9 and Cor a 14 are both abundant seed storage proteins in hazelnut. Seed storage proteins have been identified as major allergens in several other tree nuts and peanut. Sensitization to the peanut 2S albumin, Ara h 2 has been shown to be a good predictor of peanut allergy[20-22]. The clinical relevance of sensitization to seed storage proteins in tree nut allergy has not been extensively investigated. Our study convincingly shows that seed storage proteins are also relevant in hazelnut allergy, especially in the objective phenotype. A good correlation between the sum of IgE levels to nCor a 9 and rCor a 14 compared to a Cor a 1-depleted hazelnut extract ($r=0.96$, $p<0.001$ in children, $r=0.78$, $p<0.001$ in adults, data not shown), suggests limited importance of other water soluble hazelnut components, like Cor a 11[23;24], that belongs to a family of abundant and water soluble seed storage proteins, well represented in natural allergen extracts. Still, validation of the data with respect to nCor a 9 and rCor a 14 in other populations is important, not the least because sensitization profiles may vary between different geographical locations[10].

Majority of children and adults without birch pollinosis had a hazelnut allergy with objective symptoms and higher levels of IgE to nCor a 9 and rCor a 14 than those with birch pollinosis. However, thirteen percent of children and 49% adults with a hazelnut allergy with objective symptoms were sensitized to rCor a 1 without sensitization to rCor a 8, nCor a 9 and rCor a 14, compared to 14% in a previous study (in which IgE to rCor a 14 was not determined)[5]. A close correlation between IgE to hazelnut extract and rCor a 1 in these patients ($r=0.98$, $p<0.001$, data not shown) and lack of IgE binding to a Cor a 1-depleted hazelnut extract (data not shown), suggests a lack of sensitization to water extractable hazelnut components other than rCor a 1. Thus, the objective symptoms in these patients may be caused by either Cor a 1, analogous to the role of Gly m 4 in soy allergy[25], or by one or several components that are not present in aqueous hazelnut extract, such as oleosins[17], Further studies are required to resolve this issue. Hazelnut tolerant patients could not be serologically distinguished from those with a hazelnut allergy with subjective symptoms in this study as no significant difference was detected in level of sensitization to hazelnut extract or components between these groups, neither among children nor adults (data not shown).

Sensitization to rCor a 8 is important in the Mediterranean area, reported to occur in 50-71% of severe hazelnut allergic patients from Spain[10;11]. However, sensitization to rCor a 8 was hardly detected in our Dutch population and was not specific for a hazelnut allergy with objective symptoms. This is in contrast with a previous study by our group with natural Cor a 8[26].Such a discrepancy could be due to structural differences between natural and recombinant Cor a 8, or by incomplete removal of other allergens from the natural protein preparation. Recent experiments, revealing that natural Cor a 8 preparations indeed contain trace amounts of Cor a 14 and possibly other seed storage proteins (data not shown), provide a plausible explanation for the observed discrepancy.

In summary, even though the proportion of patients with a hazelnut allergy with no or subjective versus objective symptoms in our study is not fully representative of that of patients appearing at an allergy clinic, (i) a majority of children and almost half of the adults with a hazelnut allergy with objective symptoms could be identified with these tools and (ii) the majority of patients sensitized to Cor a 9 and/or Cor a 14 reacted with objective symptoms to hazelnut. Although food challenges will still be necessary in many cases, we conclude that determination of IgE to nCor a 9 and rCor a 14 has the potential to strongly improve the diagnostic work-up in cases of a suspected severe hazelnut allergy.

ACKNOWLEDGEMENTS

We would like to thank Mr. A.P.H. Jansen, MD, internist-allergologist, Arnhem, The Netherlands and Ms. E. van Zuuren, MD, Dermatologist, Leiden University Medical Center, The Netherlands for their contribution to the patient inclusion.

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Chapter 5

Clinical thresholds to hazelnut in children and adults are correlated with SPT reactivity, and IgE levels to Cor a 9 and Cor a 14

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ABSTRACT

Background: Hazelnut is often present as hidden constituent in food due to cross contamination and might lead to unexpected allergic reactions. Information on eliciting doses (EDs) will improve allergy management for patients and food industry.

Objective: Determination of EDs for both subjective and objective symptoms to hazelnut in children and adults and the influence of patient characteristics on the threshold distribution.

Method: Thresholds for both subjective and objective symptoms were established for 67 children and 81 adults, with a positive hazelnut challenge. Data on threshold doses were analyzed with Interval-Censoring Survival Analysis (ICSA) and fitted into a threshold distribution curve (TDC) from which ED values could be extracted. The effect of age, gender, atopic diseases, SPT reactivity and sensitization profiles on the threshold distribution for hazelnut was analyzed as covariates by ICSA.

Results: TDC for objective symptoms were comparable between children and adults, males and females, resulting in a pooled ED at which 5% of the allergic population is likely to respond (ED5) with objective symptoms of 10.3 mg. The threshold distribution was influenced by age, SPT reactivity, and IgE to Cor a 9 and Cor a 14. TDC for subjective symptoms was significantly higher in children than in adults and influenced by gender, SPT reactivity and IgE to Cor a 14.

Conclusion: Our data demonstrate several intrinsic factors that influence the sensitivity to hazelnut, which are useful for allergy management. The obtained ED values can improve food labeling, which may increase product choice and decrease the risk of accidental ingestions.

BACKGROUND

Hazelnut is often present as hidden constituent in food. Due to cross contamination, 25% of cookies and 75% of chocolates contained traces of hazelnut[1]. This might lead to unexpected allergic reactions. Therefore adequate information is important for allergic consumers. Nowadays an increasing amount of food products have precautionary labeling as 'may contain nuts' or similar statements[2]. This leads to a strongly reduced product choice for allergic consumers and on the other hand to ignoring of labeling information[3]. Improved food labeling for hazelnut, based on the results of eliciting dose analyses (EDs) in allergic individuals will strongly improve food safety, product choice and quality of life of hazelnut allergic individuals.

Information on ED values can further improve individual allergy management by revealing modifying factors on the sensitivity to hazelnut. Several patient characteristics may influence the individual sensitivity to hazelnut. The frequency of a severe hazelnut allergy is higher in children than in adults (manuscript submitted). A mild hazelnut allergy, as often seen in birch pollen related hazelnut allergy, is mainly observed in adults[4;5]. This is further reflected by differences in sensitization profiles between children and adults; sensitization to potent seed storage proteins in hazelnut Cor a 9 and Cor a 14 is more common in children than in adults[6;7]. Comparison of EDs to hazelnut between children and adults from one center has not been described.

Therefore, the aim of this study was to determine the threshold for objective, and subjective symptoms to hazelnut and compare ED values between children and adults, and further to study the effect of several patient characteristics on the threshold distribution for hazelnut, like gender, age, atopic diseases, SPT reactivity and sensitization profiles, on the threshold distribution for hazelnut.

METHODS

Patients

Children and adults, who underwent a positive double-blind placebo controlled hazelnut challenge in the University Medical Center Utrecht between 2000 and 2011 were retrospectively selected. Suspicion of hazelnut allergy was based on clinical history or sensitization (IgE or SPT) to hazelnut without previous ingestion.

SPT and IgE measurements

SPT was performed with commercial hazelnut extract supplied by ALK-ABELLO (Nieuwegein, The Netherlands). Hazelnut extract, histamine 1% and saline were pricked into the skin of the volar aspect of the forearm. The maximum diameter of the weal size was

determined. IgE to hazelnut extract, rCor a 1, rCor a 8, nCor a 9, rCor a 14 was determined by ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden).

Double-blind placebo-controlled food challenges

The double-blind placebo-controlled food challenge (DBPCFC) was performed as described previously[8]. Eight portions of defatted hazelnut flour were administered in series: 10 ug, 100 ug, 1 mg, 10 mg, 100 mg, 300 mg, 1 g and 3 g (protein content 15%). Whole-wheat instant cereal and apple sauce was used as matrix for the portions, cinnamon powder was added for masking. When the DBPCFC was negative an open challenge consisting of 3 gram raw hazelnuts was performed. DBPCFC was discontinued if objective symptoms occurred or was discontinued after subjective symptoms on three consecutive dosages. Subjective symptoms were oral allergy, nausea, abdominal discomfort and throat tightness; objective symptoms were generalized urticaria or angioedema, emesis, diarrhea, rhinoconjunctivitis, hoarseness, stridor and wheezing. In patients with birch pollinosis, the DBPCFC was performed outside the pollen season. Individual no observed adverse effect levels (NOAELs) and lowest observed adverse effect levels (LOAELs) for both subjective and objective symptoms were established for all patients.

Statistical analyses

Baseline characteristics were compared between children and adults, using Chi-square test (binary variables) and the Mann-Whitney U-test (continuous variables).

The threshold for objective and subjective symptoms was determined in 67 children and 81 adults using threshold dose distributions[9;10]. Data on individual NOAELs and LOAELs in the total population, children and adults, males and females were analyzed with Interval-Censoring Survival Analysis (ICSA) and fitted into a threshold distribution curve (TDC) from which EDs could be extracted[9;10]. For each subject, the true threshold lies, by definition, between the NOAEL and LOAEL dose. Individuals reacting to the first challenge dose were treated as left-censored (the NOAEL set to zero), while individuals failing to respond to the uppermost challenge dose were treated as right-censored (the LOAEL set to infinity). In cases where the challenge was stopped because subjective symptoms occurred on three successive administrations, the NOAEL for objective symptoms was set at the last consumed dose and the LOAEL was right censored. If patients developed only (objective or subjective) symptoms during open challenge with hazelnuts and not to portions with hazelnut flour the LOAEL was also right censored.

Data analyses and modelling were performed in SAS v9.1 (SAS Research Institute) using the LIFEREG procedure as previously described[10-12]. Since there is no apparent biological or mathematical basis for choosing a specific distribution, the Weibull distribution was used to fit the data, instead of the lognormal or loglogistic distribution. The

Weibull distribution has the advantage that hazard ratio's (HR) can be directly calculated using the parameter estimates. The HR indicates the hazard of a reaction from one level of a certain characteristic compared to the other level of the same characteristic. For example, an HR of 2 indicates doubling of the risk to react to a specific dose compared to the reference. The TDCs and eliciting doses for objective or subjective allergic symptoms were compared between children and adults, and males and females. The effect of age, gender, atopic dermatitis, asthma, hay fever, size of SPT, IgE to hazelnut extract, rCor a 1, nCor a 9 and rCor a 14 on the threshold distribution was analyzed as covariates by ICSA.

RESULTS

Patient characteristics

A total of 67 children and 81 adults were included. Clinical characteristics are shown in Table 1. There was a male predominance in children and female predominance in adults. Objective symptoms during the DBPCFC were more often reported in children (67%)

Table 1. Patient characteristics for total population, children and adults, p value for difference between children and adults

	Total n=148		**Children n=67**		**Adults n=81**		**P value**
	Median	**IQR**	**Median**	**IQR**	**Median**	**IQR**	
Age (y)	19	7-32	6	5-8	30	23-41	<0.01
SPT hazelnut (mm)^	7	4-10	10	7-19	6	4-10	<0.01
IgE hazelnut (kU_A/L)^	23.4	4.74-63.3	29.7	7.7-99.8	13.9	3.8-34.6	0.04
IgE rCor a 1 (kU_A/L)^	6.92	1.86-35.1	5.9	0.29-36	9.7	3.9-35.1	0.26
IgE nCor a 9 (kU_A/L)^	0.19	0.03-3.99	1.66	0.19-9.09	0.03	0.02-0.06	<0.01
IgE rCor a 14 (kU_A/L)^	0.15	0.02-14.2	7.4	0.12-31.7	0.02	0.01-0.03	<0.01
	n	**%**	**n**	**%**	**n**	**%**	
Gender (male)	67	45	42	63	25	31	<0.01
Subjective symptoms	147	99	66	99	81	100	0.45
Objective symptoms	66	45	45	67	21	26	<0.01
Asthma	77	52	34	51	43	53	0.87
Hay fever	95	64	29	43	66	82	<0.01
Atopic dermatitis	106	72	61	91	45	56	<0.01
Left censored	8	5	0	0	8	10	<0.01
Right censored	105	71	43	64	62	77	0.11

IQR, interquartile range.

^ SPT performed in 23 children and 59 adults, IgE to hazelnut, rCor a 1, nCor a 9 and rCor a 14 performed in 40 children and 27 adults.

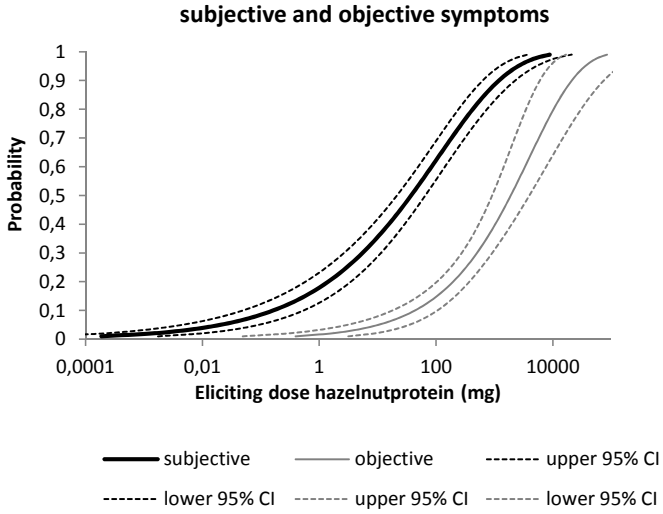


Figure 1. Threshold distribution curves for subjective and objective symptoms in pooled dataset.

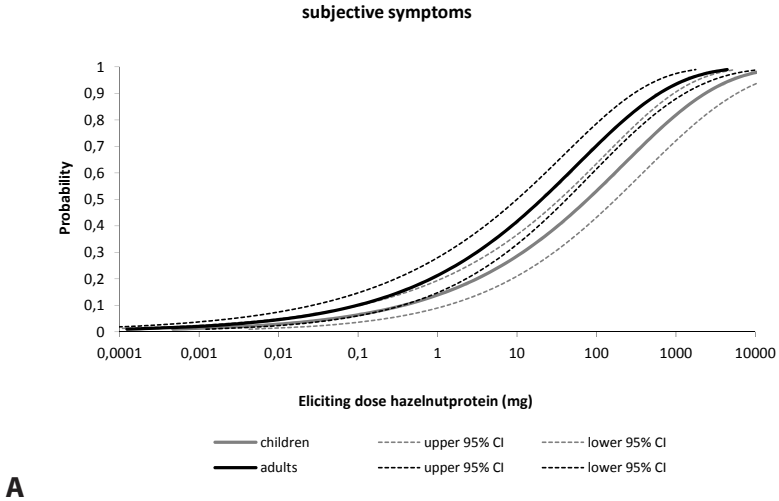
than in adults (26%). Hay fever was more common in adults than in children, whereas atopic dermatitis was more common in children than in adults. SPT reactivity to hazelnut and IgE to hazelnut extract, nCor a 9 and rCor a 14 was significantly higher in children than in adults. SPT reactivity to hazelnut was determined in 23 children and 59 adults as part of diagnostic work-up. IgE to hazelnut, rCor a 1 and rCor a 14 was determined in 40 children and 27 adults, as part of a diagnostic study[7]. These subgroups were comparable to the total population with regard to atopy (asthma, atopic dermatitis, hayfever), gender and age distribution.

ED for objective and subjective symptoms

The TDCs for the total group are shown in Figure 1 and for children or adults, and males or females separately, in Figure 2, and Figure 3. The TDC for subjective symptoms was approximately 100 fold lower than the TDC for objective symptoms. The obtained ED values for objective and subjective symptoms to hazelnut in total hazelnut allergic group, and children or adults separately are shown in Table 2. The TDC for objective symptoms was not significantly different between children and adults (p=0.07) therefore the effect of several patient characteristics on the TDC for objective symptoms to hazelnut was determined in the total group (Table 3A). The TDC for subjective symptoms was significantly lower in adults than in children (P<0.01), therefore the effect of influencing factors on the TDC for subjective symptoms was analyzed in children and adults separately (Table 3B).

Influencing factors on TDC for objective symptoms in the total group

The TDC for objective symptoms was significantly influenced by age (p=0.04, HR=0.98), SPT reactivity (p=0.02, HR=1.07) and IgE levels to nCor a 9 (p=0.03, HR=1.02) and rCor a 14 (p<0.01, HR=1.02, Table 3A). This indicates that for every additional year in age the risk to react to a specific dose with objective symptoms was decreased. For every unit increase in SPT reactivity or IgE level to nCor a 9 and rCor a 14 the risk to react to a specific dose with objective symptoms was increased. Gender, IgE level to hazelnut extract or rCor a 1, or the presence of atopic dermatitis, asthma, hay fever, did not influence the TDC for objective symptoms in the total group.



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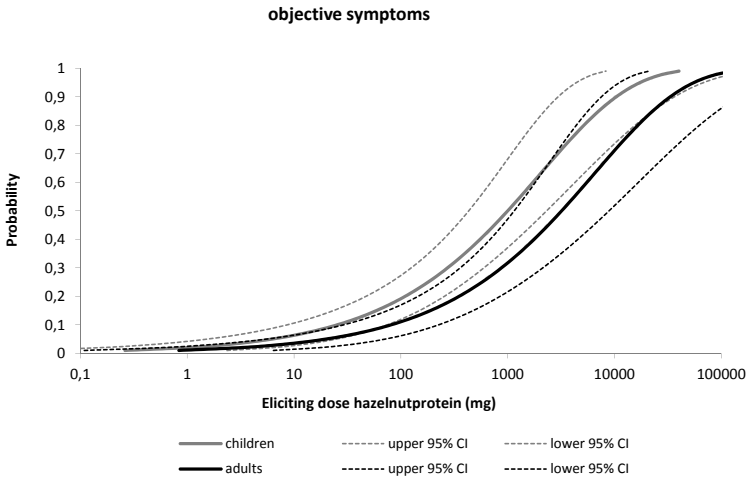
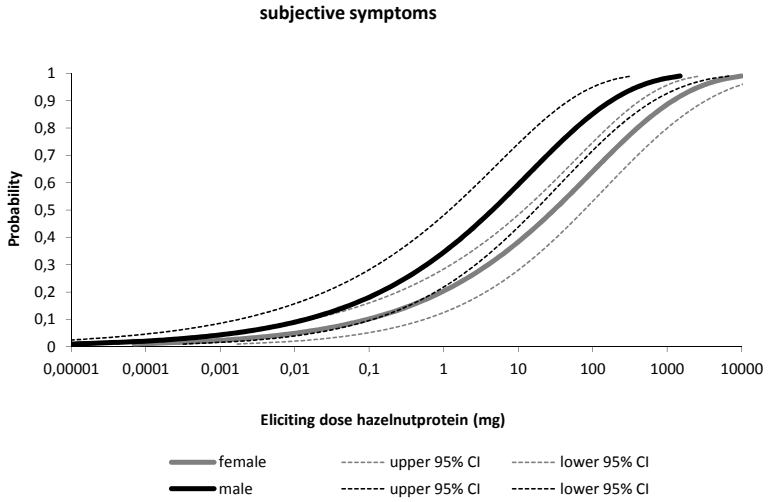


B

Figure 2. Threshold distribution curves for objective and subjective symptoms in children and adults.

Influencing factors on TDC for subjective symptoms in children and adults

Table 3B shows the effect of several factors on the TDC for subjective symptoms to hazelnut in children and adults separately. The TDC for subjective symptoms was comparable among males and females in children. However for the adult population, gender influenced the TDC for subjective symptoms. The TDC for subjective symptoms was lower in male adults than in female adults (p=0.02, HR=0.54).



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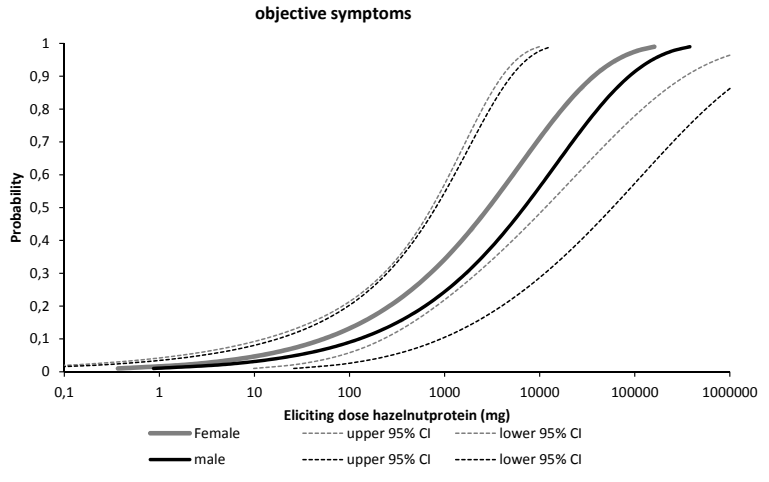

B

Figure 3. Threshold distribution curves for subjective and objective symptoms for females and males.

Table 2. ED5, ED10 and ED50 (mg protein) and the 95% confidence interval for subjective symptoms and objective symptoms in the total group, children and adults, P values for comparison of TDC for subjective or objective symptoms between children and adults

	ED5 (mg) (95% CI)	ED10 (mg) (95% CI)	ED50 (mg) (95% CI)	P value
Objective symptoms				
Total group	10.3 (3.04 - 34.9)	43.6 (18.0 - 106)	1902 (913 - 3963)	
Children	6.27 (1.57 - 25.1)	25.5 (8.67 - 74.9)	1002 (433 - 2320)	0.07
Adults	20.0 (5.59 - 71.9)	81.5 (29.3 - 227)	3207 (1153 - 8915)	0.07
Subjective symptoms				
Total group	0.02 (0.01 - 0.09)	0.16 (0.05 - 0.54)	37.4 (21.4 - 65.5)	
Children	0.05 (0.01 - 0.22)	0.37 (0.10 - 1.31)	77.5 (34.3 - 175)	<0.01
Adults	0.01 (0.003 - 0.06)	0.10 (0.03 - 0.36)	20.4 (9.97 - 41.9)	<0.01

Table 3. Effect of age, gender, atopic dermatitis, asthma, hay fever, size of SPT, IgE to hazelnut extract, rCor a 1, nCor a 9 and rCor a 14 was determined on the TDC for objective symptoms in the total group (A) and for subjective symptoms in children and adults (B). The strength of the effect is shown by a hazard ratio (HR), if p<0.05

A

	TDC for objective symptoms	
	Total group	
	HR	**P value**
Age	0.98	0.04
Gender (female)		0.81
Atopic dermatitis		0.10
Asthma		0.64
Hay fever		0.25
Size of SPT (mm)	1.07	0.02
IgE hazelnut extract		0.09
IgE rCor a 1		0.25
IgE nCor a 9	1.02	0.03
IgE rCor a 14	1.02	<0.01

B

	TDC for subjective symptoms			
	Children	**Children**	**Adults**	**Adults**
	HR	**P value**	**HR**	**P value**
Age		0.42		0.07
Gender (female)		0.90	0.54	0.02
Atopic dermatitis		Fit not ok		0.16
Asthma		0.71		0.34
Hay fever		0.73		0.71
Size of SPT (mm)		0.85	1.07	0.02
IgE hazelnut extract		0.09		0.41
IgE rCor a 1		0.35		0.53
IgE nCor a 9		0.11		0.99
IgE rCor a 14	1.01	0.03		0.15

SPT reactivity significantly influenced the TDC for subjective symptoms in adults (p=0.02, HR=1.07). The level of IgE to rCor a 14 was associated with the TDC for subjective symptoms in children (p=0.03, HR=1.01), but not in adults. Age, IgE to hazelnut extract, IgE to rCor a 1 and IgE to nCor a 9 or the presence of atopic dermatitis, asthma, hay fever, did not influence TDC for subjective symptoms in children and adults.

DISCUSSION

This study shows the threshold distributions of hazelnut in a pediatric and adult population, revealing that the threshold distribution of hazelnut for objective symptoms is similar within a pediatric or adult population, and male or female population. Therefore, pooling of all clinical threshold doses is appropriate for determination of the ED values for objective symptoms to hazelnut, which are the most important for risk assessment. Age, SPT reactivity and IgE to both Cor a 9 and Cor a 14 significantly influenced the TDC for objective symptoms.

The ED5 and ED10 for objective symptoms were 10.3 mg and 43.6 mg hazelnut protein, respectively, for the total hazelnut allergic population (both children and adults). The ED5 and ED10 tend, though statistically not significantly different, to be lower in the pediatric population (6.27 mg and 25.5 mg, respectively). This is in line with Eller et al.(8.7 mg and 15.9 mg, respectively) who performed mainly open challenges in a largely pediatric population (age mean 9.1, range 1.1-67.9 years)[13]. However, this is different from the 0.29 mg and 1.38 mg hazelnut protein reported by Blom et al. in an exclusively pediatric, but relatively small population (28 children) with a mean age of 6.5 years (range 3-17 years)[9]. The difference in ED values for hazelnut among these studies might be due to patient selection, or used challenge materials, like processing of hazelnuts[14], matrix and protocols. All hazelnut challenges were performed with unroasted hazelnuts, because roasting may reduce the allergenicity of hazelnut[15;16]. The used matrix was different among the different studies; Blom et al used baked cookies, Eller et al chocolate drink or bar and whole instant wheat cereal with apples sauce was used in our study. Baking of cookies may result in caramelization (Maillard reaction), which has been shown to increase allergenicity of peanut[17]. For hazelnut the effect of the Maillard reaction is unclear, because of conflicting data[18;19], but may explain the significantly lower thresholds to hazelnut in the study by Blom et al. A high fat matrix, like chocolate bar as used by Eller et al reduces gastric emptying and may increase thresholds for objective symptoms[20].

Clinical threshold information is important for allergen management but is hindered by insufficient data for derivation of safe threshold doses and analysis of modifying factors[21]. Our data indicate that the thresholds may be pooled between children and

adults, and males and females, because the threshold distribution of hazelnut was not statistically significantly different, which may further increase the pool of available data for safe threshold dose determination.

To get more insight into modifying factors, the effect of several patient characteristics on the TDC for objective symptoms was determined. Age did significantly influence the threshold distribution of hazelnut; increasing age was associated with an increased TDC for objective symptoms. This indicates that younger hazelnut allergic individuals are more sensitive to hazelnut than older individuals. This may be explained by a higher proportion of severe hazelnut allergy at younger age (manuscript submitted). A birch pollen related hazelnut allergy, which is generally mild, is more prevalent among adults[5].

Increased SPT reactivity and IgE titers to Cor a 9 and Cor a 14 were associated with lower thresholds for hazelnut, which indicates a lower threshold in a more severe hazelnut allergy. This association was not observed for IgE to hazelnut extract or rCor a 1. For peanut, similar results were shown between IgE levels to peanut extract, Ara h 1, Ara h 2, Ara h 3 and the thresholds for peanut[22;23]. So the potency of allergenic foods, like hazelnut and peanut, is influenced by the magnitude of the sensitization, which is related to the severity.

The TDC for subjective symptoms was approximately 100 fold lower than the TDC for objective symptoms and showed significant differences between children and adults. This is in line with earlier observations[8;24]. The TDC for subjective symptoms was significantly higher in children than in adults ($p<0.01$), which was also observed for peanut (unpublished data). But the ED5, ED10 and ED50 had overlapping confidence intervals, which does not suggest a different allergy management between children and adults. The significant difference in TDC does suggest that children develop subjective symptoms at a higher dose or might have difficulties in the recognition of subjective symptoms. Subjective symptoms can function as a warning symptom to prevent further ingestion of the culprit food. Unrecognized subjective symptoms or recognition at a higher dose than by adults, might pose children at risk for developing a more severe allergic reaction than adults. This finding is line with Van der Zee et al showing greater clinical sensitivity to subjective symptoms with increasing age[25].

Gender did not influence the TDC for subjective symptoms to hazelnut in children, but the TDC for subjective symptoms was lower in adult males than females. This indicates an increased sensitivity to recognize hazelnut in male adults, compared to female adults. This suggests that hormonal differences influence the differential recognition of allergenic foods, like hazelnut in adults.

The age and gender differences observed in recognition of subjective but not objective symptoms suggest that the TDC for objective symptoms is a better guide for allergy management than the TDC for subjective symptoms.

Sensitization to hazelnut extract and rCor a 1 and the presence of other atopic diseases like atopic dermatitis, asthma or hay fever did not significantly influence the TDC for objective nor subjective symptoms to hazelnut. Asthma and hay fever were also not associated with the threshold for peanut, only absence of atopic dermatitis was associated with a lower sensitivity for subjective symptoms to peanut[25].

In conclusion, our data demonstrate several intrinsic factors that influence the sensitivity to hazelnut. The obtained EDs can improve the food labeling, which will increase the product choice and decrease the risk of accidental ingestions for hazelnut allergic individuals.

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Chapter 6

Peanut allergy is common amongst hazelnut sensitized subjects but is not primarily the result of IgE cross-reactivity

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Submitted

ABSTRACT

Background: Hazelnut and peanut are botanically unrelated foods, but patients are often sensitized and allergic to both, for reasons that are not well understood.

Objective: To investigate molecular co-sensitization and cross-reactivity to peanut in hazelnut sensitized individuals.

Method: Children (n=81) and adults (n=80) were retrospectively selected based on sensitization to hazelnut. IgE to hazelnut extract, Cor a 1, 8, 9 and 14, to peanut extract, Ara h 1, 2, 3, 8 and 9, and to Bet v 1 was determined by ImmunoCAP. Allergy to hazelnut and peanut was established by DBPCFC and/or detailed clinical history. Patients were either tolerant or displayed subjective or objective symptoms to either food. IgE cross-reactivity between hazelnut and peanut storage proteins was assessed by reciprocal ImmunoCAP-inhibition experiments.

Results: Of the 161 hazelnut sensitized subjects, 109 (68%) were also sensitized to peanut, and 73 (45%) had clinical expression of allergy to peanut that was not associated to the presence or severity of hazelnut allergy. Instead, it was associated to IgE reactivity to peanut storage proteins, in particular Ara h 2. No cross-reactivity could be detected between Ara h 2 and Cor a 14 and 2/13 subjects displayed extensive cross-reactivity between 11S globulins, in both plasma Ara h 3 almost completely inhibited IgE binding to Cor a 9.

Conclusion: Peanut allergy is not a result of IgE cross-reactivity to hazelnut storage proteins. IgE to Cor a 14 and Ara h 2 may serve as useful markers of primary sensitization to hazelnut and peanut, respectively.

INTRODUCTION

Hazelnut is botanically closely related to several other frequently consumed tree nuts. Peanut belongs to the legumes and is botanically distant to tree nuts. Nevertheless, combined sensitization to one or more tree nuts and to peanut is very common and has been reported to occur in a majority of nut allergic children and adolescents[1] and sensitization to both hazelnut and peanut can be observed already at an early age[2]. Children and adults with peanut allergy are often also allergic to tree nuts such as hazelnut[3;4]. Pollen-induced cross-reactive IgE antibodies, in particular against the major birch pollen allergen Bet v 1, is a frequent cause of sensitization to fruits, vegetables, nuts and legumes, but these cross-reactions are generally regarded as not being involved in severe allergies to tree nuts and peanut.

It has been suggested that cross-reactivity between structurally related proteins in hazelnut and peanut, such as the major storage proteins, may be relevant in patients with more severe combined allergies to tree nuts and peanut[5]. Non-pollen related IgE cross-reactivity has indeed been described for peanut and hazelnut[6], but the molecular basis has not been elucidated in detail. Several structurally related allergenic storage proteins and pathogenesis-related proteins are present in hazelnut and peanut. Homologous regions of the 11S globulins Cor a 9 (hazelnut) and Ara h 3 (peanut) have been described[7;8]. The 2S albumin of hazelnut, Cor a 14[9], belongs to the same protein family as peanut allergens Ara h 2 and 6, two closely related proteins that play a dominant role in peanut allergy[10;11]. A study by De Leon et al.[12] reported negligible inhibition of IgE binding to Ara h 2 with hazelnut extract in three patient sera, suggesting that cross-reactivity of 2S albumins in peanut and hazelnut is possibly of limited importance. Ara h 1 and Cor a 11, belonging to the vicilin-like storage proteins[13;14], are also potential candidates for cross-reactivity between hazelnut and peanut. However, in a large pan-European study (>400 patients) carried out within the EuroPrevall project, Cor a 11 was however found to be of lower importance as compared to Cor a 9 and Cor a 14 (manuscript in preparation). This allergen was therefore not included in the study reported here. Also the homologous nonspecific lipid transfer proteins (nsLTPs) Cor a 8 and Ara h 9 are of limited importance in our central European region[15] as compared to the Mediterranean region[16]. Finally, homologous oil-body associated proteins in hazelnut and peanut, so-called oleosins, have been identified as allergens and given the names Cor a 12, Cor a 13[17], Ara h 10 and Ara h 11[18;19]. Due to the extremely hydrophobic nature of these proteins, they are very difficult to handle biochemically once taken out of their lipophilic oil-body environment. So far, no highly purified and soluble oleosin preparations, that would allow a reliable evaluation of their role in allergy to nuts and seeds, have become available.

The aim of this study was to investigate non-pollen related IgE co-sensitization and cross-reactivity between hazelnut and peanut in relation to clinical symptoms. The clinical reactivity to hazelnut and peanut was asessed by double-blind placebo-controlled food challenge (DBPCFC) and/or a detailed clinical history, which was related to component-resolved-diagnosis and reciprocal IgE inhibition experiments with the major storage protein allergens. The molecular focus of this study was on 2S albumins (Cor a 14, Ara h 2 and Ara h 6) and 11S globulins (Cor a 9 and Ara h 3).

METHODS

Patients

A group of 161 subjects in total, 81 children and 80 adults, was retrospectively included based on IgE sensitization to hazelnut extract (≥0.35 kU_A/L by ImmunoCAP). Their sensitization profiles to hazelnut allergens and clinical phenotypes have recently been described in detail[20]. Clinical relevance of sensitization to hazelnut was established by DBPCFC in all children and 56 adults. The 24 adults declining DBPCFC all had a convincing history of hazelnut allergy with objective symptoms. Of the 161 patients, 38 were tolerant, 44 only had subjective symptoms during challenge and 79 had objective symptoms (55 by challenge / 24 by history). The composition of the study population did not reflect the general hazelnut patient population because it was intentionally enriched with patients displaying objective symptoms in order to increase the power for association studies. For the present study, peanut allergy was assessed in 150/161 hazelnut-sensitized subjects by DBPCFC (n=62) or by a detailed clinical history (n=88). Eleven hazelnut-sensitized subjects had never ingested peanut to their knowledge, and were excluded from the analyses focusing on combined sensitization and allergy to hazelnut and peanut.

IgE measurements and inhibition experiments

IgE to hazelnut extract, rCor a 1.04, rCor a 8, nCor a 9, rCor a 14, peanut extract, rAra h 1, rAra h 2, rAra h 3, rAra h 8, rAra h 9 and rBet v 1 was determined by ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden). Patients with >2 kU_A/L of IgE to both hazelnut and peanut 2S albumins (Cor a 14 and Ara h 2, n=20) and/or 11S globulins (nCor a 9 and rAra h 3, n=14) were selected for reciprocal ImmunoCAP inhibition experiments, except for one of the latter 14 which could not be analysed due to shortage of plasma. Plasma samples of patients with high IgE levels (>15 kU_A/L) were diluted to a concentration of approximately 5 kU/L. To test cross-reactivity between homologous allergens, plasma samples were pre-incubated for one hour with 100 µg/mL purified rCor a 14, nCor a 9, nAra h 2, nAra h 3 and nAra h 6 (peanut allergens kindly provided by TNO, Zeist, The

Netherlands), or with buffer as a negative control, before measurement of IgE binding to the homologous peanut or hazelnut allergen and to the inhibitor allergen itself. Titrated inhibition experiments performed with two individual plasma samples or a pool of 13 plasma samples revealed that an inhibitor concentration of 100 µg/mL was required to reach maximum inhibition. Negative inhibition results were set to 0%.

Data analysis

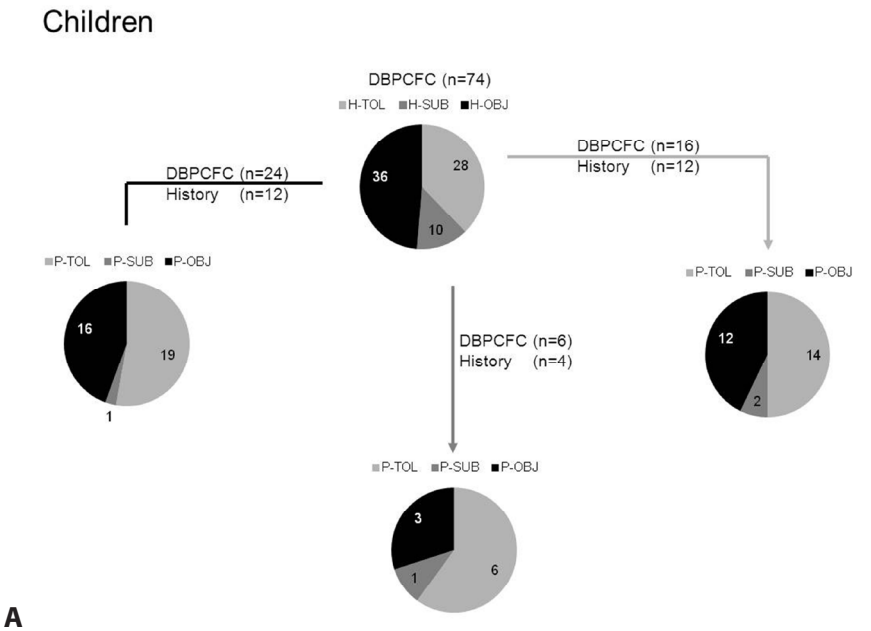
The presence and severity (subjective versus objective symptoms) of peanut allergy among hazelnut allergic and hazelnut tolerant patients were compared using the Chi-square test. To compare frequency of sensitization to hazelnut and peanut extract and their purified allergens among patients in different clinical groups, a cut-off level of 0.35 kU_A/L and the Chi-square test were used. Spearman correlation was used to analyse co-variation of IgE levels to hazelnut and peanut and their homologous purified allergens. P values <0.05 were considered significant. All statistical analyses were performed with SPSS (Version 20.0, SPSS Inc., Chicago, IL, USA).

RESULTS

Hazelnut and peanut allergy

Peanut allergy and its severity (subjective versus objective symptoms) was evaluated by DBPCFC and/or by detailed clinical history in a population of hazelnut-sensitized subjects with known clinical phenotype for hazelnut[20]. Clinical phenotype with regard to peanut was established by DBPCFC in 46 children and 16 adults and by history in 28 children and 60 adults.

No clear association between clinical phenotype for hazelnut and peanut was observed in this study population (Figure 1). Of all patients with a hazelnut allergy (n=114), 55 (48%) also had a peanut allergy, with no significant difference between children (46%) and adults (50%). The same frequency of peanut allergy, with a similar dominance of objective symptoms, occurred among children and adults that tolerated hazelnut. Of those having subjective symptoms to hazelnut, about 60% tolerated peanut while 30% of the children and 24% of the adults in this category displayed objective symptoms of peanut allergy. Peanut allergy with only subjective symptoms was rarely observed in this study population. Peanut allergy with objective symptoms was significantly more common among adults with objective (57%) than with subjective (24%) symptoms to hazelnut (p=0.01). Overall, these observations demonstrate that the occurrence and type of clinical reaction to hazelnut and peanut do not follow a common pattern in our study population.



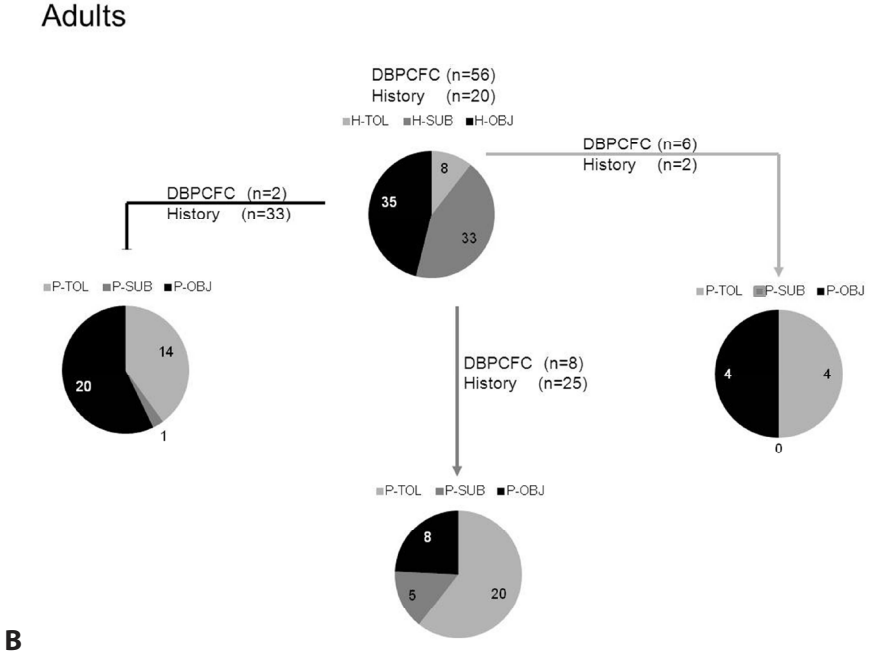


Figure 1. Severity of hazelnut allergy by DBPCFC or history (in the center) and severity of peanut allergy per severity group of hazelnut allergy in children (A) and adults (B). H, Hazelnut; P, Peanut; TOL, tolerant; SUB, subjective symptoms and OBJ, objective symptoms.

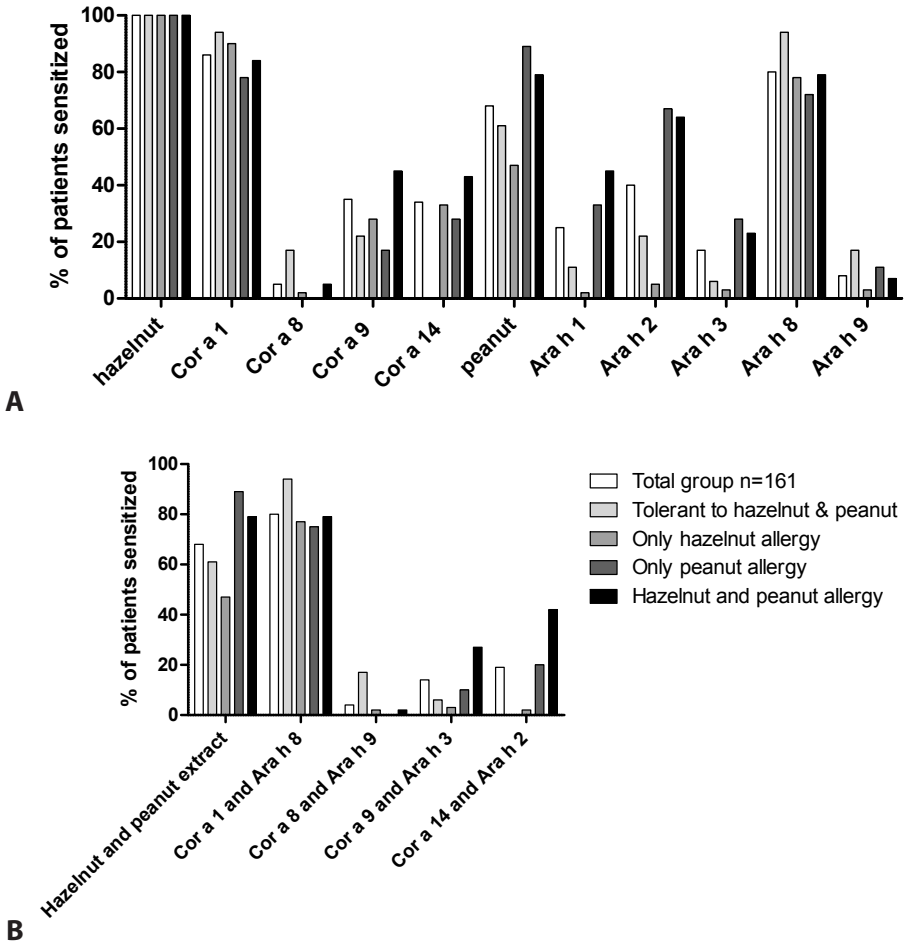


Figure 2. Percentage of patients with sensitization (≥0.35 kU_A/L) to hazelnut and peanut and their purified allergens (A) and co-sensitization to both extracts or structurally related proteins in hazelnut and peanut (B) among different clinical groups.

Sensitization to hazelnut and peanut and their components

The frequency of sensitization (specific IgE concentration ≥0.35 kU_A/L) to hazelnut, peanut and their components among all 161 subjects is displayed in Figure 2A and the occurrence of co-sensitization in Figure 2B, 3A and 3B. With regard to prevalence of detectable IgE, sensitization to hazelnut and peanut components was dominated by the PR-10 allergens Cor a 1 (86%) and Ara h 8 (80%). Eleven subjects were sensitized to Cor a 1 but not to Ara h 8. Of all patients, 87% were sensitized to rBet v 1 (data not shown). Sensitization to Cor a 9 and Cor a 14 occurred in 35% and 34% of all subjects, respectively, while 22% were sensitized to both (Figure 3B). Owing to the high frequency

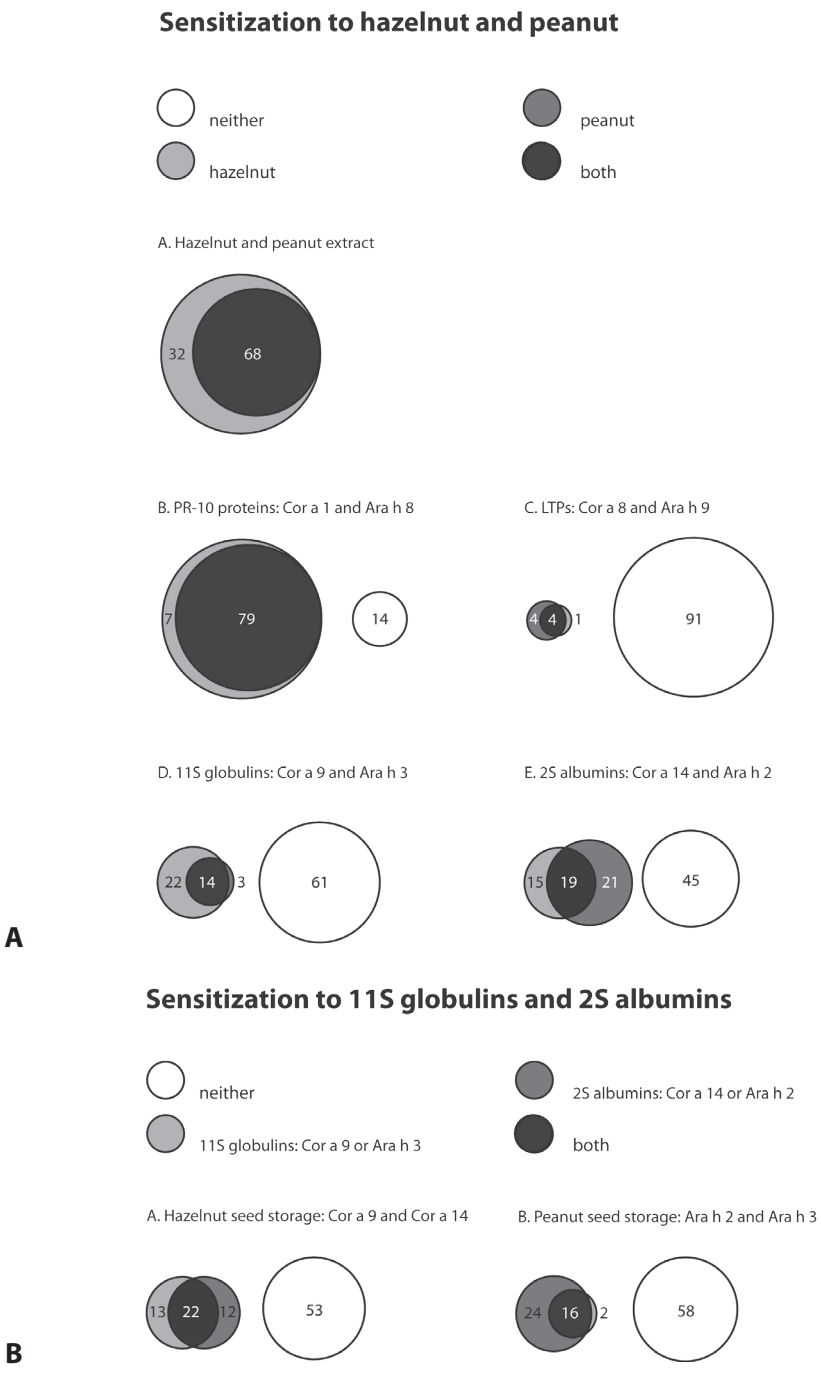


Figure 3. Percentage of subjects in the study population with sensitization to hazelnut and/or peanut and their purified allergens (n=161) (A) and sensitization to hazelnut or peanut seed storage proteins, 11S globulins and/or 2S albumins (B).

of sensitization to PR-10 proteins in the study population, 70% of Cor a 9 sensitized subjects and 80% of Cor a 14 sensitized subjects were co-sensitized to Cor a 1. Sensitization to any of the lipid transfer proteins (Cor a 8 and Ara h 9) was observed in <10% of the subjects.

A majority (68%) of all 161 hazelnut-sensitized subjects were also sensitized to peanut (Figure 3A). Co-sensitization to hazelnut and peanut 2S albumins (Cor a 14 and Ara h 2) was found in 19%, and to the 11S globulins (Cor a 9 and Ara h 3) in 14% of the subjects (Figure 2B and 3A). Sensitization to both Ara h 2 and Ara h 3 occurred in 16% (Figure 3B).

Spearman rank correlation analyses of concentrations of IgE antibodies to hazelnut, peanut and their pairs of homologous components (Figure 4) demonstrated, as expected, that IgE responses against Cor a 1 and Ara h 8 were very closely correlated (r=0.90, 95% CI 0.87-0.93), and both correlated even stronger with IgE responses against Bet v 1 (r=0.96, 95% CI 0.95-0.97, and r=0.91, 95% CI 0.88-0.94, respectively). Concentrations of IgE against lipid transfer proteins Cor a 8 and Ara h 9 correlated moderately (r=0.71, 95% CI 0.62-0.78). Correlations between IgE responses to 11S and 2S storage proteins from hazelnut and peanut were clearly weaker (r=0.58, 95% CI 0.47-0.68, and r=0.50, 95% CI 0.37-0.61, respectively), and between IgE levels to hazelnut and peanut extracts even more so (r=0.40, 95% CI 0.26-0.53). The order of observed correlations in IgE binding was related to the level of amino acid sequence identity between the pairs of homologous allergens: Cor a 1/Bet v 1 - 68%, Cor a 1/Ara h 8 - 53%, Ara h 8/Bet v 1 - 46%, Cor a 8/Ara h 9 - 55%, Cor a 9/Ara h 3 - 47% and Cor a 14/Ara h 2 - 29%.

Cross-reactivity between hazelnut and peanut storage proteins

Plasma samples from a total of 25 subjects were selected for reciprocal IgE inhibition experiments (clinical characteristics are summarized in Table 1). Consistent with the weak correlation between IgE responses to hazelnut and peanut 2S albumins, ImmunoCAP inhibitions (n=20) showed no significant inhibition of IgE binding to rCor a 14 by nAra h 2 at saturating inhibitor concentrations (100 µg/ml, Figure 5A, E1). The same result was obtained when nAra h 6 was used as inhibitor instead of nAra h 2 (not shown). Also the reverse inhibition experiment, i.e. preincubation of plasma with rCor a 14 prior to measurement of IgE binding to rAra h 2, provided no evidence for cross-reactivity between these two allergens. In both cases, self-inhibition resulted in virtually complete abrogation of IgE binding (Figure 5A).

In the corresponding experiments with 11S globulins, no significant inhibition of IgE binding to rAra h 3 by nCor a 9 occurred in any of the 13 subjects tested (Figure 5B and C). A somewhat more diverse picture was seen in the reverse inhibition experiment (Figure 5B). In plasma of two subjects (HN142A and HN147A), preincubation with nAra h 3 caused almost complete inhibition (83% and 95%) of IgE binding to nCor a 9 (Figure 5C). In two additional cases (HN001C and HN082C), IgE binding to nCor a 9 was more

effectively inhibited by nAra h 3 (38% and 39%) than by nCor a 9 itself, (21% and 27%). Overall, the cross-inhibition experiments with the two 11S globulins showed variable degrees of blockage of IgE binding (Figure 5B), even at 100 µg/ml of inhibitor, ranging from negligible (8%) to almost complete inhibition (95%).

Sensitization profiles and clinical phenotypes of hazelnut and peanut allergy

Sensitization to Cor a 9 and Cor a 14 was previously shown to be associated with objective symptoms to hazelnut in the population studied here[20]. Among the individuals with objective hazelnut allergy (n=63), 60% had IgE to nCor a 9 and 54% to rCor a 14. In the present study, focusing on co-existing peanut allergy, none of the IgE responses to individual hazelnut allergens were predictive for having peanut allergy, nor for its clinical presentation (subjective versus objective symptoms). Interestingly, the same was true for sensitization to Ara h 8 and Ara h 9 in this study population. Instead, peanut allergy was associated with sensitization to the known clinically relevant peanut allergens (Ara h 1, Ara h 2 and Ara h 3), especially in regard to those presenting with an objective phenotype of peanut allergy. Among individuals with objective peanut allergy, 43% had IgE to rAra h 1, 67% IgE to rAra h 2 and 25% IgE to rAra h 3. In particular, all but one peanut tolerant patient (n=77, 99%) had IgE to Ara h 2 <5 kU_A/L.

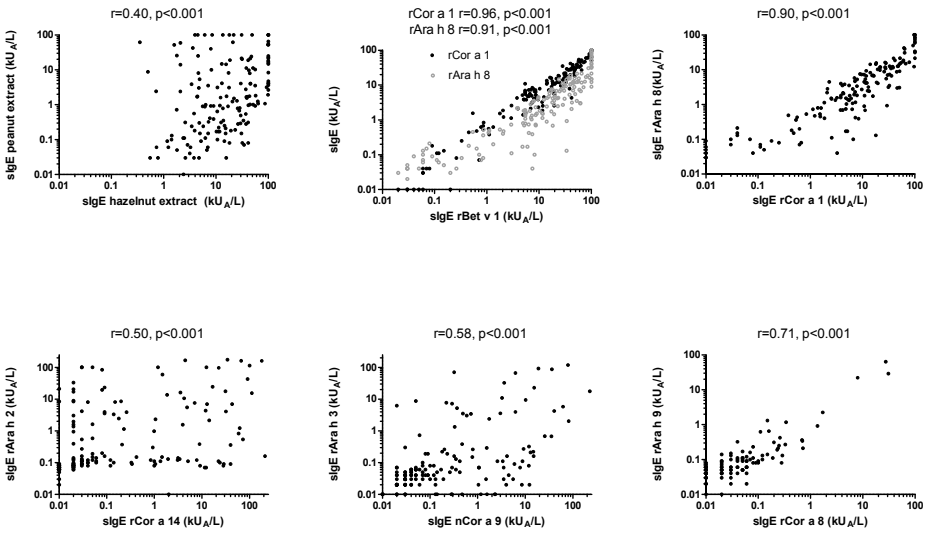


Figure 4. Correlation between IgE levels to hazelnut and peanut extract and several homologous allergens in hazelnut, peanut and birch pollen.

Table 1. Clinical characteristics of 25 patients selected for ImmunoCAP inhibition experiments

Studynr	Age	Gender	Hazelnut allergy		Peanut allergy		ImmunoCAP inhibition for	
			severity	by	severity	by	Cor a 14 Ara h 2/6	Cor a 9 Ara h 3
HN001C	10	M	No	DBPCFC	No	DBPCFC	No	Yes
HN011C	10	F	Obj	DBPCFC	Obj	DBPCFC	Yes	Yes
HN017C	7	M	Obj	DBPCFC	Obj	History	Yes	No
HN035C	15	M	Obj	DBPCFC	Unknown		Yes	Yes
HN041C	12	F	No	DBPCFC	Obj	DBPCFC	Yes	No
HN066C	10	M	No	DBPCFC	Obj	History	Yes	No
HN070C	12	F	Obj	DBPCFC	Unknown		Yes	No
HN075C	4	M	Obj	DBPCFC	Obj	DBPCFC	Yes	No
HN078C	14	F	Obj	DBPCFC	Obj	History	Yes	No
HN082C	13	F	Obj	DBPCFC	Obj	DBPCFC	No	Yes
HN094C	7	M	Obj	DBPCFC	Obj	History	Yes	Yes
HN100C	8	M	Obj	DBPCFC	Unknown		Yes	Yes
HN121C	7	F	Obj	DBPCFC	Unknown		Yes	No
HN131C	6	M	Obj	DBPCFC	Obj	DBPCFC	Yes	Yes
HN134C	3	F	Obj	DBPCFC	Obj	DBPCFC	Yes	No
HN158C	1	M	Obj	DBPCFC	No	DBPCFC	No	Yes
HN164C	5	M	Obj	DBPCFC	Obj	History	Yes	Yes
HN114A	22	M	Obj	DBPCFC	Unknown		Yes	Yes
HN118A	40	M	Obj	History	Obj	History	Yes	Yes
HN126A	29	F	Obj	History	Obj	History	Yes	No
HN142A	20	M	Obj	History	Obj	History	No	Yes
HN143A	21	F	Obj	History	Obj	History	Yes	No
HN145A	18	M	Obj	DBPCFC	Obj	DBPCFC	Yes	No
HN147A	27	F	Obj	History	Obj	History	No	Yes
HN155A	26	F	Obj	History	Unknown		Yes	No

Obj, objective symptoms; Subj, subjective symptoms; No, no symptoms
C, child; A, adult
M, male; F, female

DISCUSSION

In this study, we observed frequent sensitization and allergy to peanut in a population of hazelnut sensitized subjects. The clinical manifestation of peanut allergy was associated to the level of IgE to peanut storage proteins, in particular Ara h 2, as previously described[10;11], but not to the subjects' hazelnut allergy; neither their symptoms nor their pattern of sensitization to hazelnut allergens. Although about half of the peanut

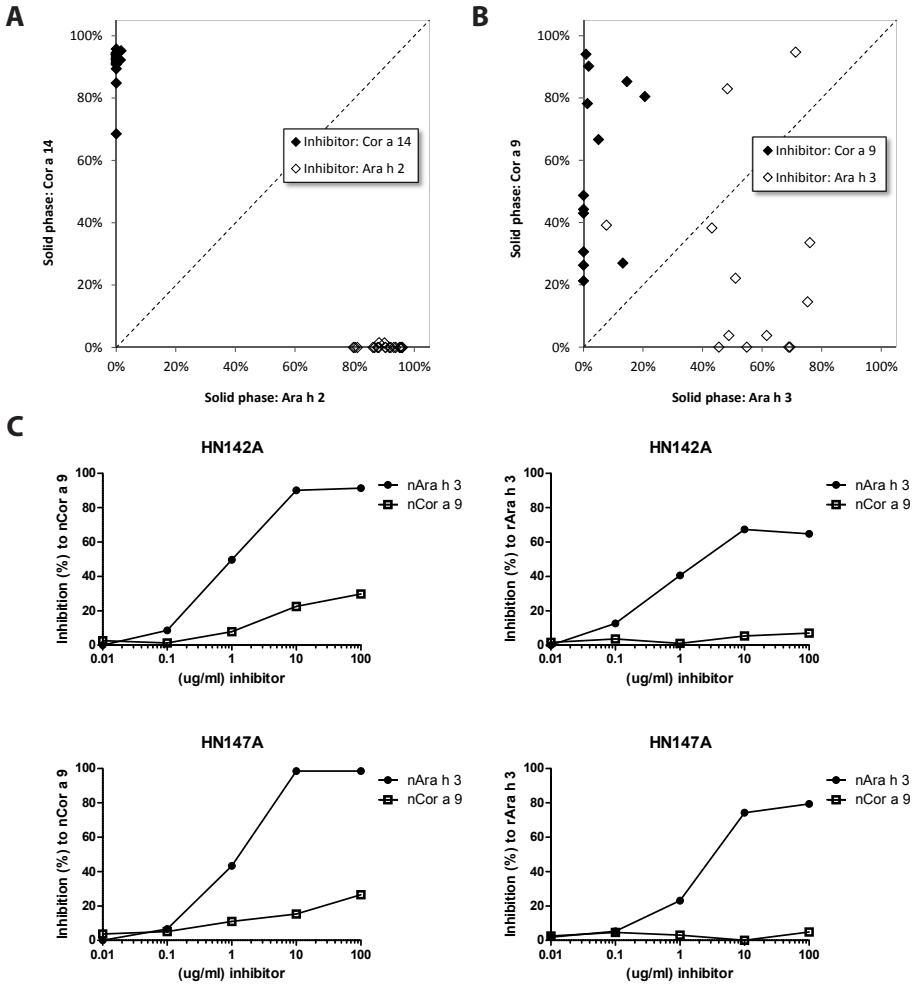


Figure 5. ImmunoCAP inhibition of 2S albumins in twenty plasma samples (A) and 11S globulins in thirteen plasma (B) between hazelnut and peanut. Solid phase is shown at x- and y-axis and the inhibitor in squares. Titrated inhibitions are shown for two patients whose IgE response to 11S globulins displayed extensive co-recognition of nCor a 9 and nAra h 3 (C).

allergic patients also had IgE to hazelnut 2S albumin, the absence of demonstrable cross-reactivity between 2S albumins from peanut and hazelnut suggests that primary peanut sensitization rather than cross-reactivity with hazelnut was the cause of peanut allergy in these patients.

IgE antibodies to Ara h 3 and Cor a 9 were only rarely cross-reactive in this study population. In plasma of two patients (HN142A and HN147A), however, Ara h 3 could almost completely outcompete the IgE binding to Cor a 9. The IgE levels were also far higher to Ara h 3 than to Cor a 9 in these plasma, indicating that these patients had a primary sensitization to Ara h 3 and a cross-reactive response to Cor a 9. Neither of

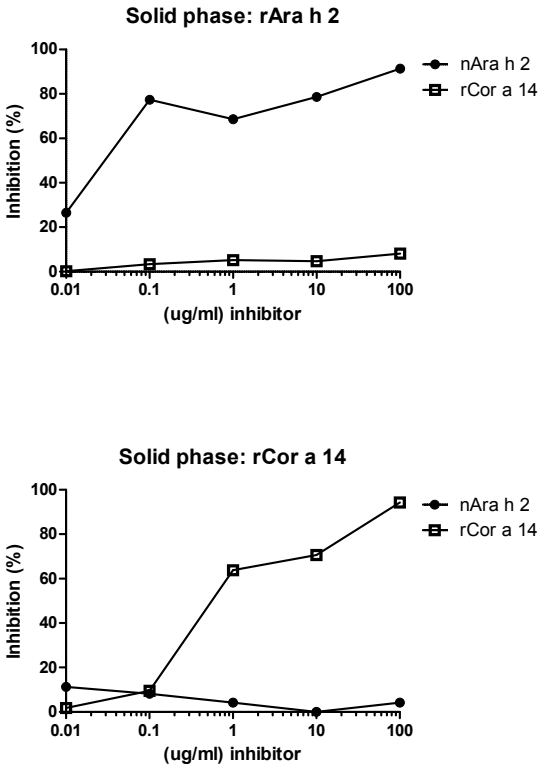


Figure 6. ImmunoCAP inhibition with rAra h 2 and rCor a 14 on solid phase inhibited with nAra h 2 and rCor a 14 in a plasma pool (n=13)

the two patients had IgE reactivity to other hazelnut components. The fact that both displayed objective symptoms to hazelnut suggests that primary sensitization to Ara h 3 may in rare cases cause severe hazelnut allergy through cross-reactive IgE recognition of Cor a 9.

Based on our findings, we conclude that sensitization to peanut and hazelnut 2S albumins and 11S globulins are largely independent events. In comparison to Cor a 14 and Cor a 9, the 7S globulin Cor a 11 appears to play a less prominent role in hazelnut allergy. Even though a link between hazelnut and peanut allergy through 7S globulins Cor a 11 and Ara h 1 cannot be excluded, we find it unlikely that it would be a major determinant. Murine data suggested 7S globulin cross-reactivity between cashew, walnut and peanut, but the cross-reactive clinical response to peanut was limited[21;22]. No experimental evidence regarding cross-reactivity between Cor a 11 and Ara h 1 is available, whereas their amino acid sequences show a modest level of similarity and other members of this protein family have been found not to share IgE binding epitopes with one another[13;14].

While this study has focused on examining allergenic relationships between major hazelnut and peanut storage protein and demonstrated a very limited degree thereof, it is likely that a more extensive pattern of cross-reactions exist among different tree nuts. Indeed, cross-reactivity has been demonstrated between Cor a 9 and the 11S globulins from walnut (Jug r 4)[23] and also between the 2S albumins of these two nuts (unpublished observations). In contrast, a study by Rosenfeld and co-workers could not demonstrate cross-reactivity between walnut and peanut when analyzing linear IgE epitopes of Ara h 1, Ara h 2 and Ara h 3[24]. Future studies utilizing pure major allergen components from a variety of tree nuts will shed light on causes underlying allergies to multiple nuts.

This study has clearly demonstrated that pollen-associated and pollen-independent tree nut and peanut co-sensitization are extensively overlapping serotypes. The common division of tree nut and peanut allergic patients into two distinct categories, pollen-associated and non-pollen associated[25], therefore appears insufficient and should be complemented with a third category representing a combination of these two types.

Most hazelnut sensitized subjects were co-sensitized to the Bet v 1-related allergens Cor a 1 and Ara h 8 (80%). Although we did not formally demonstrate that the observed IgE responses to Cor a 1 and Ara h 8 were cross-reactive, their very close correlation and even closer correlation to IgE responses to Bet v 1 are highly suggestive of strong cross-reactivity.

The hazelnut and peanut PR-10 proteins (Cor a 1 and Ara h 8) and LTPs (Cor a 8 and Ara h 9) have a similar level of amino acid sequence identity, 53% and 55%, respectively. Yet, the IgE binding to Cor a 1 and Ara h 8 showed much higher correlation than the IgE binding to Cor a 8 and Ara h 9, indicating higher conservation of IgE binding structures between the PR-10 proteins than between the LTPs in relation to the entire amino acid sequences of the proteins. In addition, another PR-10 protein Mal d 1 from apple showed also higher similarity with Bet v 1 in 3-dimensional structure, than in linear sequence identity[26].

It is important to emphasize that the patient selection in the present study was biased towards sensitization to hazelnut. It is possible that the use of other inclusion criteria, such as sensitization to peanut, would have resulted in slightly different observations with respect to cross-reactivity and clinical phenotypes. Nevertheless, the data strongly support the conclusion that co-sensitization and combined allergy to hazelnut and peanut is not a phenomenon primarily explained by cross-reactivity of IgE against hazelnut and peanut storage proteins. Sensitization to Bet v 1-related allergens Cor a 1 and Ara h 8 was not found to be predictive for hazelnut and peanut allergy, respectively, in this group of patients. The only serum marker found to be differentially associated with objective hazelnut and peanut allergy was sensitization to the storage proteins.

Despite the fact that IgE against 2S albumins and/or 11S globulins has been identified as risk factor for objective (severe) symptoms[20], a substantial number of adult patients with objective symptoms in this study population were negative to both the 2S albumins and the 11S globulins but positive to Cor a 1 and/or Ara h 8: 30% of subjects with objective symptoms to hazelnut had IgE only to Cor a 1, 20% of subjects with objective symptoms to peanut had IgE only to Ara h 8. The association between Ara h 8 and objective peanut allergy might be overrepresented in this study, because of the patient selection. For hazelnut, the observation suggests a role for Cor a 1 also in more severe symptoms, as discussed in our recent paper on hazelnut allergy[20] and already described for PR-10 proteins in soy and apple[27;28].

It appears from this study that peanut sensitization and allergy is common in subjects with sensitization and/or allergy to hazelnut. Interestingly, the clinical presentation with regard to hazelnut (tolerant, subjective or objective symptoms) had no predictive value with regard to peanut allergy. For identification of hazelnut and/or peanut allergic subjects, sensitization to the hazelnut and peanut specific seed storage proteins appeared helpful. The rare occurrence of cross-reactivity between hazelnut and peanut seed storage proteins makes them good markers for primary sensitization to each of these foods.

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Chapter 7

Major hazelnut and peanut allergens are potent in basophil activation and cross-react at T cell level

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Submitted

ABSTRACT

Background: The importance of individual hazelnut allergens in effector and immune-regulatory responses of hazelnut allergy is largely unknown.

Objective: To assess and compare the potency of hazelnut and peanut allergens at effector and T cell level.

Method: Basophil activation test was performed using natural extracts and purified allergen components of hazelnut, peanut and birch pollen on basophils passively sensitized with plasma from eleven hazelnut allergic patients. Short term hazelnut specific (n=22) and peanut specific (n=13) T cell lines (TCLs) were stimulated with extracts and a selection of individual components of hazelnut and peanut.

Results: rCor a 14 was more potent than nCor a 9 (p=0.02) and rCor a 1 (p=0.08) in basophil activation. A corresponding pattern was observed for peanut allergens, nAra h 2 and nAra h 6 being more potent than nAra h 3 and rAra h 8. The majority of hazelnut specific TCLs (n=22) responded to rCor a 1 (n=16, 73%), nCor a 9 (n=12, 55%), rCor a 14 (n=13, 59%), peanut extract (n=22, 100%), nAra h 3 (n=14, 64%) and nAra h 6 (n=17, 77%). A hazelnut extract size fraction containing a high molecular weight protein, possibly Cor a 11, induced the strongest T cell proliferation. Peanut specific TCLs showed a similar pattern of responses to hazelnut and peanut extract and their individual components.

Conclusion: Hazelnut and peanut 2S albumins are potent in basophil activation. At T cell level, cross-reactivity between hazelnut and peanut was observed.

INTRODUCTION

Hazelnut and peanut allergy are potentially life threatening conditions[1] and are seldom outgrown[2]. They often occur together, frequently in the presence of allergies to other tree nuts[3-5]. Sensitization to hazelnut and peanut can occur already early in life, before hazelnut and peanut have been introduced into the diet[6;7]. Cross-reactions between structurally related proteins in hazelnut and peanut could play a role in this pattern of concomitant sensitization. Cross-reactions may be induced at IgE and T cell level.

While effector and immune-regulatory responses to peanut allergens have been extensively studied, little is known about hazelnut allergens in this regard. For peanut it was shown that Ara h 2 and Ara h 6 were much more potent than Ara h 1 and Ara h 3 in induction of basophil activation[8-11]. T cells have an important role in the induction of allergic responses. For peanut, allergen specific responses to Ara h 1, 2, 3 and 6 have already been shown[5;12]. Basophil activation and T cell responses to different hazelnut allergens in comparison to peanut allergens will provide insight into the induction of hazelnut and peanut allergy.

Several allergens have been identified in hazelnut. The birch pollen-related hazelnut allergen Cor a 1 (PR-10 protein family) has mainly been associated with mild and local symptoms[13-15]. The lipid transfer protein Cor a 8 has been described particularly in patients from the Mediterranean area, who frequently display severe reactions to hazelnut[14;16]. T cell cross-reactivity was shown with Pru p 3 from peach, which dominated the response[17]. It was recently shown that IgE to the 11S globulin nCor a 9 and 2S albumin rCor a 14 was highly specific for an objective hazelnut allergy[18]. Furthermore, recognition of purified Cor a 11 was recently reported in children with severe hazelnut allergy[19]. However, little information is available regarding the potency of these allergens in the induction of IgE or T cell responses. Only for Cor a 11, the potency to induce basophil degranulation was shown to be low[19;20].

The aim of this study was to assess the potency of the response to hazelnut and peanut allergens in basophil activation and at T cell level in a hazelnut sensitized population, and to evaluate potential T cell cross-reactivity between hazelnut and peanut.

METHODS

Patients

Patients (16 children and 9 adults) with a sensitization to hazelnut extract ≥0.35 kU_A/L (n=25) were selected for this study (Table 1, 2). A double-blind placebo-controlled food challenge (DBPCFC) for hazelnut was performed in 24 patients. Of these, two were hazelnut tolerant, four developed subjective symptoms and eighteen objective symp-

toms during challenge. One patient had a convincing history of objective symptoms to hazelnut and declined challenge. Twenty patients also underwent a DBPCFC for peanut; eight were tolerant, one had subjective and eleven had objective symptoms during challenge. Five patients had an elimination diet based on sensitization and therefore never ingested peanut, nor underwent a DBPCFC. The IgE levels to rAra h 2 in those five patients ranged from 4.08-43.10 kU_A/L.

Table 1 Patients' characteristics n=25 (n=11 BAT, n=22 TCL) and performed experiments

Study nr	Age	Gender	Hazelnut allergy		Peanut allergy		BAT	T cells
			severity	History/DBPCFC	severity	History/DBPCFC		
HN002C*	14	M**	Subj^	DBPCFC	Unknown			HN
HN004C	9	M	No	DBPCFC	Obj	DBPCFC		HN-PN
HN008C	8	M	Obj	DBPCFC	Obj	DBPCFC	Yes	HN-PN
HN011C	10	F	Obj	DBPCFC	Obj	DBPCFC	Yes	
HN017C	7	M	Obj	DBPCFC	Obj	History	Yes	HN
HN018C	7	M	Obj	DBPCFC	No	DBPCFC		HN-PN
HN021A	18	M	Subj	DBPCFC	No	DBPCFC		HN-PN
HN023C	9	M	Subj	DBPCFC	No	History		HN-PN
HN026C	9	M	No	DBPCFC	Unknown			HN
HN035C	15	M	Obj	DBPCFC	Unknown		Yes	HN-PN
HN037C	7	F	Obj	DBPCFC	Obj	DBPCFC	Yes	HN-PN
HN038C	7	M	Obj	DBPCFC	No	DBPCFC		HN-PN
HN070C	12	F	Obj	DBPCFC	Unknown		Yes	HN-PN
HN082C	13	F	Obj	DBPCFC	Obj	DBPCFC		HN
HN094C	7	M	Obj	DBPCFC	Obj	History	Yes	HN-PN
HN100C	8	M	Obj	DBPCFC	Unknown		Yes	HN
HN135C	4	F	Obj	DBPCFC	No	DBPCFC	Yes	
HN045A	37	F	Subj	DBPCFC	No	DBPCFC		HN-PN
HN046A	22	F	Obj	DBPCFC	No	History		HN-PN
HN056A	21	F	Obj	DBPCFC	Subj	History	Yes	
HN116A	22	F	Obj	DBPCFC	Obj	History		HN
HN117A	37	M	Obj	DBPCFC	Obj	DBPCFC		HN
HN144A	36	F	Obj	History	Obj	History		HN
HN145A	18	M	Obj	DBPCFC	Obj	DBPCFC		HN-PN
HN157A	23	F	Obj	DBPCFC	No	History	Yes	HN

* C: child, A: adult

** M: male, F: female

^ Subj: subjective symptoms, No: no symptoms, Obj: objective symptoms

ImmunoCAP

IgE to hazelnut extract, rCor a 1.04, nCor a 9, rCor a 14, peanut extract, rAra h 1, rAra h 2, rAra h 3, rAra h 8, and birch pollen and rBet v 1 was determined by ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden).

Allergens

Hazelnut extract was prepared from non-roasted, defatted hazelnut flour (2 gram) by stirring in 20 mL of phosphate buffered saline (PBS) with 0.01% phenol for one hour at room temperature and overnight dialysis against PBS at 4°C on a shaker. Peanut extract

Table 2 IgE levels for hazelnut, peanut and birch pollen extract and their purified allergens

Studynr	IgE (kU_A/L)										
	Hazelnut extract	Cor a 1	Cor a 9	Cor a 14	Peanut extract	Ara h 1	Ara h 2	Ara h 3	Ara h 8	Birch pollen extract	Bet v 1
HN002C*	1.8	1.1	0.37	0.15	25.7	19.9	8.1	5.2	0.73	2.9	2.9
HN004C	39.0	39.3	<0.10	<0.10	1.6	<0.10	<0.10	<0.10	21.3	62.1	53.1
HN008C	93.5	4.8	8.5	72.9	3.1	<0.10	0.54	<0.10	0.17	7.2	6.0
HN011C	>100	>100	78.9	67.0	>100	>100	162	118	86.4	>100	>100
HN017C	>100	>100	0.47	7.03	15.8	0.64	7.6	0.38	59.2	>100	>100
HN018C	31.9	<0.10	2.4	28.2	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
HN021A	56.1	64.1	<0.10	<0.10	0.92	<0.10	0.12	<0.10	39.6	>100	>100
HN023C	45.6	48.2	<0.10	1.0	4.0	<0.10	<0.10	<0.10	12.6	68.3	56.7
HN026C	47.6	54.0	<0.10	<0.10	24.1	<0.10	20.5	<0.10	15.5	59.7	57.7
HN035C	>100	>100	6.2	91.0	64.8	8.5	43.1	3.9	>100	>100	>100
HN037C	>100	0.11	35.5	62.1	4.1	0.16	1.2	0.67	<0.10	0.16	0.11
HN038C	15.3	0.13	4.0	11.5	0.15	<0.10	<0.10	<0.10	<0.10	0.23	0.15
HN070C	44.5	<0.10	8.8	31.9	8.0	<0.10	4.1	0.11	0.15	0.21	<0.10
HN082C	6.1	<0.10	6.2	<0.10	>100	90.0	>100	66.8	0.10	0.16	0.71
HN094C	>100	33.0	15.4	181	>100	>100	161	22.8	7.3	51.0	59.2
HN100C	>100	37.5	62.0	112	22.8	0.34	15.5	5.8	12.3	55.3	53.2
HN135C	>100	6.9	228	212	21.6	0.31	0.16	17.6	1.9	9.6	5.4
HN045A	3.5	3.8	<0.10	<0.10	0.24	<0.10	<0.10	<0.10	0.69	5.7	5.2
HN046A	33.5	12.5	<0.10	28.7	0.35	<0.10	<0.10	<0.10	2.5	17.5	22.9
HN056A	>100	93.0	12.9	59.1	8.4	0.51	0.82	0.21	49.7	>100	>100
HN116A	42.7	46.9	<0.10	<0.10	9.8	7.6	3.1	0.30	12.5	81.4	75.5
HN117A	2.1	1.6	0.29	<0.10	59.0	39.4	21.2	7.2	0.79	5.3	5.5
HN144A	45.0	51.3	<0.10	0.14	1.0	<0.10	0.10	<0.10	9.0	>100	>100
HN145A	>100	>100	0.23	4.2	17.3	<0.10	10.6	0.19	63.4	>100	>100
HN157A	30.7	11.4	3.5	23.9	0.15	<0.10	0.10	<0.10	4.2	19.3	18.4

* C: child, A: adult

was prepared from lightly roasted, defatted peanut flour (10 gram) by stirring in 50 mL of 20 mM Tris/150mM NaCl buffer (pH 7.2) for three hours at room temperature. The hazelnut and peanut extract were centrifuged at 3000 rpm for 30 min and filtered over a 0.20 µm filter. Protein content was determined using a BCA protein assay kit (Thermo Fisher Scientific, Rockford, USA). Recombinant and natural hazelnut allergens were produced as previously described[16]. Purified nAra h 1, nAra h 2, nAra h 3 and nAra h 6[11] were kindly provided by TNO, Zeist, The Netherlands. Birch pollen extract was obtained from ALK Abello A/S, Hørsholm, Denmark, and rBet v 1 from Biomay, Vienna, Austria. The hazelnut extract was fractionated by size exclusion chromatography on a HR 10/30 column packed with superdex 75 (GE Healthcare, Uppsala, Sweden) and fractions of 0.6 ml were collected.

LPS removal

Lipopolysaccharide (LPS) content of allergen preparations was analyzed using the Limulus Amebocyte Lysate (LAL) assay (Lonza, Walkersville, USA). Hazelnut and peanut extract and purified peanut allergens was below 0.005 EU/mg protein. Purified hazelnut allergens (rCor a 1, nCor a 9 and rCor a 14) contained higher levels of LPS which were reduced to below 0.0025 EU/mg protein using Detoxi-Gel Endotoxin Removing Columns (Thermofisher Scientific, Rockford, USA).

Basophil activation

The basophil activation test was performed with passively sensitized basophils as described previously[10]. In short, healthy donor PBMC were isolated by Ficoll density centrifugation and cell-bound IgE was removed by lactic acid stripping. Basophils were then loaded with patient IgE by incubation with patient plasma for 60 min at 37°C. Cells were diluted to a final concentration of 1 x 10^6 per 75ul in RPMI medium and stimulated with equal volumes of RPMI/IL-3 (2 ng/ml, R&D Systems Minneapolis, MN, USA)/0.5% HSA (Sanquin, Amsterdam, The Netherlands), with 10-fold serial dilutions of allergen (from 0.1 ng/ml to 1 µg/ml) or with a goat anti-human IgE antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) as a positive control. The reaction was stopped by addition of 25 uL of cold PBS/20 mM EDTA. Cells were stained for expression of CD63, CD123, CD203c (Biolegend San Diego, CA, USA). Activation was analyzed by flow cytometry on a FACSCanto II (BD Biosciences, San José, CA, USA) using FACSDiva software. Activated basophils were identified as CD63+ within the $CD203c^+CD123^+$ cells.

Definition of CD-sens

The biological activity of different allergens was studied by basophil activation. The basophil threshold sensitivity was determined as the lowest allergen concentration (µg/mL) resulting in a half-maximum CD63 up-regulation of the dose-response curve.

CD-sens[21] was defined as the inverted value of the concentration required for half maximum activation. CD-sens of basophil activation below 5% was set to 1.

Generation of allergen specific T cell lines

Short-term conglutin-specific T cell lines (TCLs) were generated as described before[12]. TCLs were stimulated with hazelnut or peanut extract, rCor a 1, nCor a 9, rCor a 14, nAra h 3 and Ara h 6 at 50 µg/mL (titration experiments showed that proliferation was optimal at a concentration of 50 µg/mL, data not shown), in the presence of irradiated autologous PBMC. After 48 hr, supernatants were harvested and stored at -20°C for subsequent cytokine measurements. TCL proliferation was measured by tritiated thymidine incorporation ([^{3}H]-TdR, 1 µCi/well; Amersham, Aylesbury, UK) using a Microbeta2 counter (Perkin Elmer, Waltham MA, USA). The stimulation index (SI, allergen-specific divided by background proliferation) was considered positive when the SI ≥2.0. Proliferation with SI<1 was set to 0.1.

Determination of cytokines

Cytokines in the culture supernatants of short-term TCLs (IL-10, IL-13 and IFN-γ) were measured by ELISA, according to the manufacturer's recommendations (Sanquin, Amsterdam, the Netherlands). The detection limit was 1.2 pg/mL for IL-10, 0.5 pg/mL for IL-13, 2.0 pg/mL for IFN-γ.

Data analysis

To compare the IgE levels, CD-sens, T cell responses (SI) and cytokine levels between the different components or clinical groups the Wilcoxon matched-pairs signed rank and ANOVA was used. P<0.05 was considered significant. All analyses were performed with GraphPad Prism version 5.04 for Windows (GraphPad Software, La Jolla, CA, USA).

RESULTS

IgE binding and basophil activation

The basophil activation test was performed with basophils passively sensitized with plasma from 11 patients (median age 8 years, range 4-23 years) with a hazelnut allergy with objective symptoms in DBPCFC. Patients were selected on high levels of sensitization to the different hazelnut allergens. Five patients also had a peanut allergy with objective symptoms (Table 1).

In the 11 patients, IgE to hazelnut extract was significantly higher than to different purified hazelnut and peanut allergens, peanut and birch pollen extract and rBet v 1. IgE

to rCor a 14 was significantly higher than IgE to nCor a 9 ($p<0.01$), while IgE to rCor a 1 was not significantly different from IgE to nCor a 9 or rCor a 14 (Figure 1A).

Next to the potency of the different allergens to bind IgE, we also examined the potency to activate effector cells by IgE crosslinking on basophils after stimulation with hazelnut, peanut and birch pollen allergens. Figure 1B shows a representative example of the % CD63 positivity for the hazelnut allergens in one child (HN037C). The allergen concentration at half maximum activation was calculated in all 11 patients for the different allergens, as illustrated in Figure 1B. The inverted value at half maximum degranulation, the CD-sens, is illustrated in Figure 1C. Basophil activation showed that rCor a 14 was the most potent hazelnut allergen, followed by rCor a 1 ($p=0.08$) and nCor a 9 ($p=0.02$) (Figure 1C). In two patients with higher IgE levels to Cor a 9 than to Cor a 14 (HN011C and HN135C), Cor a 14 was still more potent in basophil activation (data not shown). In line with Cor a 14 in hazelnut, Ara h 2 and 6 in peanut were the most potent peanut allergens. Ara h 2 ($p=0.08$) and Ara h 6 ($p=0.03$) were more potent than peanut extract. Cor a 9 tended to be more potent than Ara h 3 and Ara h 1 in basophil activation, although statistically not significantly different.

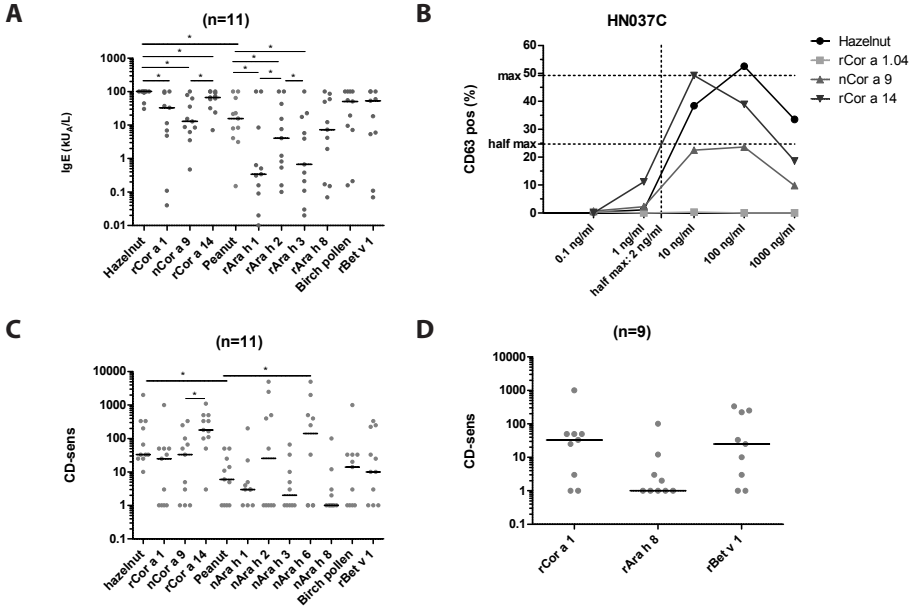

Figure 1. (A) Levels of IgE to hazelnut, peanut and birch pollen and their purified allergens in eleven hazelnut allergic patients. (B) One representative example of CD63 positivity for different concentrations of the hazelnut allergens, to illustrate CD sens. (C) CD-sens to hazelnut, peanut and birch pollen and their purified allergens in eleven hazelnut allergic patients. (D) CD-sens for PR-10 proteins, in nine individuals sensitized to rCor a 1, rAra h 8 and rBet v 1. Horizontal bars indicate median levels.

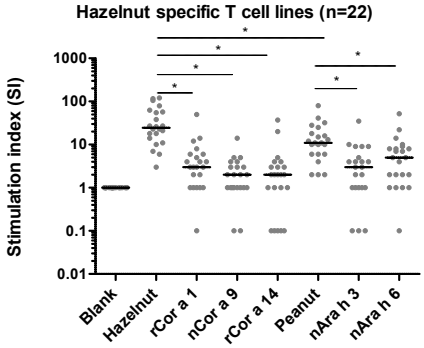

Figure 2. (A) Proliferation of hazelnut specific TCLs upon stimulation with hazelnut or peanut extract and their purified allergens. Horizontal bars indicate median levels.

The response to Ara h 8 appeared lower than to Cor a 1 and Bet v 1, though the difference did not reach significance (Figure 1D). The response to hazelnut and peanut extract and their purified allergens in individuals with an objective allergy to both hazelnut and peanut was comparable (data not shown). The potency of allergens in basophil activation correlated positively with the IgE levels (data not shown).

Allergen specific T cell lines

Hazelnut and peanut specific TCLs were generated from PBMCs from 32 hazelnut sensitized patients. TCLs from patients with a proliferation to hazelnut extract >1000 counts per minute (CPM) and an SI ≥2.0 were selected for this study. This resulted in 22 hazelnut specific TCLs (median donor age 12.5 years, range 7-37) and 13 peanut specific TCLs (median donor age 9 years, range 7-37). The TCLs were specific, as evidenced by their unresponsiveness to casein, an unrelated allergen from cow's milk (data not shown). All selected hazelnut specific TCLs (n=22) also showed a significant response to peanut extract SI ≥2.0 (Figure 2). However, the response to hazelnut extract was significantly higher than to peanut extract (p=0.02). The majority of the hazelnut specific TCLs had a response with SI ≥2.0 to rCor a 1 16/22 (73%), to nCor a 9 12/22 (55%), to rCor a 14 13/22 (59%), to nAra h 3 14/22 (64%) and to nAra h 6 17/22 (77%). The magnitude of the response to the purified hazelnut and peanut allergens was significantly lower than to hazelnut and peanut extract (Figure 2A).

Almost all selected peanut specific TCLs 12/13 (92%) also showed a significant response to hazelnut extract. The magnitude of their response was significantly higher to peanut extract than to hazelnut extract (p<0.01). A response to rCor a 1 was shown in 6/13 (46%), to nCor a 9 by 6/13 (46%), to rCor a 14 by 6/13 (46%), to nAra h 3 by 13/13 (100%) and to nAra h 6 by 12/13 (92%) TCLs. Again, the response to the purified hazelnut

and peanut allergens was significantly lower than to the hazelnut and peanut extract (Online repository E1).

The response to Ara h 2 was not tested in our TCLs. Because previous experiments showed limited responses to Ara h 2, but good responses to Ara h 6, only Ara h 6 was used in the current study[12]. Ara h 1 was not studied, because its homologue Cor a 11 from hazelnut was not available as a highly purified protein.

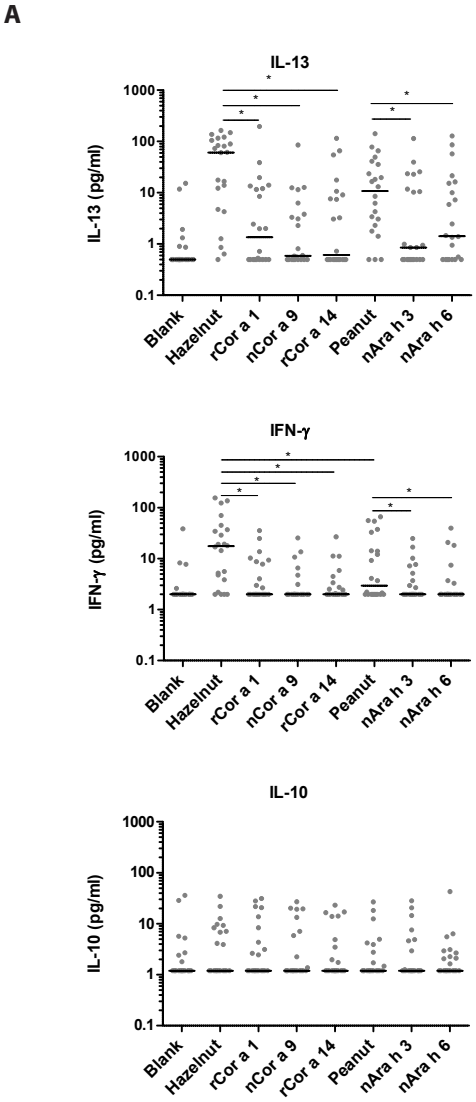

Figure 2. (B) Cytokine response by hazelnut specific TCLs (n=22) upon stimulation with hazelnut extract, rCor a 1, nCor a 9 and rCor a 14, peanut extract, nAra h 3 and nAra h 6. Horizontal bars indicate median levels.

Upon stimulation with hazelnut extract, the hazelnut specific TCLs produced mainly IL-13, but also IFN-γ and limited amounts of IL-10 (Figure 2B). The stimulation with hazelnut extract resulted in a significantly higher production of IL-13 and IFN-γ than stimulation with the purified hazelnut allergens, which correlated with the extent of proliferation. The same was true for peanut and its purified allergens (data not shown).

The T cell response to the different allergens was compared between children and adults (Figure 3A). The magnitude of the response to rCor a 1 was significantly higher in

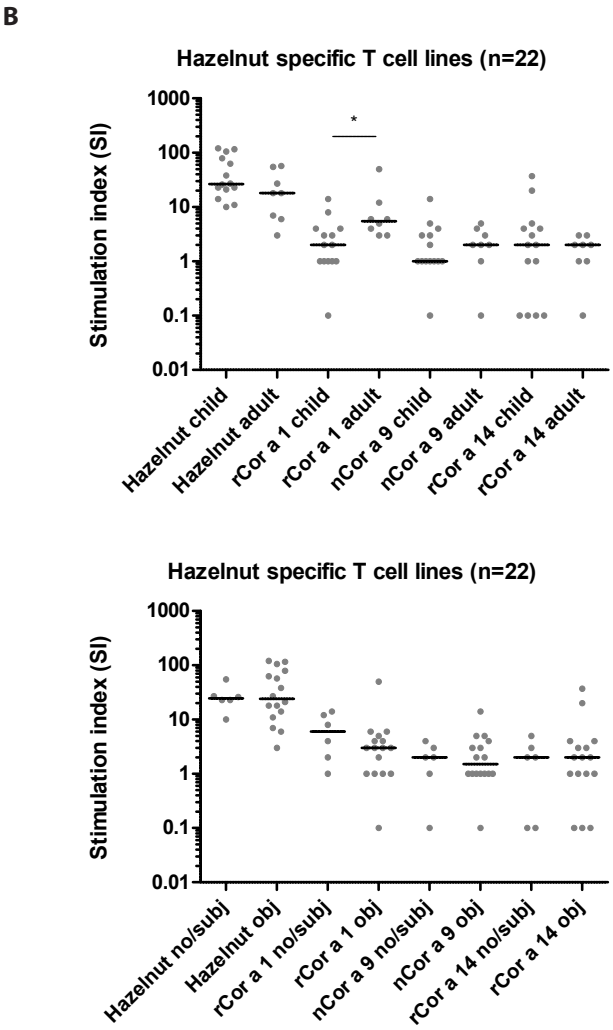

Figure 3. Proliferation of hazelnut specific TCLs upon stimulation with hazelnut and their purified allergens in (A) children versus adults and in (B) patients without or with a hazelnut allergy with subjective symptoms versus objective symptoms. Horizontal bars indicate median levels.

hazelnut specific TCLs from adults than from children (p=0.01). The responses to other allergens and the responses in the peanut specific TCLs were not significantly different between children and adults.

The response to the allergens in the hazelnut and peanut specific TCLs was not significantly different between hazelnut-sensitized donors with objective symptoms to hazelnut, or donors that had subjective symptoms or tolerated hazelnut (Figure 3B).

Dissection of the response to hazelnut extract

Hazelnut extract stimulated proliferation to a much larger extent than the major allergens. Therefore, the response to hazelnut extract was studied in more detail. To analyze whether the strong response to hazelnut extract was not due to an additive effect of the response to the major allergens, since the combination of Cor a 1, Cor a 9 and Cor a 14 did not result in enhanced proliferation compared to the separate stimulations (data not shown). To study whether the unexpectedly low response to Cor a 14 was due to the fact that this was a recombinant protein, the response to natural Cor a 14 was evaluated as well in eight hazelnut specific TCLs. However, no difference in proliferation between recombinant and natural Cor a 14 was observed (Figure 4A).

To characterize the strong T cell stimulating activity evidently present in hazelnut extract but not represented by the available components, the extract was subjected to size exclusion chromatography and selected fractions (Fr) were analyzed by SDS-PAGE

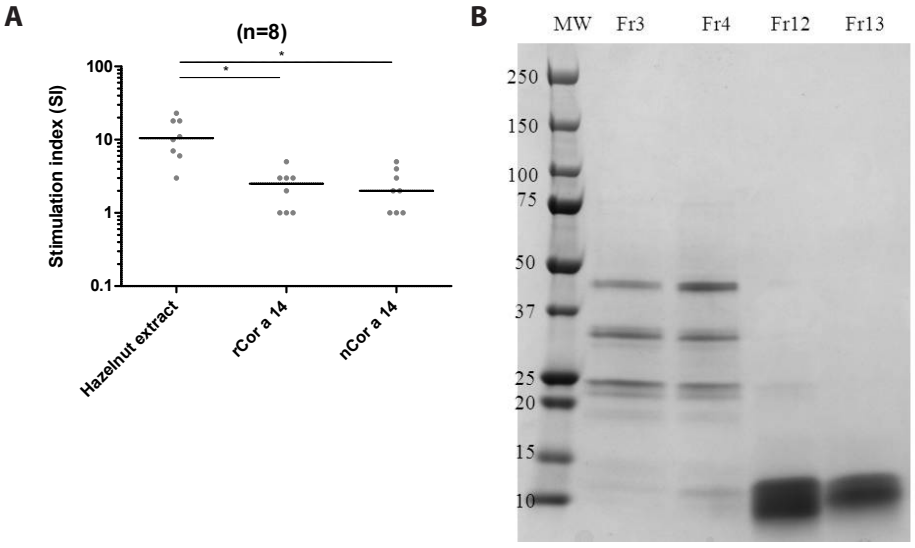

Figure 4. The proliferation of hazelnut specific TCLs upon stimulation with (A) recombinant versus natural Cor a 14. (B) Coomassie blue staining of the different fractions of hazelnut after size exclusion chromatography. (C) The proliferation of hazelnut specific TCLs upon stimulation with different fractions of hazelnut extract (representative example from 3 experiments). Horizontal bars indicate median levels.

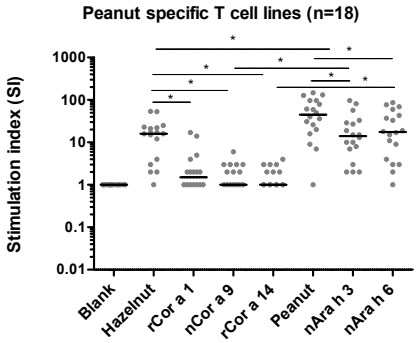

Figure 5. Proliferation of peanut specific TCLs upon stimulation with hazelnut or peanut extract and their purified allergens. Horizontal bars indicate median levels.

(Figure 4B) and tested for TCL stimulation activity (Figure 4C). In Fr3 and Fr4, several proteins of different molecular weights were present; a 48 kD band, possibly representing Cor a 11, several bands between 19-35 kD, likely belonging to the basic and acidic subunits of Cor a 9, and bands between 12-14 kD, possibly Cor a 14 (Figure 4B). Fr12 and Fr13 showed bands around 10 kD, possibly belonging to Cor a 14. Three fractions, Fr3, Fr 12 and Fr13 were tested in three different TCLs. A representative example is shown in Figure 4B. Fr3 induced a strong T cell stimulation, comparable to the proliferation with hazelnut extract (Figure 4C). Also a peanut specific TCL showed a significant response after stimulation with Fr3 (data not shown). In contrast, Fr12 and Fr13 induced hardly any proliferation in the hazelnut and peanut specific TCLs (Figure 4C and data not shown).

DISCUSSION

This study showed that the 2S albumin Cor a 14 was more potent in IgE-mediated basophil activation than the PR-10 protein Cor a 1 and the 11S globulin Cor a 9. This pattern was equivalent to that of peanut allergens, for which the 2S albumins in peanut Ara h 2 and Ara h 6 were the most potent. Peanut extract induced proliferation of hazelnut specific TCLs, and hazelnut extract induced proliferation of peanut specific TCLs. Furthermore, the three tested hazelnut allergens Cor a 1, Cor a 9 and Cor a 14, as well as the tested peanut allergens Ara h 3 and Ara h 6, induced proliferation of both hazelnut and peanut specific TCLs, suggesting cross-reactivity.

The finding that 2S albumins are the most potent elicitors of basophil activation among hazelnut and peanut allergens is in line with previous studies showing that Ara h 2 and Ara h 6 were more potent than Ara h 1 and 3[8;9]. The difference in potency between allergens could in part be explained by differences in IgE levels, as shown between Cor a 14 and Cor a 9 (Figure 1A). However, the observation that Cor a 14 was also more potent

in patients with lower IgE to Cor a 14 than to Cor a 9, suggests that Cor a 14 is intrinsically more potent in basophil activation. IgE levels to 2S albumins have been shown to be strong predictors of hazelnut or peanut allergy[18;22;23]. The potency of 2S albumins in basophil activation further confirms the relevance of IgE to Cor a 14 or Ara h 2 in the diagnostic work-up of hazelnut and peanut allergy.

Cor a 11 was already shown to be a weak inducer of basophil degranulation[20]. Cor a 9 seemed more potent than Ara h 3. Cor a 9 has been shown to be a good marker of severity in contrast to Ara h 3[22;23]. Cor a 1 and Bet v 1 seemed more potent than Ara h 8, although this did not reach significance in this study. A limited potency of Ara h 8 is in line with a study by Asarnoj et al who showed that children with an isolated Ara h 8 sensitization tolerated (roasted) peanuts, in which Ara h 8 is likely denatured[24].

T cell responses are more difficult to study than IgE responses. Since only a small fraction of the CD4+ T cell pool is allergen specific, stimulation of PBMCs with purified peanut allergens was unsuccessful as no significant proliferation was shown[12]. Therefore, T cell responses were studied in allergen specific TCLs.

Majority of both hazelnut and peanut specific TCLs showed a significant proliferation to both hazelnut and peanut extract, as well as their major allergens. This suggests that hazelnut and peanut allergens induced an allergen specific cross-reactive T cell stimulation. Cross-reactivity at T cell level between hazelnut and peanut has been described previously in five patients[5], partially driven by Ara h 1 or Ara h 2. Cross-reactivity at T cell level in mice has been shown between cashew, walnut and peanut extracts although no causal allergen was identified in that study[25], but at IgE level, the 7S globulins, including Ara h 1 seemed cross-reactive[25]. In contrast to the observed cross-reactivity at T cell level between hazelnut and peanut, a previous study by our group could not show any IgE cross-reactivity between Cor a 14 and Ara h 2 or 6 and only limited cross-reactivity between Cor a 9 and Ara h 3 (manuscript submitted). T cell epitopes are linear structures, whereas IgE epitopes can be both linear as well as conformational[26]. The absence of cross-reactivity at IgE level raises the possibility that similar T cells may induce discrete sets of B cells, producing antibodies specific for either hazelnut or peanut allergens. The observation that these IgE antibodies were not able to cross-react might be due to differences in tertiary structures of the related hazelnut and peanut allergens. Cross-reactivity at T cell level might be an explanation for multiple peanut and tree nut sensitizations that can be observed early in life, even without previous known ingestion of the culprit nuts.

Seed storage proteins and proteins extracts induced comparable T cell responses in children and adults. However, the PR-10 protein in hazelnut Cor a 1 induced a stronger proliferation in hazelnut specific TCLs from adults than from children. Bet v 1 is the likely inducer of the Cor a 1 specific T cells, as has been shown in several studies[27-29]. Bet v 1-specific T cells have been shown to cross-react with Cor a 1 from hazelnut[29]. More

frequent exposure to birch pollen in adults than in children might have further favoured expansion of Cor a 1-cross-reactive T cells[28].

Stimulation of hazelnut specific TCLs with a fraction containing several high and low molecular weight proteins resulted in strong proliferation. The component causing the strong T cell proliferation was not identified. Cor a 1, Cor a 9 and Cor a 14 could be excluded based on the TCL profileration assays in a pure form. A major band with a molecular weight consistent with Cor a 11 was present in that fraction. Cor a 11 is a glycosylated protein belonging to the 7S vicilin protein family, to which also Ara h 1 from peanut belongs[30]. Glycan structures of Ara h 1 have been shown to stimulate the innate immune system by DC-SIGN signalling, which results in a Th2-skewing environment and enhanced proliferation[31]. The glycan structure of Cor a 11 might have a similar ability to induce Th2-skewing and enhance proliferation. Another possibility is that the T cell stimulation was caused by lectins, which stimulate T cells in a T cell receptor independent way [32]. Since, cow's milk specific T cell lines did not respond to hazelnut extract (data not shown), cross-reactive T cell responses between hazelnut and peanut caused by non-allergen specific lectin stimulation seems unlikely. Further studies are needed to identify the strong T cell stimulating component in hazelnut extract.

In conclusion, our data indicate that Cor a 14 is the most potent hazelnut allergen in basophil activation in vitro, comparable in importance to Ara h 2 and 6 in peanut. At T cell level, a high molecular weight fraction, possibly containing Cor a 11 was the strongest inducer of T cell proliferation. In contrast to IgE cross-reactivity, the data show that T cell cross-reactivity exists between hazelnut and peanut. This could in part explain the frequently observed concomitant occurrence of hazelnut and peanut allergy.

ACKNOWLEDGEMENTS

We would like to thank Aard de Jong and Ron van den Dool, TNO, Zeist, The Netherlands for the fractionation of hazelnut extract.

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Chapter 8

A systematic review of the effect of thermal processing on the allergenicity of tree nuts

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Allergy 2013;Aug;68(8):983-93

ABSTRACT

Background: Allergenicity of foods can be influenced by processing. Tree nuts are an important source of nutrition and increasingly consumed, however, processing methods are quite variable and data is currently lacking on the effects of processing on allergenicity.

Objective: To perform a systematic literature review on the effects of food processing on the allergenicity of tree nuts.

Methods: A systematic literature search of PubMed and Embase databases was performed, with screening of references, related articles and citations. Studies were included if they assessed the allergenicity or immunogenicity of processed nuts.

Results: The search resulted in 32 articles suitable for analysis. Clinical studies indicate that roasting reduces the allergenicity of hazelnut in individuals with a birch pollen allergy and reactivity to raw hazelnut. Thermal processing may reduce the allergenicity of the PR-10 protein in hazelnut and almond in vitro. The majority of the in vitro studies investigating the allergenicity of nonspecific lipid transfer proteins and seed storage proteins in hazelnut, almond , cashew nut, Brazil nut, walnut, pecan nut and pistachio nut, show heat stability towards different thermal processing methods.

Conclusion: Thermal processing may reduce allergenicity of PR-10 proteins in hazelnut and almond, in contrast to nonspecific lipid transfer proteins and seed storage proteins. This has important implications for source materials used for IgE testing and food challenges and diet advise.

INTRODUCTION

Tree nuts (e.g. hazelnut, walnut) are an important source of nutrients. A diet rich of tree nuts has been shown to improve cardiovascular risk markers[1]. On the other hand tree nuts are a major cause of food allergy[2]. Tree nut allergy can result from cross-reactivity after primary sensitization to birch pollen (BP). The major allergen in these foods appeared structurally related to the major allergen in BP, Bet v 1, and belongs to the pathogenesis-related protein 10 (PR-10) family. Of BP allergic individuals, 73% reported a BP-related food allergy[3] involving Rosacea fruits and nuts and in addition vegetables, legumes and seeds (almond)[4]. Hazelnut allergy is one of the most frequently reported BP-related food allergies and leads often to mild oral allergy symptoms[3;5]. Allergy for almond and walnut often accompanies a hazelnut allergy in our allergy clinic, suggesting a relation with BP. The BP-related allergens Cor a 1 (hazelnut)[6] and Pru du 1 (almond) are PR-10 proteins and Cor a 2 and Pru du 4[7] are profilins (panallergens which are present in most pollens and fruits). Non-BP related allergens such as nonspecific lipid transfer proteins (nsLTPs) and seed storage proteins might be associated with a severe allergy[8-10] For hazelnut Cor a 8, 9, 11, 12, 13 and 14[11-14] have been described. For almond: Pru du 2S, 3, 5 and 6[15;16] were identified. Table 1 shows the major allergens from tree nuts, including their structural relationship.

Table 1. Major allergens and structural relation for different tree nuts, according to allergome.org. Estimated molecular weight (kD) of each allergen is indicated in brackets

	PR-10 Protein MW (kD)	Profilin MW (kD)	Ribosomal Protein MW (kD)	Nonspecifc Lipid transfer protein LTP MW (kD)	11S Globulin MW (kD)	7S Globulin MW (kD)	Oleosin MW (kD)	2S Albumin MW (kD)
Hazelnut	Cor a 1 (17)	Cor a 2 (14)		Cor a 8 (9)	Cor a 9 (30-40)	Cor a 11 (48)	Cor a 12, 13 (14-17)	Cor a 14 (12-14 17)
Almond	Pru du 1	Pru du 4 (14)	Pru du 5 (10)	Pru du 3 (9)	Pru du 6 (41)			Pru du 2S (12)
Cashew					Ana o 2 (52)	Ana o 1 (50)		Ana o 3 (14)
Pecan					Car i 4 (55)			Car i 1 (16)
Pistachio					Pis v 2 (53) Pis v 5 (36)	Pis v 3 (55)		Pis v 1 (17)
Walnut		Jug r 5		Jug r 3 (9)	Jug r 4 (58)	Jug r 2 (44)		Jug r 1 (14)
Brazil nut					Ber e 2 (52)			Ber e 1 (9)

Tree nut consumption shows geographical differences in Europe, with highest consumption in Mediterranean countries. Walnut is the most popular nut, followed by almond and hazelnuts, respectively[17]. The ingestion of tree nuts increased over the last decades and raw nuts are increasingly available and consumed[17;18]. This might contribute to the severity of allergic reactions in tree nut allergic patients, since raw nuts might be more allergenic than processed nuts. Processing, such as heating might lead to denaturation of food allergens and disruption of conformational IgE epitopes[19], while linear T cell epitopes may preserve. For peanut a decreased allergenicity was observed after boiling, while roasting increased the allergenicity[20].

The influence of processing on the allergenicity of tree nuts is largely unknown. Different heating methods are used when processing various tree nuts, of which an overview is shown in Table 2. Information about the effect of processing on the allergenicity of tree nuts is vital in the diagnosis and treatment advice provided to tree nut allergic patients. Therefore we performed a systematic literature search to evaluate the current knowledge on the influence of processing on the allergenicity and immunoreactivity of tree nuts.

METHODS

Search strategy

A systematic search of the the PubMed and Embase databases was performed by two reviewers using the terms 'processing' and 'nuts' and synonyms, with screening of references, related articles and citations (Web of Science and SCOPUS) (Figure 1.) From the major list of tree nuts[18] according to the FDA official list, seven (hazelnut, almond, cashew nut, Brazil nut, pecan nut, walnut and pistachio nut) are described in this study, because the others have hardly been studied or have no established allergenicity.

Study selection

Studies were included if they assessed the allergenicity or immunogenicity of processed nuts. Included studies were published in peer-reviewed journals and written in English. Reviews and case reports were excluded along with studies of which full text articles were not available (Figure 1.). Screening for eligibility was performed independently by two reviewers.

Data collection

Data on patient characteristics (age, hazelnut and BP sensitization, skin prick test, food challenge), source and type of antibodies, tree nut variety, temperature of process, duration and way of processing, and in vitro techniques to assess allergenicity or im-

Table 2. Usual processing methods in the food industry

Tree nut	Usual processing methods	Temperature
Hazelnut	Raw	
	Blanched^	100 °C[1]
	Dry roasted	Quickly roasted to remove skin at 100 °C for 4-5 min[3] Further roasted until 160 °C[1]
	Fried~	Fried at 150-160 °C for 1-4 min[2]
Almond	Raw	
	Pasteurised (not entirely raw)	Superficial: till 70 °C for 30 min, quick: high temperatures for short duration (e.g. 135 °C for 2 sec).[5]
	Blanched^	100 °C[1]
	Dry roasted	Roasted until 160 °C[1] or 120°C for 20-25 min[3]
	Fried~	Soaked in water, blanched and dried in heated cabinet. at 70 °C gradually increasing to 115 °C for 25 min[4] or 150-160 °C for 1-3 min[2]
Cashew	Raw	First heated till 150 °C for 20-35 min to remove the shell.[1]
	Dry roasted (US)	Roasted until 160 °C[1] or 120°C for 20 min[3]
	Fried~ (The Netherlands)	Slowly fried: 93 °C gradually increasing to 135 °C in 35-40 min[4] or 150-160 °C for 1-3 min[2]
Brazil	Raw	First heated till 150 °C for 20-35 min to remove the shell[1]
Walnut	Raw	No prior heating to break open the shell[2]
Pecan	Raw	
	Dry roasted	Roasted until 160 °C[1] or quickly, because of delicacy of the nut: 120°C for 10 min[3]
	Fried~	Fried at 80 °C gradually increasing to 115 °C in 15-18 min[4] or 150-160 °C for 1-3 min[2]
Pistachio	Raw	
	Dry roasted	Roasted until 160 °C[1] with shell: 140-150°C for 20-30 min[3] peeled: 120°C for 15 min[3]
	Fried~	Fried at 150-160 °C for 1-3 min[2]

^ Blanched by means of steam or quickly roasted in an oven to remove the skin.[1]

~ Duration of frying is dependent on the size of the oven used[2]

[1] *Delinuts, Ede, The Netherlands, H. Budding, personal communication, April 18th and May 11th, 2011.*

[2] *De NotenBeurs bv, Zevenhuizen, The Netherlands, personal communication October 25th 2011.*

[3] *Hazel Noten & Zuidvruchten, Amsterdam, The Netherlands, personal communication October 25th 2011.*

[4] *Blumenthal S. Food manufacturing; a compendium of food information, with practical factory-tested commercial formulae for the food manufacturer, chemist, technologist, in the canning, flavouring, beverage, confectionery, essence, condiment, dairy products, meat and fish, and allied industries. Chemical Publishing Company, Inc. Brooklyn, New York, U.S.A.; 1942, p. 279-281.*

[5] *http://www.naturalnews.com/021776.html*

munogenicity were collected independently by two reviewers. Data were discussed and interpreted by both reviewers. Disagreements were discussed to reach consensus, if needed a third reviewer was consulted. Clinical studies with food challenges were given highest strength of evidence score, followed by in vitro studies measuring IgE reactivity (allergenicity), and the lowest score was given to studies measuring in vitro IgG reactivity (immunogenicity).

Database	Search[a]
Pubmed	#1: roast OR roasting OR heat OR heating OR cook OR cooking OR boil OR boiling OR frying OR fry OR bake OR baked OR microwave heating OR microwave OR roasted OR heated OR cooked OR boiled OR baking OR autoclaving OR autoclave OR blanch OR blanching OR blanched OR dry OR drying OR thermal processing
	#2: nut OR nuts OR tree nut OR tree nuts OR hazelnut OR hazelnuts OR filbert OR filbert OR Corylus OR walnut OR walnuts OR Juglan OR Juglans OR pecan OR pecans OR hickory OR hickory nuts OR Carya OR Carya illinoensis OR almond OR almonds OR Prunus dulcis OR Prunus amygdalus OR cashew OR cashews OR Anacardium OR Anacardium occidentale OR pistachio OR pistachios OR Pistacia OR Pistacia vera OR macadamia OR macadamias OR Brazil nut OR Bertholletia excelsa
	#3: #1 AND #2 Field: Title/Abstract
Embase	As in Pubmed Field: ti,ab; AND [embase]/lim NOT [medline]/lim
[a] Unfound terms are not shown	

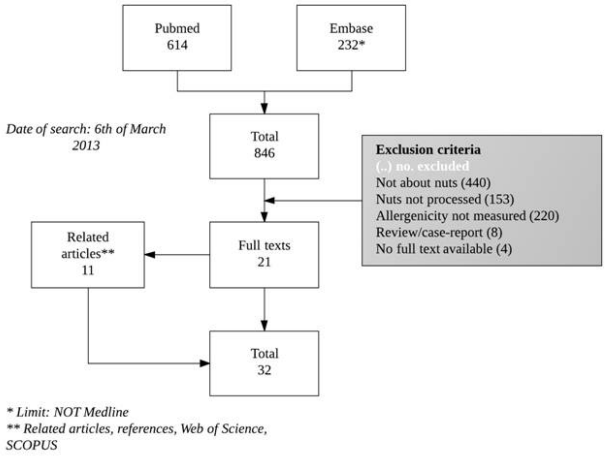

Figure 1. Overview of systemic search method.

RESULTS

The systematic search of the literature resulted in 846 articles. Of these 846 articles, 825 articles did not meet our inclusion criteria and were excluded (Figure 1). After thoroughly screening related articles of the 21 articles that initially met our inclusion criteria, an additional 11 articles about allergenicity or immunogenicity of processed nuts were found and included in our review. This resulted in 32 articles for our final analysis (Figure 1.) A summary of the results from clinical studies and measured IgE reactivity is shown in Table 3. A detailed summary of the effects of processing on the allergenicity of each tree nut is provided in the following sections.

Decreased allergenicity of hazelnut by roasting in individuals with a hazelnut and BP allergy

Two clinical studies investigated the influence of roasting on the allergenicity of hazelnut by double-blind placebo-controlled food challenges (DBPCFCs). Both studies reported a decreased allergenicity.

Hansen et al. performed a DBPCFC with roasted hazelnut (140 °C, 40 min) in 17 patients with a BP allergy and a DBPCFC-confirmed food allergy to raw hazelnut. Serum IgE from 94% (16/17) of patients in the study recognized rCor a 1, 41% (7/17) rCor a 2 and none recognized rCor a 8 on immunoblot. All 17 patients experienced oral symptoms and three of them reported additional symptoms such as asthma, rhinitis and gastrointestinal discomfort after consumption of raw hazelnut. Five patients (29%) experienced oral symptoms with roasted hazelnut consumption; one of them also experienced rhinoconjunctivitis. Eliciting doses were elevated after roasting (median eliciting doses were at least doubled). More than 50% of patients lost reactivity to prick-to-prick and sIgE with roasted hazelnut compared to raw hazelnut. In addition, histamine release test (HRT) reactivity was significantly reduced as well as EAST inhibition. Together these results indicate decreased allergenicity of hazelnut after roasting, however clinical symptoms were not completely alleviated in all patients[21]. Worm et al. performed a DBPCFC with roasted hazelnut (144 °C, duration unknown) in 20 patients (with BP allergy) who were previously challenged with raw hazelnut. Seventeen patients (85%) developed oral symptoms during the challenge with roasted hazelnut. Eliciting doses were elevated compared to the eliciting dose of raw hazelnut in the majority of patients (median eliciting doses were doubled). SPT and basophil reactivity was decreased to roasted hazelnut[22]. A thorough component resolved evaluation of the patients against other hazelnut allergens was not conducted. It is not described whether patients that experienced clinical symptoms upon consumption of roasted hazelnut may have had some reactivity to hazelnut allergens that remain stable during heat processing.

Table 3. (A+B) Summary of the results after often used processing methods. (A) Decreased allergenicity after processing, (B) conflicting data about effect of processing or limited effect shown after processing. The strength of evidence is based on the source data: from clinical studies (+++), measured IgE reactivity (++) in more than 2 patients, IgE reactivity measured in only 1 or 2 patients (+), IgG data are not depicted in this table.

A. Decreased allergenicity after processing

Tree nut	Allergens involved	Processing method	Conditions	Magnitude of effect after processing compared to raw or native tree nut*	Strenght of evidence
Hazelnut	Cor a 1	Roasting	140°C – 40 min (21) 144°C -duration unknown (22)	15-71% of patients reacted to roasted hazelnuts in DBPCFC, median eliciting dose were doubled (21,22)	+++
			140°C – 40 min (21,23-25) 144°C -duration unknown (22) 170-185°C – 15 min (26)	<50% of patients reacted to sIgE, SPT or Prick-to-prick with roasted hazelnut (21,22) 50% reduction histamine release after roasting (21) IC50 increased 10-100 times (21,24,26) AC50 increased 50 times (22) IC 50 not reached (23) B-hexosaminidase release 50% reduction after roasting in RBL-cell assay (25)	++
	Cor a 1 Cor a 2	Processing into commercial hazelnut products	Unknown, commercially processed (27)	IC50 increased 5-20 times (27)	++
Almond	Pru du 1	Blanching Roasting	Unknown (35,36) Unknown (35,36)	Complete loss of recognition on Western blot of Pru du 1 after blacnhing and roasting (31,32)	++

Table 3. (Continued)

B. Conflicting data about effect of processing				
Tree nut	**Allergens involved**	**Processing method**	**Conditions**	**Strenght of evidence**
Hazelnut	Cor a 1 Cor a 9 Cor a 11	Maillard	145°C – 20 min (31) 70°C – 48 hours (32-43)	++
Limited effect shown after processing				
Allergen	**Allergens involved**	**Processing method**	**Conditions**	**Strenght of evidence**
Hazelnut	Cor a 8 Cor a 9 Cor a 11 Cor a 14	Roasting	140°C – 20-40 min (23-25) 170-185°C – 10-15 min (26,28,29)	++
	Cor a 9	Processing into commercial hazelnut products	Unknown, commercial hazelnut paste (29)	++
	Not shown Cor a 9	Storage	19 weeks (26) Six months ((29))	++
Almond	Pru du 6	Blanching Roasting	Unknown (35,36,37) 180°C – 15 min (28) Unknown (35,36,37)	++
	Pru du 1 Pru du 6	Processing into almond butter	Unknown (35)	++
Cashew nut	Not shown	Roasting	180°C – 15 min (28)	+
Brazil nut	Not shown	Roasting	180°C – 15 min (28)	+
Pecan nut	Car i 1 Car i 4	Blanching Roasting	100°C – 10 min 148°C – 30 min 172°C – 12 min (49)	++
Pistachio	Unknown	Roasting	37-150°C – 8 hours (52)	++

DBPCFC: Double-blind placebo-controlled food challenge, sIgE: Specific IgE, SPT: skin prick test, IC50: concentration of inhibitor to reach 50% inhibition of native protein extract by EAST inhibition, AC50: concentration of inhibitor to reach 50% inhibition of native protein extract by basophil activation test, RBL-cell assay: rat basophilic leukemia cell assay

PR-10 proteins and profilins in hazelnut

Two in vitro studies[23;24] found decreased allergenicity after roasting (140 °C, 40 min) by EAST inhibition using sera from patients with a hazelnut and BP allergy. Furthermore, recognition of Cor a 1 was completely lost after roasting[23;25] at 140 °C for 20-40 min. Wigotzki et al[26] demonstrated that Cor a 1 was heat resistant to treatment at 100 °C for up to 90 min, using immunoblots and an EAST inhibition assay with sera from 19 patients, whereas at 185 °C Cor a 1 (18 kD) and Cor a 2 (14 kD) detection was lost. Decreased allergenicity of hazelnut by EAST inhibition assay was reported after processing into hazelnut chocolates, nougat products, hazelnut cake, hazelnut cookies and hazelnut croquants. Furthermore detection of Cor a 1 and Cor a 2 on immunoblot was reduced[27]. One study reported decreased rat basophilic leukemia cell (RBL) activity after roasting[25].

Together, data from clinical and in vitro studies indicate that roasting reduces the allergenicity of PR-10 proteins and profilins in hazelnut.

NsLTPs and seed storage proteinsin hazelnut

No DBPCFC studies in patients with NBPR hazelnut allergy recognizing nsLTPs or seed storage proteins in hazelnut were found. Seven in vitro studies were published. Patients with a hazelnut allergy without birch pollinosis showed similar reactivity to raw and roasted hazelnut (140 °C, 40 min) by EAST inhibition[23;24], while BP extract showed no significant inhibition, indicating involvement of heat resistant nsLTPs or seed storage proteins. In a hazelnut allergy with recognition of PR-10 proteins combined with nsLTPs or seed storage proteins, roasting resulted in a decreased allergenicity but this decrease was less pronounced then in a hazelnut allergy with PR-10 protein recognition only[24]. The decreased IgE binding by IgE blotting and EAST inhibition may be caused by a decreased solubility.

IgE binding to roasted and unroasted hazelnut was comparable in two different studies: two patients with severe hazelnut allergy (180 °C, 15 min, by ELISA)[28] and five patients sensitized to Cor a 9 (±170 °C, 10 min, using ELISA and immunoblot). Intact Cor a 9 was detected in roasted hazelnut paste[29]. Another study showed that Cor a 9 and Cor a 11 and an allergen <14 kD (Cor a 14) were stable after roasting at 185 °C for 15 min[26]. Müller et al. showed heat stability (140°C, 20-40 min) of a 12-14 kD allergen (Cor a 14) and Cor a 11[25]. Heat stability of Cor a 8 (140°C, 40 min) was shown by EAST inhibition[23]. One study investigated the effect of autoclaving and showed decreased IgE binding on Western blot and protein bands on SDS-PAGE after autoclaving (138°C, 15-30 min), most likely due to decreased solubility[30].

Together, these in vitro studies indicate that roasting does not affect the allergenicity of nsLTPs or seed storage proteins in hazelnut.

Maillard reaction of hazelnut

The effect of Maillard reaction or caramelization, a chemical reaction between an amino acid and a reducing sugar, usually caused by heat, is not unequivocal.

A decreased immunoreactivity of Cor a 11 was shown after glycation (heated at 145 °C in the presence of glucose) by SDS PAGE, immuno-dot blot and IgG on ELISA. However, RBL activity was increased. Such a discrepancy might be caused by precipitation of glycosylated Cor a 11 in the RBL assay[31]. This was confirmed by Cucu et al. who showed a decrease in intensity of the 49 kD band (Cor a 11) on a SDS-PAGE gel after glycation of hazelnut at 70 °C which caused precipitation. In addition, Cor a 9 was unaffected and appeared stable, while a Cor a 1 showed only some decrease[32;33]. Cucu et al recently showed in six patients with systemic reactions to hazelnut that glycation of hazelnut enhanced (2/6) or decreased (3/6) the allergenic property of hazelnut in the basophil activation test[34].

Effect of storage on the allergenicity of hazelnut

Storage of hazelnuts for 1-19 weeks at room temperature had no effect on the protein pattern of hazelnut as investigated by SDS-PAGE and immunoblot. The EAST inhibition assay showed very little difference in the C50 values over the 19-week storage period[26]. Dooper et al. found a decrease in detection of a Cor a 9 using ELISA and immunoblot after storage of more than six months, likely due to loss of solubility of the protein than a true decrease in allergenicity[29].

Almond

Clinical studies of patients with almond allergy have not been published. In vitro studies reported that heat reduces the allergenicity of a 15-17 kD protein, which may be the Bet v 1 homologue, Pru du 1 after blanching and roasting[35;36]. Immunoblot recognition was similar for almond butter and raw almond, suggesting that processing into almond butter (no extreme heat required) did not influence allergenicity[35].

De Leon et al. found no difference in IgE binding to roasted and unroasted almond (180 °C, 15 min), by ELISA in one patient with an almond allergy[28].

The effect of different heating methods on the allergenicity of 11S globulin, Pru du 6 (also known as amandin, or almond major protein, 37-66 kD protein bands on Western blot)[16] was investigated in six in vitro studies. Most bands were very stable towards blanching and roasting[28;35-37], except for two bands between 55-65 kD[35-37]. This thermo stability of amandin was also illustrated in three studies with polyclonal IgG antibodies[36-38]. No major changes in secondary structure were found with circular dichroism spectroscopy after heating amandin from 13 to 77 °C[39]. However, fluorescence spectroscopy revealed significant changes in secondary structure of amandin, after heating to 100 °C for 10 min, whereas immunoreactivity was not effected on dot blot[40]. Acosta et al

showed conflicting data with a decrease (up to 87%) in immunoreactivity after blanching, moist heat >100°C, roasting and processing into almond paste with competitive ELISA, not confirmed by SDS-PAGE and western blot[41]. In agreement with the 15-17 kd almond allergen, Bargman et al. showed similar IgE-binding patterns of almond butter compared to raw almond on electro- and immunoblot with sera obtained from eight almond allergic patients[35].

Summarizing, most in vitro studies indicate that Pru du 1 but not Pru du 6 is affected by blanching and roasting.

Cashew nut

Only one study investigated the effect of roasting of cashew nut using patient sera (one allergic and one only sensitized). No significant effect on IgE binding was found after roasting at 180 °C for 15 min[28]. Several in vitro studies investigated binding of monoclonal and polyclonal antibodies (IgG) to cashew major protein (CMP or Ana o 2), Ana o 1 and Ana o 3 after different heating methods. Roasting resulted in a slight decreased immunoreactivity of Ana o 1 and Ana o 3. Immunoreactivity of Ana o 2 was not affected by heating, although more extreme roasting conditions (160 °C for 30 min or 200 °C for 15 min) resulted in a decrease[38;42]. The effect of blanching was limited and primary due to leakage of proteins in the blanching water. Cashew frying (at 191 °C for 1 min) showed no significant effect on Ana o 2[38].

Microwave heating and autoclaving resulted in conflicting data[38;42].

The available data showed a limited effect of roasting on the allergenicity of cashew nut withAna o 2 seemingly more heat stable than Ana o 1 and Ana o 3.

Brazil nut

De Leon et al found no significant effect of roasting at 180 °C for 15 min on the IgE binding of Brazil nut in two patients (one allergic, one only sensitized)[28]. Brazil nut consists of two major allergens, Ber a 1 (30%) and Ber e 2 (60%). IC50 determinations suggested that Ber e 1 is less immunogenic than Ber e 2[43]. It was shown that an irreversible denaturation of Ber e 1 starts at temperatures above 110 °C[44-48]. Denaturing conditions for Ber e 2 have not been published yet.

One in vitro study confirmed the limited effect of different heating methods on the immunoreactivity. The measured effect was not consistent with the different methods (ELISA, dot blot and Western blot). No effect or only a slight decrease (3-36%) was reported after blanching for 3 and 10 min, roasting, autoclaving for 30 min and frying. In contrast to this, the ELISA showed an increased immunoreactivity of 32% after microwave heating at 500 Watt for 3 min[43].

Overall, in vitro data (human IgE and rabbit IgG) showed only limited effect of different heating methods on the allergenicity and immunoreactivity of Brazil nut.

Pecan nut

A limited effect of heating on pecan nut was detected by Western blot using pooled patient sera. Most protein bands of Car i 1 and Car i 4 seemed very stable or showed some decrease after blanching for 10 min, roasting at 148 °C, 30 min or 172 °C, 12 min and autoclaving for 5 min. Some subunits of Car i 4 almost disappeared after blanching, roasting and autoclaving, likely due to irreversible loss of protein solubility rather than protein epitope destruction. Polyclonal antibodies showed also stability towards blanching and roasting, with a significant decrease after roasting at 160°C, 20 and 30 min and autoclaving[49] or microwave heating for 15 min[50]. These processing conditions resulted in a dark unappealing external appearance, so it is unlikely that these extreme conditions are used in commercial pecan processing and thus would not be representative of the type of pecan that allergic consumers may be exposed to[49]. The decrease in immunogenicity due to extreme conditions could be due to the loss of protein solubility.

Walnut

The effect of heating on the allergenicity of walnut was studied by circular dichroism spectra and polyclonal IgG antibodies. Sordet et al. showed that the protein structure of nJug r 1 exhibited good resistance to heating at 90 °C[51]. One in vitro study showed that blanching for 5 to 10 min did not show a significant effect on immunoreactivity of walnut glutenin (WG), the major storage glutenin fraction in walnut (Jug r 4 and Jug r 2). Roasting at different conditions, frying (191 °C) and microwave heating also showed no significant effect on immunoreactivity of walnut. Autoclaving did not effect immunoreactivity tested in ELISA, however, Western blot showed a decreased recognition of 42-45 kD proteins (Jug r 2) and 45-66 kD bands (Jug r 4), not shown after blanching and roasting[38]. Concluding, the two studies found showed a limited effect of heating on the immunoreactivity of walnut allergens, however human studies should be performed to show the clinical relevance of these findings.

Pistachio nut

One study showed the effect of processing in pistachio nut allergy. A limited effect of roasting (dry) was shown on IgE-binding in two human serum pools with SDS-PAGE, Western blot and ELISA inhibitions, however steam-roasting strongly reduced the IgE-binding in these assays. Steam-roast processing resulted in protein aggregation which contributed to the decrease in IgE binding but it is unknown if this form of processing decreases the allergenicity of pistachio nut[52].

DISCUSSION

The aim of this study was to review the influence of different heating methods on the allergenicity and immunoreactivity of tree nuts to improve the diagnosis and treatment (diet advises) of tree nut allergic patients. A key factor in evaluating the effect of processing on the allergenity of tree nuts is the consideration of the solubility of the tree nut allergens after processing. In vitro analysis using sera IgE in conjunction with immunoblotting or inhibition ELISAs should be followed up with clinical oral challenge trials to confirm a decrease or removal of the allergenicity before it is determined that processing results in hypo-allergenic tree nuts. The two available clinical studies have shown a decreased allergenicity of hazelnut after roasting in patients with a BP allergy and reactivity to raw hazelnut[21;22]. This was confirmed by in vitro studies, illustrating a decrease in Cor a 1 reactivity[23-27]. A similar phenomenon was reported for almond Pru du 1[35;36]. In contrast, nsLTPs and seed storage proteins in hazelnut and almond appeared very stable[23-26;28;29;35-40]. Studies examining the effect of thermal processing on the allergenicity of cashew nut, Brazil nut, pecan nut, walnut and pistachio were scarce and limited in their scope. They all show stability of these foods to different heating methods[28;38;40;42-49;51].

PR-10 proteins are the most important BP related allergens and share homology in their tertiary structures (conformational epitopes). PR-10 proteins are generally heat labile, as described for hazelnut[21], celery[53;54], apple[55], carrot[56] and peanut[57]. Heating might lead to unfolding and disruption of conformational epitopes[19]. Bohle et al showed unfolding of PR-10 proteins in BP (Bet v 1), celery (Api g 1), carrot (Dau c 1) and apple (Mal d 1) upon cooking between 50 °C and 80 °C. The structure of Mal d 1 and Dau c 1 remained unfolded upon cooling, whereas the unfolding of Bet v 1 and Api g 1 seemed partly reversible[56]. Such a mechanism might explain the retained reactivity to roasted hazelnut in 29-85% of the hazelnut allergic patients[21;22], although for Cor a 1 (hazelnut) folding experiments have not been published. Recognition of nsLTPs or seed storage proteins in addition to Cor a 1 and Cor a 2 might also lead to a remained reactivity in some patients. Another explanation might be that the hazelnut core was not heated sufficiently as reported for baking of crumps, in which the temperature did not exceed 100 °C during baking at 180 °C to 230 °C, due to the water content inside[48]. However, the presence of water is required for denaturation. Dry heat treatment like roasting makes proteins more thermostable than moist heat treatments like blanching, cooking or steam-roasting[52;58]. Understanding how heat influences allergenicity and whether this is reversible, could lead to strategies to reduce allergenicity in food production.

A 15-17 kD protein in almond (Pru du 1) was also found to be heat labile. The clinical relevance of this finding has not been confirmed yet. Further insight might broaden the product choice of almond allergic patients or reveal processing methods that might

eliminate or reduce the allergenicity, like the eliminated allergenicity of Mal d 1 in apple after microwave heating.

The limited effect of heating on the allergenicity of hazelnut, almond (nsLTPs or seed storage proteins), cashew nut, Brazil nut, pecan nut, walnut and pistachio nut might be due to heat stabile allergens like the seed storage proteins. Heat stability has been illustrated for the 2S albumins: Ber e 1 in Brazil nut until 110 °C[44-48] and Jug r 1 in walnut until 90 °C[51] and the 7S and 11S globulins in soy: 7S globulins until 70-75°C and 11S globulins until 94°C[59]. Heat stability might also be expected for cross-reactive sensitizations to 11S globulins for example[60]. However, some subunits of 11S globulins seem heat labile after denaturing on SDS-PAGE, which has been shown for almond, Brazil nut, pecan nut and walnut. This is likely due to irreversible loss of protein solubility, which does not necessarily indicate a decrease in allergenicity. If allergic consumers eat a tree nut, they would be exposed to both the soluble and insoluble forms of the proteins. Gastric digestion may aid in the resolubilization of some of the allergens, however, little information is currently known about the effect of ingestion and digestion on the resolubilization of allergens in the human body.

The impact of factors like matrix[61] and stability to digestion[62] might further influence the allergenicity of tree nuts and are not discussed in this review. Allergens that are stable to digestion reach the intestinal mucosa intact (nsLTPs in cherry), where absorption and sensitization can occur in contrast to the labile allergens (PR-10 proteins and profilins in cherry)[63;64]. The warranted clinical studies with processed nuts could also provide insight to the contribution of these factors to the allergenicity in tree nut allergy.

In contrast to decreased allergenicity, an increased immunogenicity has been described after microwave heating of nuts[42;43]. For peanut, an enhanced allergenic property has been described for Ara h 1 and Ara h 2 after roasting or browning (Maillard reaction)[20;65]. The effect of Maillard reaction on the allergenicity of hazelnut is not clear yet, since the data found were not consistent. This might be due to precipitation of the proteins. Further investigation to determine the effect of the Maillard reaction is requisite.

Storage of hazelnut up to 19 weeks had little effect on allergenicity[26], storage for more than six months resulted in decreased detection of Cor a 9, likely due to a decreased solubility[29]. Data on the storage of other tree nuts are lacking.

The decreased allergenicity after processing of tree nuts has important implications for clinical practice. Clinicians should perform a thorough history including reactivity to raw (directly from tree) or processed (blanched or roasted) tree nuts. Reacting to raw or unprocessed tree nuts, without symptoms to heated tree nuts, might results in a modified dietary advice. Reactivity to processed and unprocessed hazelnuts in combination with more severe symptoms makes reactivity to nsLTPs or seed storage proteins more likely, a risk for more severe or anaphylactic reactions (9). These findings further influ-

ence the advices concerning consumption of nut products. Raw tree nuts are a risk for all tree nut allergic patients, although processed nuts might be tolerated by patients recognizing only the PR-10 proteins. Unfortunately, it is not possible to discriminate between both groups with current diagnostic in vivo or in vitro tests. The hazelnut oral challenge with roasted hazelnuts should be the most reliable test however, the processing conditions of nuts in daily life are not standardized and therefore the clinical reaction will be difficult to predict. The component-resolved-diagnosis will give more insight in the specific sensitization pattern of the patient and might lead to an individual advice concerning ingestion of processed nuts. If it is possible in the future to totally eliminate the allergenicity of Cor a 1 in hazelnut or Pru du 1 in almond after heating or processing, patients recognizing solely this PR-10 protein, could take advantage of this.

Furthermore, the heat lability, storage effect and influence of processing of tree nut allergens warrants us to use fresh, raw nuts for diagnostic food challenges for patients with aPR-10 protein-related tree nut allergy. False negative outcomes may increase the risk for unexpected allergic reactions during introduction of the nut into the diet.

In conclusion, this study shows that heating and processing may reduce allergenicity of PR-10 proteins in hazelnut and almond. In the work-up of tree nut allergy, reactivity to raw versus processed nuts should be discussed for diagnosis and dietary advice. In PR-10 protein-related tree nut allergies, raw nuts should be used as source materials for IgE tests and hazelnut challenges. In the future, information on the influence of processing on the allergenicity might lead to the development of hypo-allergenic tree nuts or tree nut products.

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Chapter 9

General discussion

1. DIAGNOSING HAZELNUT ALLERGY IN CHILDREN AND ADULTS

Allergic reactions to hazelnut are common and range in severity from local and mild to systemic and severe[1;2]. In diagnostic workup, accurate identification of patients at risk of severe reactions is necessary to advise appropriate but not excessive dietary restrictions and other precautions. Several aspect of diagnostic workup will be discussed.

I. HISTORY

a. Previous ingestion

b. Allergic symptoms

c. Birch pollen allergy

d. Asthma

e. Atopic dermatitis

II. SENSITIZATION

a. Specific IgE

b. SPT

c. Allergen specific IgE and SPT in relation to clinical severity

d. Component-resolved-diagnosis

III. DOUBLE-BLIND PLACEBO-CONTROLLED FOOD CHALLENGE

IV. CLINICAL IMPLICATIONS

I. HISTORY

a. Previous ingestion

A large discrepancy between self-diagnosed and true allergy[3] makes a thorough evaluation of a suspected food allergy important. Evaluation starts with a detailed clinical history. The clinical history is focused on the time relation between occurrence of allergic symptoms and the intake of the suspected food. IgE-mediated allergic symptoms develop quickly after ingestion of the culprit food, starting within minutes or even seconds, up to two hours (occasionally several hours longer). In adults previous ingestion of hazelnut with allergic symptoms is highly predictive for a hazelnut allergy (28/30, PPV 93%). In children with a suspected hazelnut allergy, previous ingestion is often unknown, because many children have never ingested hazelnut. We showed that even if hazelnut was identified as the culprit nut (15%) only 61% of these children appeared to be hazelnut allergic during DBPCFC. This indicates that the diagnostic value of previous ingestion in children is limited (Chapter 2). Most adults have ingested nuts previously. But still, only in a minority of cases (35%) can the accidental ingestion be specifically attributed to hazelnut. Many adults have difficulty identifying the exact culprit nut. Hazelnut may be a hidden ingredient or ingested in combination with other nuts. Furthermore, hazelnut seems more difficult to identify than other tree nuts or peanut in both children and adults[4;5]. Inability to recognize the intake of hazelnut, may place hazelnut allergic individuals at a higher risk for unexpected allergic reactions than individuals allergic to other tree nuts or to peanut. On the other hand, most tree nut allergic individuals eliminate all tree nuts from their diet and recognition of different nuts is less important to prevent allergic reactions. Our data indicate that the predictive value of previous ingestion is often limited in children, but important in adults for the diagnosis.

b. Allergic symptoms

Food allergic symptoms may vary from itch in the oral cavity, lips and throat, cutaneous symptoms, and rhino-conjunctivitis to gastro-intestinal, respiratory or cardiovascular symptoms. Most frequently reported symptoms of tree nut allergy in children are urticaria, angioedema, and dyspnoea[6]. Severe pharyngeal edema was more associated with tree nut than peanut allergy[7]. The occurrence of respiratory symptoms after accidental ingestion of hazelnut was associated with the severity of symptoms after DBPCFC in adults (chapter 3). However, oral allergy symptoms are reported by most hazelnut allergic adults, both with a mild and with a severe hazelnut allergy, and are therefore not discriminative for the severity of the allergic response. In adults oral symptoms may function as an early warning signal to prevent further ingestion of the culprit food, which may prevent progression to more severe allergic symptoms. Oral allergy symptoms were rarely reported by children. It is unknown whether children develop oral allergy symp-

toms less often or have difficulties in recognizing them as allergic symptoms. This is suggested by the observation that subjective symptoms during a DBPCFC for hazelnut are reported at a significantly higher threshold in children than in adults (chapter 5). This has also been shown for peanut (unpublished data).The absence or delayed perception of oral symptoms as an early warning signal[8] might predispose children at risk for more severe allergic reactions to hazelnut. In conclusion, the most frequently reported allergic symptoms to nuts differ between children and adults. Children frequently develop urticaria, angioedema and dyspnea, while most adults report oral allergy symptoms.

c. Birch pollen allergy

Hazelnut allergy, like many other food allergies, occurs often in the presence of other atopic diseases, like hay fever. Birch pollen allergy may result in pollen-induced cross-reactive IgE antibodies to hazelnut[9], which is generally associated with a hazelnut allergy with mild and local symptoms[10]. However, birch pollinosis is not exclusively present in individuals with a mild hazelnut allergy. Many children and adults with a severe hazelnut allergy also report birch pollinosis (chapter 4). Furthermore, birch pollinosis is more common in adults (86%), than in children (39%) with a hazelnut allergy (chapter 3). This could be related to the severity of the hazelnut allergy. Children more often have a severe hazelnut allergy (63%-67%)[11] than do adults (7-33%)[2;12] (chapter 2, 3). The age of children and adults with severe hazelnut allergy is significantly lower compared to children and adults with a mild hazelnut allergy (chapter3). A likely explanation for the association between age and severity in hazelnut allergy is the increasing prevalence with age of individuals with a mild (birch pollen related) hazelnut allergy. A mild hazelnut allergy develops frequently after childhood, as a consequence of birch pollen related cross-reactivity, which is much more common in adults than in children. In contrast to hazelnut, a clear association between age and severity could not be shown for peanut[13;14]. In patients with birch pollen allergy peanut allergy (24%) is less frequently reported than hazelnut allergy (59%)[15]. A likely explanation is that the birch pollen related peanut allergen (Ara h 8) is less homologous to Bet v 1 than the birch pollen related hazelnut allergen (Cor a 1). Furthermore, Ara h 8 is less potent than Cora 1 in basophil activation (chapter 7). Therefore, the supposed dilution effect with increasing age in hazelnut allergy seems of limited importance in peanut allergy. In conclusion, there is no evidence that the severity of the hazelnut allergy decreases with increasing age as an explanation for the association between age and severity of hazelnut allergy. We suppose that the population of individuals with a severe hazelnut allergy becomes diluted by the increasing numbers of individuals with a birch pollen related mild hazelnut allergy. Furthermore, the presence of birch pollen allergy does not always indicate the presence of a mild phenotype of hazelnut allergy.

d. Asthma

Asthma is frequently observed in food-allergic individuals, in 49% of both children and adults with a hazelnut allergy (Chapter 2, 3) and in 58% of individuals with a suspicion of a tree nut or peanut allergy[16]. This is also true for other food allergies: asthma was observed in more than two thirds of adults with cow's milk allergy[17]. Asthma was more frequently present in adults with a severe than with a mild hazelnut allergy, whereas this was not seen in children (Chapter 4). Also in peanut allergic children, the severity of peanut allergy was not associated with the presence of asthma[13]. However, for several food allergies in children, including peanut, it was shown that asthma was a risk factor for severe allergic reactions[18;19]. An evaluation of asthma is important in tree nut allergic individuals, because it has been shown that tree nut allergy is an independent risk factor for asthma in children[20]. Furthermore, the presence of severe asthma was associated with life-threatening bronchospasms in tree nut and peanut anaphylaxis in a population of mainly children[7]. Therefore, adequate diagnosis and treatment of asthma is essential in tree nut allergic individuals, to reduce the risk of severe allergic reactions. Severe wheezing is a prominent feature of severe reactions. An evaluation of asthma is indicated in the standard diagnostic work-up of tree nut allergy.

e. Atopic dermatitis

Atopic dermatitis is very common (94%) in children with a hazelnut allergy, but also in adults with a hazelnut allergy (58%)(Chapter 2, 3). The prevalence of atopic dermatitis may be overrepresented, because inclusion occurred mainly via the department of Dermatology. Still, in another pediatric allergy clinic the prevalence of atopic dermatitis was 57% in individuals with a suspicion of peanut and or tree nut allergy[16]. Several other studies have also shown that tree nut, including hazelnut, and peanut allergy is most prevalent in children with (moderate to severe) atopic dermatitis[21-25].The presence of severe atopic dermatitis was associated with severe allergic reactions, affecting systemic circulation in tree nut and peanut anaphylaxis[7]. In our study no association between severity of atopic dermatitis by SCORAD and severity of hazelnut allergy by DBPCFC/history was observed (data not shown). However, severity of atopic dermatitis by SCORAD at three months of age was significantly associated with peanut sensitization[26]. This suggests in combination with the high prevalence of atopic dermatitis in children with a tree nut or peanut allergy that evaluation of a tree nut and peanut allergy is indicated in children with moderate to severe atopic dermatitis early in life.

II. SENSITIZATION

Hazelnut specific IgE and skin prick test (SPT) with hazelnut extract are the currently used tests in the diagnostic work-up of a hazelnut allergy. However, they should be interpreted in the context of a detailed history. The presence of allergen specific IgE and mast cell degranulation shows sensitization, but not clinical allergy.

a. Specific IgE

Diagnostic tests are valuable for clinical practice, if they show a useful level of discrimination between allergic and tolerant subjects. For tree nuts, allergen specific IgE≥15 kU_A/L was reported to give a 95% chance of having a suggestive history of tree nut allergy[27]. However, we could not find a relevant specific IgE cut off level for hazelnut as illustrated in our studies (chapter 2, 3), in line with another study[16]. In children the negative predictive value (NPV) of hazelnut specific IgE<0.35 kU_A/L was 100% and useful for exclusion of hazelnut allergy (chapter 2). In adults the NPV of hazelnut specific IgE was very low and not useful for exclusion of hazelnut allergy (chapter 3). The difference in NPV between children and adults may be caused by population differences. Most children with a suspicion of a hazelnut allergy seemed hazelnut tolerant, which makes exclusion of a hazelnut allergy easier than in adults, who seemed mainly hazelnut allergic. The reliability of the history results in a higher suspicion of hazelnut allergy in adults than in children. In birch-endemic areas, discrimination between sensitization to hazelnut and birch pollen based on hazelnut specific IgE is difficult, because Bet v 1 is homologous to Cor a 1[28]. The spiking of hazelnut extract with Cor a 1 in 2007, to improve the sensitivity[29], further impaired the discrimination between hazelnut and birch pollen sensitization. Therefore, sensitization to hazelnut extract is frequently false positive in birch-endemic areas, especially in the absence of a reliable history. Absence of a reliable history in children with a suspicion of a hazelnut allergy was frequently observed (74%) in our studies (chapter 2). Only 25% of the children with a suspected hazelnut allergy based on the presence of hazelnut specific IgE developed allergic symptoms during a DBPCFC for hazelnut, compared to 59% of children with a previous ingestion of hazelnut. So, 75% of the elimination diets in children with a suspicion based on sensitization only are unnecessary. Comparable results were shown by Fleisher et al, who showed that elimination diets primarily based upon the presence of allergen specific IgE were frequently unnecessary. The eliminated food could be reintroduced to the diet in 84%-93% of the cases after an oral food challenge[30]. Another study showed many (84%) unnecessary milk elimination diets in children with atopic dermatitis. Of these children, 24% had an elimination diet, while only 4% appeared milk allergic by DBPCFC[31]. Unnecessary elimination diets pose a considerable risk of developing acute allergic symptoms after reintroduction. This has been shown for children with atopic

dermatitis, who previously ingested cow's milk without problems, and who developed allergic symptoms upon exposure to cow's milk after a period of elimination[32]. In general, allergy testing is not necessary in children with atopic dermatitis not yet exposed to a potential culprit food[33] or with a negative history for acute reactions. Together, this indicates that test results to determine sensitization should be interpreted with caution, especially in the absence of a reliable history. In the absence of a reliable history or unknown previous ingestion, the need for an elimination diet should not be primarily determined by food specific IgE only, especially in children with atopic dermatitis[30]. Our data indicate that in birch-endemic areas hazelnut specific IgE should not be used to screen for hazelnut allergy without a suspect history. This prevents the prescription of many unnecessary elimination diets. However, in specific cases, like children with moderate to severe atopic dermatitis early in life, screening could be considered with highly specific tests (component-resolved-diagnosis), as described in clinical implications. Individuals with sensitization to hazelnut extract, but without previous ingestion should undergo DBPCFC for diagnosis.

b. SPT

The SPT is considered to be more reliable than specific IgE in diagnosing nut allergy[34]. This was confirmed for SPT to hazelnut extract in children (Chapter 2), whereas, SPT to hazelnut extract had limited diagnostic value in adults (Chapter 3). Children more frequently have a severe and adults a mild hazelnut allergy, which may possibly be reflected in SPT responses (Chapter 3). In both children and adults large SPT wheal sizes (≥15-17 mm) were highly specific for a hazelnut allergy, but accounted only for a minority of the patients (3-10%). Another study using a different commercial extract showed that SPT wheal sizes≥8 mm diameter predicted hazelnut allergy by oral challenge in 95% of the children. This accounted for only 8% of hazelnut allergic children[35]. Clark et al also showed that SPT responses ≥8 mm were almost always diagnostic for a tree nut or peanut allergy[34]. In children, the negative predictive value (NPV) of a wheal <3mm was 100%, making it a useful tool for exclusion of hazelnut allergy. However, this accounted for only 19-36% of the children with a suspected hazelnut allergy, as illustrated in our study (chapter 2) and in one study using a different extract[3;35]. Still, less than 30% of the children could be correctly diagnosed by SPT for hazelnut (Chapter 2). In adults the NPV of a wheal <3mm was very low, and therefore not useful for exclusion of a hazelnut allergy. In adults, prick-to-prick tests performed with fresh nuts, which are even more difficult to standardize than SPT, showed a better level of discrimination between hazelnut allergic and tolerant adults[12;36]. Prick-to-prick tests with fresh nuts have however not been studied in children. SPT and prick-to-prick tests have several disadvantages, which limit their performance and comparison between different centers. For example, the composition of extracts from different batches and companies may be quite different[37].

Therefore, extrapolation of results to other commercial extracts may not be possible. Furthermore, the SPT and prick-to-prick methods are difficult to standardize and should be performed by trained personnel. For example, in patients with atopic dermatitis flares may limit the performance of SPT and prick-to-prick tests on unaffected skin. Our data indicate that SPT to hazelnut extract performs better than specific IgE to hazelnut extract in children; this could however not be shown in adults. Hazelnut allergy may be correctly diagnosed by SPT with hazelnut extract in almost 30% of children in contrast to only 3% in adults.

c. Specific IgE and SPT in relation to clinical severity

To identify individuals at risk of more severe allergic reactions, both size of SPTs and levels of allergen specific serum IgE were not good predictors of a severe hazelnut (chapter 2, 3) or other tree nut allergy[34]. Similar results were seen for specific serum IgE to egg, milk, peanut, wheat, soy and fish[38]. In our cohort of children, SPT was predictive for a severe hazelnut allergy, but only at group level. No useful cut off values for clinical practice were identified (chapter2). In conclusion, our data indicate that routine diagnostic tools are not good predictors for the severity of the hazelnut allergy.

d. Component-resolved-diagnosis

Previous studies have shown that IgE reactivity to specific allergens or components in hazelnut extract might be a better predictor of severity than total hazelnut extract[42-45]. Sensitization to the seed storage protein Cor a 9 has been observed frequently in children and adults with a severe hazelnut allergy in the USA and Europe[39;40]. Chapter 4 shows that, in addition to sensitization to Cor a 9, sensitization to Cor a 14 is also frequently observed in Dutch children and adults with a severe hazelnut allergy. The majority of children and almost half of the adults with a severe hazelnut allergy could be identified with these tools. Furthermore, IgE levels to Cor a 9 and Cor a 14 were also correlated with a lower threshold for objective symptoms to hazelnut during DBPCFC. This was not shown for IgE levels to Cor a 1 (chapter 5). This makes IgE to Cor a 9 and Cor a 14 good markers for severe hazelnut allergy. The absence of cross-reactivity between Cor a 14 and its homologues in peanut Ara h 2 and Ara h 6 (chapter 6), makes Cor a 14 also a good marker for primary sensitization to hazelnut and could be used, in combination with Cor a 9, to identify severe hazelnut allergic patients.

In general absence of IgE to Cor a 1 was predictive for hazelnut allergy with objective symptoms, but hardly observed in Dutch children and adults from a birch-endemic area. Some children (13%) and half of adults (49%) with objective symptoms to hazelnut were not sensitized to Cor a 9 nor to Cor a 14, but only to Cor a 1[40]. So, sensitization to Cor a 1 was not only associated with a mild phenotype of hazelnut allergy, as has been suggested previously[10;28]. Sensitization to Cor a 1 was observed among all the

different clinical groups in children and adults: hazelnut tolerant as well as hazelnut allergic with subjective or objective symptoms (chapter 4). Severe symptoms caused by Cor a 1 seem unlikely, because Cor a 1 is highly susceptible to low pH during gastric digestion[41]. Cor a 1 is probably already inactivated in the stomach, before reaching the intestine for further digestion and systemic uptake. However, a role for PR-10 proteins in severe symptoms, similar to Cor a 1 has also been described for other food allergens, such as soy and apple. Severe symptoms to soy drinks or desserts upon first exposure was shown in six patients without sensitization to soy, but only sensitization to Gly m 4, the soy PR-10 protein[42]. Mal d 1, a PR-10 protein in apple was recognized in individuals with anaphylactic symptoms[43]. These data imply that PR-10 proteins may be involved in severe symptoms, and that this may occur also in a subgroup of patients with a hazelnut allergy. Factors influencing the pH in the stomach, like antacids or large amounts of neutralizing fluids, could contribute to the allergenicity of PR-10 proteins. Antacid use in children has been associated with the occurrence of food allergy[44]. Another possibility is recognition of not yet identified allergens, like the oleosins[45], also present in soy[46], in individuals with severe symptoms to hazelnut without sensitization to seed storage proteins. Future studies should focus on the identification of the component causing the severe symptoms in individuals with a severe hazelnut allergy, without IgE to Cor a 9 or Cor a 14. To study the allergenic potential of PR-10 proteins, food challenges with purified PR-10 proteins have to be performed. The effect of antacids and large amounts of neutralizing fluids may also be evaluated.

The importance of other hazelnut allergens has been studied in several studies. The role of sensitization to Cor a 11 seems to be limited, because sensitization was almost never observed in >400 individuals from the Europrevall study(unpublished data). However, sensitization to purified Cor a 11 has been described in children with a severe hazelnut allergy[47]. Sensitization to recombinant Cor a 8 was hardly observed in hazelnut allergic individuals from our study (chapter 4) and in three other studies[28;40;43] from northern Europe. This is in contrast to a previous study from our own group showing recognition of natural Cor a 8 in children with a severe hazelnut allergy[48]. Recent inhibition experiments revealed trace amounts of Cor a 14 in the natural Cor a 8 preparation. The trace amounts of the Cor a 14 contamination were still IgE reactive (unpublished data). This further suggests limited importance of Cor a 8 in northern Europe. This is in contrast to the Mediterranean area, where sensitization to rCor a 8 is frequently reported and associated with severe symptoms[28;49]. The role of oleosins, the oil-body associated hazelnut allergens, has to be further investigated[45].

Component-resolved-diagnosis (CRD) has hardly been described for other tree nuts; therefore diagnosis still relies on a combination of a detailed history, sensitization to allergen extracts and DBPCFC. A good example of successful CRD is sensitization to the seed storage protein Ara h 2 in peanut allergy, which was strongly related to clinical

symptoms, but not to their severity[50;51]. The lack of prediction of severity may be due to the fact that peanut allergy is generally severe and is rarely limited to only mild symptoms, like hazelnut allergy. Another example is isolated sensitization to Ara h 8, which was associated with tolerance to roasted peanuts[52].Furthermore, for kiwi fruit allergy sensitization to Act d 1 was a strong risk factor for a severe kiwi allergy[53].In conclusion our data have shown that IgE to Cor a 9 and Cor a 14 are useful tools to identify children and adults with a severe hazelnut allergy. Our data further suggest that isolated sensitization to Cor a 1 is possible in a severe hazelnut allergy.

III. DOUBLE-BLIND PLACEBO-CONTROLLED FOOD CHALLENGE

The DBPCFC for hazelnut is the 'gold' standard to diagnose hazelnut allergy. But still, several reasons for false positive or negative food challenge are present. A DBPCFC can be false positive if misleading subjective symptoms or allergic symptoms to the matrix instead of the allergen occur. False negative DBPCFCs can occur due to inadvertent drug use like antihistamines, to a temporary short-term specific oral tolerance induction during titrated ingestion of allergen, leading to clinical tolerance[54], missed late phase reactions because of too short observation periods[55] or due to inactivation, absence or low amount of important allergens in the recipe[56;57]. These aspects in combination with the costs, required facilities and risk for potentially severe allergic reactions, makes the DBPCFC not an ideal test. On the other hand, a DBPCFC can improve food-related quality of life and reduce anxiety in the following months even in those with a positive challenge[58;59]. Exposure to the feared stimulus in a controlled situation and learning appropriate skills to deal with the stressor can reduce the anxiety and improve the quality of life. A negative test results in a more pronounced improvement of food allergy-related quality of life than after a positive DBPCFC[60].

IV. CLINICAL IMPLICATIONS

This thesis shows that the diagnostic work-up of a hazelnut allergy shows differences between children and adults. Determination of sensitization to Cor a 9 and Cor a 14 may play a central role in the diagnostic work-up of hazelnut allergy in children and a substantial proportion of the adults. These data can be extrapolated to clinical practice as follows, which is illustrated in Figure 1.

In children, the contribution of information about previous ingestion is often limited. Therefore, additional testing remains the mainstay in diagnosing food allergy. The SPT with hazelnut extract is a good predictor of hazelnut allergy in children, with an AUC

of 0.87. However, it yields a correct diagnosis in less than 30% of the children. This will therefore hardly reduce the number of DBPCFCs. The IgE to hazelnut extract after spiking has a low positive predictive value (PPV). If IgE to hazelnut extract will be used to diagnose a hazelnut allergy in children, this will result in many (62%) unnecessary elimination diets for hazelnut. Furthermore, the clinical relevance of the sensitization to hazelnut extract cannot be determined before the children reach the age of four to five years, because before that age they are not able to undergo a DBPCFC. To reduce the number of unnecessary elimination diets and DBPCFCs, the diagnostic work-up in children should be focused on identifying severe hazelnut allergic cases. Most children with a severe hazelnut allergy (87%) can be identified by the presence of IgE to Cor a 9 and Cor a 14 (Figure 1). Translating this to the total outpatient population in our center, this implies that about 18% of children (87% of the 21% of children with severe symptoms) do not need to undergo a DBPCFC. The majority of the children (82%) should still undergo a DBPCFC for correct diagnosis. To reduce the number of children with unnecessary elimination diets and DBPCFCs considerably, identification of the children with a severe hazelnut allergy with IgE to Cor a 9 and Cor a 14 could be considered. 13% of the children with a severe hazelnut allergy could not be identified with these tools. When this percentage is extrapolated to the total group of children with a suspicion of a hazelnut allergy(13% of the 21% of children with a severe hazelnut allergy), only 3% of the total group, would be missed when using IgE to Cor a 9 and Cor a 14 as a diagnostic tool to diagnose a severe hazelnut allergy. These children cannot be identified as having a severe hazelnut allergy by serology, because they have an isolated Cor a 1 sensitization and cannot be discriminated from the children with an isolated Cor a 1 sensitization without or with only mild symptoms to hazelnut. Therefore, diagnosis and exclusion of a severe hazelnut allergy by IgE to Cor a 9 and Cor a 14 could be considered an acceptable alternative. In children without IgE to Cor a 9 and Cor a 14 hazelnut can be introduced into their diet; an exception should be made in children with a very suspect history of acute allergic symptoms to hazelnut, where a DBPCFC or elimination diet would be recommended.

In adults, the number of DBPCFCs can be strongly reduced, because a history with only oral symptoms to hazelnut in combination with birch pollinosis is common in adults (49%) and is a good predictor of hazelnut allergy. This could be used to diagnose a hazelnut allergy in adults without further testing, also in different settings. Also for other birch pollen related foods, these diagnostic features were predictive of food allergy, in both a tertiary center and in a community population (unpublished data from Europrevall, Thesis of TM Le, MD PhD). In adults with sensitization to Cor a 9 or Cor a 14 >0.35 kU_A/L an elimination diet to hazelnut is indicated, because this is already diagnostic for severe hazelnut allergy. In adults without IgE to Cor a 9 and Cor a 14, but IgE to Cor a 1 and doubt about the culprit nut or doubt about presence of allergic symptoms to

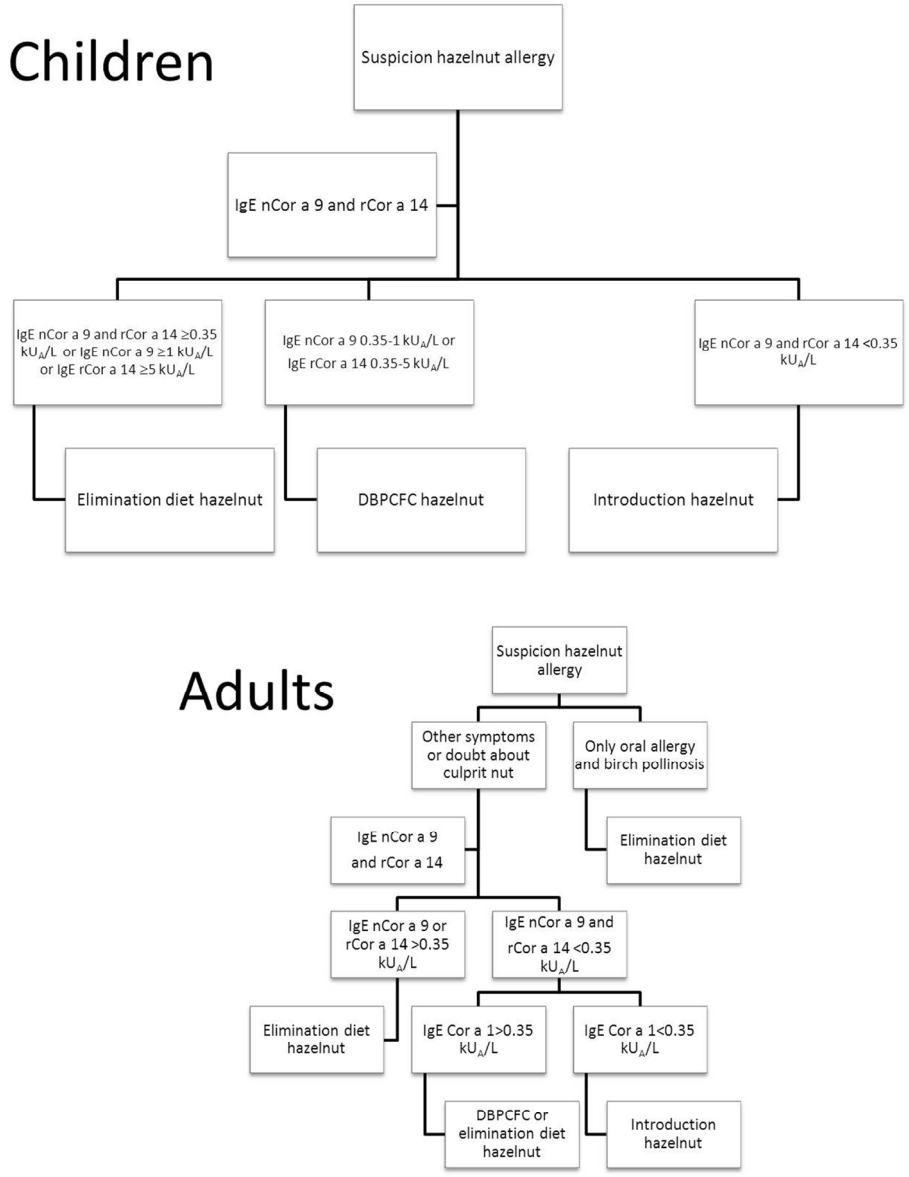

Figure 1. Diagnostic work-up of hazelnut allergy in children and adults.

hazelnut, a DBPCFC could be performed for diagnosis. At this moment the severity of allergic symptoms after previous ingestion or during DBPCFC is used to determine the required rescue medication. If a hazelnut allergy is diagnosed with IgE to Cor a 9 and Cor a 14, strict avoidance in combination with an epinephrine prescription would be appropriate advice. Increased IgE levels to Cor a 9 and Cor a 14 are associated with a low eliciting dose to hazelnut and both allergens are involved in objective allergic reactions, which may lead to potentially anaphylactic allergic reactions.

2. HAZELNUT SENSITIZATION AND ALLERGY IN RELATION TO PEANUT AND TREE NUT ALLERGY

Children and adults with a tree nut and/or peanut allergy are currently advised to avoid all tree nuts and peanut. Cross-contamination, unclear labeling and difficulties in identification make a strong case for total avoidance. What is actually known about the risk of another tree nut or peanut allergy in the presence of a hazelnut, other tree nut or peanut allergy?

I. CLINICAL SYMPTOMS AND SENSITIZATION PATTERN TO TREE NUTS AND PEANUT

II. CROSS-REACTIVITY BETWEEN MULTIPLE TREE NUTS AND PEANUT?

a. PR-10 proteins

b. Seed storage proteins

c. Cross-reactions at T cell level

III. ROUTE OF SENSITIZATION

IV. CLINICAL IMPLICATIONS

I. CLINICAL SYMPTOMS AND SENSITIZATION PATTERN TO TREE NUTS AND PEANUT

Sensitization to multiple nuts is frequently observed in nut allergic children and expands with increasing age. The majority of nut allergic children (72%-92%) is sensitized to more than one nut[16;34;61]. Clinical reactivity to several tree nuts and peanut is also often reported in nut allergic individuals. Allergic symptoms to tree nuts (37%-60%)[16;62;63], such as hazelnut (49%)[63], are frequently reported by children and adults with a peanut allergy. In children and adults with a hazelnut allergy, 48% also report a peanut allergy; the percentages are comparable between children and adults (chapter 6). In this thesis, clinical symptoms to hazelnut and peanut in children and adults were studied in detail. They did not follow a common clinical pattern. The risk of a severe peanut allergy was only higher in adults with a severe (57%), rather than a mild (24%), hazelnut allergy, but this difference was not observed in children. This finding might reflect a power problem, because only a few children had mild symptoms to hazelnut. Still, the magnitude of sensitization to other tree nuts, and sensitization to more species of nuts, have been shown to be associated with the severity of hazelnut allergy[11]. Furthermore, sensitization to multiple nuts, like hazelnut, Brazil nut and peanut can already be observed at an early age[64], which might point to cross-reactions between structurally related storage proteins.

II. CROSS-REACTIVITY BETWEEN MULTIPLE TREE NUTS AND PEANUT?

Cross-reactivity is difficult to predict, but may follow botanical families. The botanical relation between the different tree nuts and peanuts is shown in the phylogenetic tree (Figure 2). The structural relation between different identified allergens in tree nuts and peanut are shown in Table 1.

a. PR-10 proteins

Cross-reactivity with birch pollen has been described for several tree nuts and peanut. IgE antibodies primarily directed to Bet v 1(PR-10 protein) or Bet v 2 (profilin) can cross-react to structurally related allergens in tree nuts and peanut. Hazelnut, almond and peanut contain PR-10 proteins: Cor a 1 in hazelnut, Pru du 1 in almond and Ara h 8 in peanut (Table 1). Cross-reactivity between Bet v 1 and Cor a 1[10;65] and Ara h 8[66] has been demonstrated in patients with a combined birch pollen and hazelnut and peanut allergy, respectively. Cross-reactive antibodies to Bet v 2 are considered clinically irrelevant, because sensitization to profilins seems clinically inert[67]. Sensitization to the PR-10 proteins is generally considered to lead to only mild symptoms or clinical tolerance, but chapter 4 does discuss isolated Cor a 1 sensitization in individuals with a severe hazelnut allergy. The allergenicity of PR-10 proteins is generally considered to be limited, because PR-10 proteins are labile to heat and digestion. The allergenicity of PR-10 proteins can be decreased after heating, because their tertiary IgE binding epit-

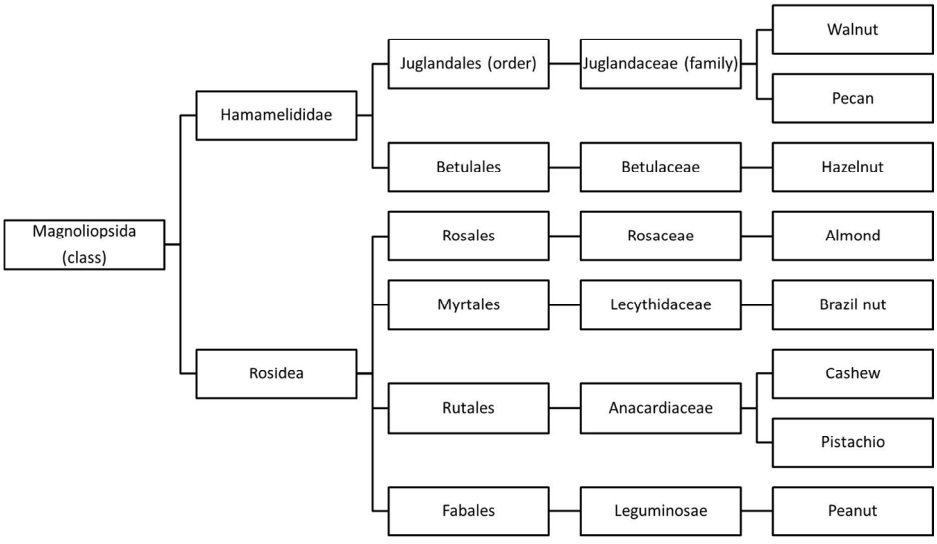

Figure 2. Taxonomy of tree nuts and peanut.

Table 1. Structural relation between identified allergens in hazelnut, birch pollen, other tree nuts and peanut, according to allergome.org and uniprot database. Percentage of identical amino acids compared to the homologue in hazelnut is shown

			Seed storage proteins		
	PR-10 Protein	**Lipid transfer protein**	**11S Globulin**	**7S Globulin**	**2S Albumin**
Hazelnut	**Cor a 1**	**Cor a 8**	**Cor a 9**	**Cor a 11**	**Cor a 14**
Birch pollen	Bet v 1 68%	ni	ni	ni	ni
Walnut	ni	Jug r 3 60%	Jug r 4 73%	Jug r 2 47%	Jug r 1 65%
Pecan	ni	ni	Car i 4 72%	Car i 2 46%	Car i 1 62%
Almond	Pru du 1 64%	Pru du 3 60%	Pru du 6 51%	ni	ni
Cashew	ni	ni	Ana o 2 55%	Ana o 1 53%	Ana o 3 43%
Pistachio	ni	ni	Pis v 2 Pis v 5 47%	Pis v 3 51%	Pis v 1 39%
Brazilnut	ni	ni	Ber e 2 50%	ni	Ber e 1 47%
Peanut	Ara h 8 53%	Ara h 9 55%	Ara h 3 47%	Ara h 1 34%	Ara h 2, 6 29%-35%

ni= not indentified

opes will unfold upon heating (Chapter 8). It has been described that the allergenicity of Cor a 1 and Ara h 8 will be decreased after roasting[10;66;68;69]. DBPCFC studies with roasted hazelnuts showed that most individuals with a hazelnut allergy to raw hazelnut in combination with birch pollen allergy did not report allergic symptoms to roasted hazelnut[56;57]. Also, all individuals with an isolated Ara h 8 sensitization tolerated roasted peanuts[52]. Reactivity to raw peanut seems irrelevant, because raw peanuts are not part of regular food. In contrast, hazelnuts are frequently consumed raw. This could explain the fact that mild peanut allergy is rarely observed(9-16%)[70]in Central Europe, despite the highly abundant sensitization to PR-10 proteins, whereas mild hazelnut allergy (67%) is frequently reported in adults. Another possible explanation for the rarely observed mild phenotype of peanut allergy is the stronger sequence homology between Bet v 1 and Cor a 1, than between Bet v 1 and Ara h 8. This suggests that cross-reactive Bet v 1 antibodies are more prone to bind to Cor a 1 than to Ara h 8. A third explanation could be the observation that Cor a 1 is more potent than Ara h 8 in basophil activation, which may reflect their different potential to induce allergic symptoms (chapter 7). The potency of Cora 1 in basophil activation may also further explain its potential to induce severe allergic reactions.

PR-10 sensitization shows geographical differences and is common in Central Europe, which is a birch abundant environment. Most hazelnut allergic individuals from Central Europe (86-100%) are sensitized to Cor a 1[12;28]. Cor a 1 sensitization is less often observed in individuals with a hazelnut allergy from the USA (50%)[39] and is rarely observed in individuals from the Mediterranean (4-14%)[49;68].

b. Seed storage proteins

Structurally related seed storage proteins are described in different tree nuts and peanut (Table 1).

In general, the strongest cross-reactions follow taxonomic families (Figure 2): walnut and pecan from the Juglandaceae and cashew and pistachio from the Anacardiaceae[71]. Cross-reactivity has been shown between the 2S albumins and 11S globulins in pecan and walnut[72;73].

Cashew showed cross-reactivity with pistachio, but also with almond and to some degree with peanut[74]. The 7S globulins were cross-reactive between cashew and pistachio. Clinical history indicated that cashew was the primary sensitizer in some cases[75]. In mice, cross-reactions between the 7S globulins from cashew, walnut and peanut were demonstrated[76].

Cross-reactivity between almond, hazelnut and walnut has been shown (2S albumin and conglutinγ)[77]. Furthermore, the 2S albumins from cashew and walnut share considerable homology[78], which may result in cross-reactivity.

The 11S globulin (Jug r 4) from walnut showed further cross-reactivity with hazelnut and cashew[79]. Shared 'hotspots' have been shown between 11S globulins from hazelnut (Cor a 9), walnut (Jug r 4), cashew (Ana o 2), peanut (Ara h 3) and soybean (Gly m 6) [80;81]. However, 11S globulins were rarely cross-reactive between tree nuts and peanut. This thesis demonstrated that IgE to Cor a 9 from hazelnut can be a cross-reactive result of primary sensitization to Ara h 3 from peanut, but this was rarely observed (chapter 6). Cross-reactivity between the 11S globulins from walnut and peanut was also not shown[82]. This may indicate that the impact of shared 'hotspots' on cross-reactivity is difficult to predict. Another possibility is that IgE cross-reactivity is stronger among tree nuts than between tree nuts and peanut. The sequence homology is also much higher for 11S globulins between hazelnut and walnut (73%) or pecan (72%), than between hazelnut and peanut (47%) (Table 1).

Peanut did show cross-reactivity with almond, Brazil nut and hazelnut[83], but not with walnut[82;84]. However, only almond and Brazil nut were able to induce degranulation of basophils sensitized with affinity purified peanut specific IgE[85]. IgE binding to Ara h 2 from peanut could be inhibited by almond and Brazil nut, but not by hazelnut[86]. Cross-reactivity between Cor a 14 from hazelnut and Ara h 2 and Ara h 6 peanut was not observed (chapter 6).

Therefore, cross-reactivity among tree nuts, like hazelnut, walnut or pecan may be more likely than that between hazelnut and peanut. Future studies could specifically focus on the possible cross-reactivity between hazelnut and walnut or pecan. Identification of the primary sensitizer or driven force will further reveal the pathogenesis of hazelnut allergy and may help to identify a potential therapeutic target.

c. Cross-reactions at T cell level

Antibodies are involved in effector responses, while T cells are important regulators of the immune response. Information about T cell reactivity and cross-reactivity among tree nuts and peanut can provide information about the development of multiple nut allergies and may result in the identification of potential therapeutic targets[76].

At T cell level, cross-reactivity has been shown between hazelnut and peanut. Several allergens seem to be involved, such as Ara h 1 and Ara h 2 from peanut[63]. Chapter 7 shows that also Ara h 3, Ara h 6 from peanut and, vice versa, Cor a 1, Cor a 9 and Cor a 14 from hazelnut seem cross-reactive. This confirms that allergen specific T cell cross-reactivity occurs between hazelnut and peanut. Several identical segments were identified after alignment of 2S albumins, 7S globulins and 11S globulins from hazelnut and peanut, which could be responsible for the cross-reactions at T cell level. In mice, T cell cross-reactivity was shown between cashew, walnut and peanut[87]. T cell cross-reactivity was also shown for birch pollen related allergens, between bet v 1 from birch pollen and several birch pollen related food allergens like hazelnut (Cor a 1), apple (Mal d 1), cherry (Pruav 1), celery (Api g 1), carrot (Dau c 1) and soybean (Gly m 4)[88;89]. Cross-reactivity between Bet v 1 and Cor a 1 has been illustrated at T cell level, but also at IgE level[88]. This is in contrast to the observed T cell cross-reactivity between the seed storage proteins from tree nuts and peanut as shown in this thesis, which is hardly accompanied by IgE cross-reactivity. IgE antibodies to seed storage proteins showed little cross-reactivity between hazelnut and peanut (chapter 6) and also not between walnut and peanut[82]. In mice IgE antibodies between cashew or walnut and peanut were also not cross-reactive, while IgE antibodies were cross-reactive between cashew and walnut in mice[87]. This suggests that IgE cross-reactivity may be present among different tree nuts, but is less important between tree nuts and peanut. Additional experiments revealed that the induction of the cross-reactivity at T cell level between tree nuts and peanut is allergen specific. Human cow's milk specific T cell lines did not respond to hazelnut extract, and the cashew specific T cells in mice responded very little to macadamia and not at all to egg. A less specific effect was shown for Ara h 1 from peanut. Glycan structures of Ara h 1stimulate DC-SIGN on dendritic cells, which results in a Th2-skewing environment[90]. This Th2-skewing effect may provide an environment prone to the development of multiple sensitizations. Cor a 11 from hazelnut, which is also glycosylated, might have a similar effect. A strong T cell stimulation was shown after

stimulation with high molecular weight molecules, possibly Cor a 11 (chapter 7). Other strong T cell stimulating components present in tree nuts and peanut are the lectins. Lectins are strong carbohydrate-binding proteins and might have an adjuvant effect during sensitization. The Th-2 skewing caused by sensitization may induce sensitization spreading, as has been illustrated in a mouse model, which showed that primary ovalbumin sensitization enhanced sensitization to a secondary unrelated allergen (latex)[91]. But also house dust mite induced inflammation facilitated neosensitization to a second unrelated inhaled allergen (ovalbumin) following a single mucosal exposure[92]. This indicates that sensitization spreading is induced by primary sensitization or ongoing inflammation. Preventative measures, like immunotherapy should already be considered early in life to prevent multiple sensitizations.

The aim of allergen specific immunotherapy is modulation of the T cell response. In mice it has already been shown that single tree nut immunotherapy can effectively decrease the responses to cross-reactive nuts as well[76]. So, more insight into the cross-reactivity between allergens could be used to develop an appropriate therapeutic target for immunotherapy. This could result in an effective treatment for individuals with tree nut and peanut allergy. Our data show cross-reactivity between hazelnut and peanut at T cell level, and suggest that single immunotherapy could be developed that also decreases the response to the cross-reactive nut.

III. ROUTE OF SENSITIZATION

To develop allergen specific antibodies (sensitization), previous exposure to a culprit allergen is needed. Two routes of sensitization are generally considered to take place in hazelnut allergy; ingestion or inhalation of pollen in mild hazelnut allergy, or uptake of primary hazelnut allergens via the gastro-intestinal tract in severe hazelnut allergy. But several studies have indicated that many children develop severe allergic symptoms to nuts upon the first known ingestion[24;61;93;94]. This suggests that other routes of sensitization to primary allergens may be involved than just the oral route. The cutaneous route may be involved, since sensitization to primary hazelnut allergens Cor a 9 and Cor a 14 is associated with early onset atopic dermatitis (data not shown). Several studies have supported the hypothesis of cutaneous sensitization. Mice studies have shown that the cutaneous route of sensitization induces Th2-responses during sensitization to peanut[95] or hazelnut[96]. For peanut it was even shown in mice that epicutaneous sensitization prevented the development of oral tolerance[97]. These mouse data indicate that the skin might be an important inductive site for allergic sensitization. Human data showed that the prevalence of peanut allergy was 10-fold higher in Jewish schoolchildren from the UK than from Israel[98;99]. The children from Israel were already orally exposed to

peanut early in life, because of the ingestion of popular peanut snacks that are given to infants even before six months of age[99]. This is in contrast to the UK, where children were advised not to ingest peanut before four years of age. This was accompanied by a strong increase in peanut allergy in the UK. Human studies have further shown that a high household peanut consumption[100] was a risk factor for the development of peanut allergy. The peanut protein levels in house dust in infants' sheets and playrooms were correlated to the household peanut consumption. The allergens in these samples were biologically active, because they were able to stimulate basophils from children with a peanut allergy[101]. It was suggested that because peanut allergens are very stable, reservoirs could be build up in mattresses after months. The high stability of biologically active peanut proteins in house dust provides a logical source of environmental peanut, necessary for sensitization. T cell experiments have suggested involvement of skin T cells in the sensitization to peanut. It was shown that skin homing T cells (cutaneous lymphocyte antigen positive) from children with a peanut allergy proliferated more strongly after stimulation with peanut extract than gut homing T cells ($\alpha 4\beta 7$ positive) and more strongly than skin homing T cells from children tolerant to peanut. Skin homing T cells from allergic children were Th2-skewed, while gut homing T cells from peanut tolerant children were Th1-skewed[102]. These data suggest that the cutaneous route of sensitization might explain the strong link between atopic dermatitis and severe tree nut and peanut allergy. Skin may provide sensitization to seed storage proteins, which are potent allergens, like sensitization to the seed storage proteins in hazelnut which was associated with early onset atopic dermatitis (unpublished data). Mouse studies have already shown that skin is an inductive site for sensitization to hazelnut. Human studies are needed to confirm whether the cutaneous route of sensitization is involved in the development of a severe hazelnut or other tree nut allergy. Future studies may provide a basis for preventative strategies, like induction of oral tolerance before cutaneous sensitization occurs. The window of opportunity to introduce potentially allergenic foods safely into the diet is unknown. The study by Levy et al suggests that introduction of peanut within the first six months of life may reduce the risk of a peanut allergy[99]. Until this is further elucidated, adequate treatment of atopic dermatitis to restore the barrier defect early in life may be the best strategy to reduce the risk of cutaneous sensitization.

IV. CLINICAL IMPLICATIONS

Sensitizations and allergies to multiple nuts are frequently observed. Individuals with a mild hazelnut allergy in the presence of birch pollen allergy, frequently report mild allergic symptoms to almond and walnut as well, while individuals with a more severe hazelnut or tree nut allergy are more prone to develop multiple sensitizations and more

severe symptoms to other nuts and peanut[11]. Elimination diets for all tree nuts and peanut are indicated in nut allergic individuals, especially in those with severe symptoms.

The additional value of diagnostic testing for different tree nuts may be limited, because many individuals will have multiple nut allergies. Furthermore, the elimination of a single nut in daily life is difficult, because allergic individuals are faced with the risk of cross-contamination, unclear labeling and difficulties in the identification of different tree nuts and peanut[4;5]. In individual cases more complete information about all nut allergies might help to reduce the psychological impact. An elimination diet for all tree nuts can further increase the already high psychological impact of eliminating one single nut[103]. And an unnecessarily restrictive elimination diet may lead to increased risk-taking behavior[104]. If reactivity to different tree nuts or peanut is tested, this could be combined with training patients to recognize the different tree nuts and peanut. For now, diagnosing reactivity to different tree nuts strongly relies on DBPCFCs, but future developments in component-resolved-diagnosis may improve the diagnostic work-up. In the case of a low suspicion of different nut allergies testing with a nut mixture during challenge could be considered, with the intention to introduce all nuts into the diet. The underlying mechanism of sensitization and allergies to multiple nuts seems to be induced already at T cell level and not always to be accompanied by cross-reactive IgE antibodies. The observed T cell cross-reactivity between tree nuts and peanut provides the opportunity to develop a single immunotherapeutic agent, which may be effective for treatment of different tree nut allergies and peanut allergy and may prevent progression to multiple sensitizations. Preventive measures, like induction of oral tolerance, should already be considered early in life, but the exact window of opportunity has to be elucidated. Another way to prevent sensitization might be restoration of the skin barrier in children with atopic dermatitis to prevent the cutaneous route of sensitization.

GENERAL CONCLUSIONS

This thesis shows that children more frequently have a severe and adults a mild hazelnut allergy. So far no data suggest that the severity of a hazelnut allergy decreases with increasing age, but the prevalence of a mild birch pollen related hazelnut allergy seems to rise in the older age groups. This is further reflected by the fact that most adults report oral allergy symptoms to previous ingestion of hazelnut. Oral symptoms were hardly reported by children, but difficulties in recognition of oral symptoms as allergic symptoms may lead to an underrepresentation of them in children. The diagnostic work-up of a hazelnut allergy starts with a detailed clinical history. For many children and adults identification of the exact culprit nut is difficult, but this will hardly influence their diet, since the majority of them will eliminate all nuts from their diet. In children, a previous

ingestion of hazelnut is often unknown and the diagnosis strongly relies upon sensitization and DBPCFC. In half of the adults hazelnut allergy may be diagnosed with a detailed history with only oral symptoms to previous ingestion of hazelnut and the presence of birch pollinosis. However, the presence of birch pollen allergy is not only associated with a mild hazelnut allergy, but also frequently present in children and adults with a severe hazelnut allergy. Asthma is frequently observed in hazelnut allergic individuals. Evaluation of asthma could be considered in hazelnut allergy, because adequate treatment of asthma may prevent severe allergic reactions. Atopic dermatitis is present in almost all children and many adults with hazelnut allergy. The association between atopic dermatitis and hazelnut allergy may suggest that skin is a potential route of sensitization to hazelnut. Therefore, screening for a hazelnut allergy could be considered in children with moderate to severe atopic dermatitis early in life. However, our data indicate that in birch-endemic areas hazelnut specific IgE should not be used to screen for hazelnut allergy without acute allergic symptoms, to prevent many unnecessary elimination diets. SPT to hazelnut extract performs better than IgE to hazelnut extract in children; this was not shown in adults. Yet less than 30% of the children will be correctly diagnosed by SPT. And neither hazelnut specific IgE nor SPT are good predictors for the severity of the hazelnut allergy. Our data indicate that IgE to Cor a 9 and Cor a 14 are useful tools to diagnose a hazelnut allergy. Our data further suggest that isolated sensitization to Cor a 1 is possible in a severe hazelnut allergy.

In this thesis, it was shown that half of the children and adults with a hazelnut allergy also suffered from a peanut allergy. The clinical symptoms to hazelnut and peanut did not follow a common clinical pattern; only a severe peanut allergy was more common in adults with a severe (57%) rather than a mild (24%) hazelnut allergy, something which was not observed in children. Inhibition experiments revealed that IgE cross-reactivity was hardly observed between hazelnut and peanut seed storage proteins. But our data showed cross-reactivity between hazelnut and peanut at T cell level, suggesting that T cell cross-reactivity is not always accompanied by cross-reactivity at IgE level. The observed cross-reactivity at T cell level may explain the frequently observed sensitization to multiple tree nuts and peanut and provide a basis to develop an immunotherapeutic target.

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Chapter 10

Summary/Samenvatting

SUMMARY

Hazelnut is a frequently consumed tree nut, because of the taste and price. Unfortunately, allergic symptoms to hazelnut are common, especially in adults from Northern Europe. Allergic symptoms to hazelnut are highly variable, from mild symptoms limited to the oral cavity to severe and even anaphylactic reactions. The frequency of a severe hazelnut allergy shows differences between children and adults, as described in Chapter 3. A hazelnut allergy in adults is generally limited to mild and local symptoms in the oral cavity and associated with birch pollen allergy. In children, a hazelnut allergy is more severe and often accompanied by atopic dermatitis. Frequently reported allergic symptoms in children are angio-edema, urticaria and dyspnoea. The diagnosis of a food allergy has a great impact on daily life, because allergic individuals are continuously confronted with the risk of unexpected allergic reactions. This influences not only the life of the allergic individuals and their parents, but also their environment (i. e. friends, school). Therefore, a correct diagnosis is important, so that appropriate advise on dietary restrictions and emergency medication can be given. To diagnose a hazelnut allergy in adults, a history with only oral symptoms to hazelnut in combination with birch pollinosis is generally convincing to diagnose a hazelnut allergy, without the need to perform additional tests. In other cases, additional tests, including a double-blind placebo-controlled food challenge (DBPCFC), are often needed for a correct diagnosis. Chapter 2 describes the diagnostic value of hazelnut allergy tests in children. A history of allergic reactions with previous ingestion of hazelnut is often unknown in children, because many children have an elimination diet solely based on sensitization. Therefore, the diagnosis of a hazelnut allergy in children strongly relies on diagnostic tests. The skin prick test (SPT) with hazelnut extract is a better predictor of a hazelnut allergy in children than laboratory tests for specific IgE to hazelnut. The SPT with hazelnut extract can reduce the number of DBPCFCs in less than 30% of the children, but a DBPCFC remains necessary in many children to diagnose a hazelnut allergy. Chapter 4 shows the value of IgE to specific hazelnut components in the diagnostic work-up of a severe hazelnut allergy. IgE to hazelnut seed storage proteins Cor a 9 and Cor a 14 was highly specific for a severe hazelnut allergy in children and adults. Determination of IgE to these components may identify almost all children and half of the adults with a severe hazelnut allergy. In children, this may reduce the number of DBPCFCs considerably. Chapter 7 evaluates the potency of the major hazelnut allergen in basophil activation in comparison to the peanut allergens. Cor a 14 is the most potent hazelnut allergen in basophil activation, comparable to the 2S albumins in peanut (Ara h 2 and Ara h 6) which are most potent. This fits with the observation that IgE to Cor a 14 is highly specific for severe allergic reactions. Chapter 4 also shows that some children and half of the adults with a severe hazelnut allergy have an isolated Cor a 1 sensitization. This may suggest that Cor a 1 can be involved in

severe symptoms to hazelnut in some individuals, but is generally involved in hazelnut tolerance or mild symptoms to hazelnut. In line with this, chapter 7 illustrates a large variation in basophil activation with Cor a 1 among different individuals.

The amount of hazelnut needed to elicit symptoms in hazelnut allergic children and adults was studied in chapter 5. The thresholds for objective symptoms to hazelnut (considered as a severe hazelnut allergy in this thesis) were comparable between children and adults. Several patient characteristics like age, SPT to hazelnut extract and IgE to Cor a 9 and Cor a 14 influenced the threshold distribution curve (TDC) to hazelnut. The obtained eliciting dose values from this chapter may improve the food labeling. The product choice of hazelnut allergic individuals is strongly reduced, because an increasing number of products may contain hazelnut. Labeling information based on eliciting doses may improve the product choice of hazelnut allergic individuals. Hazelnut can be consumed after processing which is generally the case in industrially prepared food, but raw consumption directly from the tree is increasingly popular. Chapter 8 is a systematic review of the literature about the influence of processing on the allergenicity of tree nuts, including hazelnut. Roasting reduces the allergenicity of the PR-10 protein Cor a 1 in hazelnut. Sensitization to Cor a 1 is frequently observed in individuals with a hazelnut and birch pollen allergy, as a cross-reactive response after primary sensitization to Bet v 1 from birch pollen. Clinical studies with DBPCFCs have confirmed the reduced allergenicity of hazelnut after roasting in individuals with a hazelnut and birch pollen allergy. Still, the allergic symptoms were not absent in all individuals after roasting of hazelnuts, which suggests that other allergens are involved in the allergic reaction in these patients. The *in vitro* heat lability of the birch pollen related hazelnut allergen and the reduced *in vivo* allergenicity indicate that raw hazelnut is more allergenic than roasted hazelnut in individuals with a birch pollen related tree nut allergy. The heat lability of the PR-10 proteins in hazelnut has important implications for source material used for IgE testing, SPT and DBPCFCs and diet advises. Allergens not related to birch pollen, the lipid transfer proteins and seed storage proteins, present in different tree nuts (hazelnut, cashew, walnut etcetera), are generally heat stable, which suggests that processing may not influence their allergenicity.

Allergy to peanut, which is a legume and not a tree nut, is common in individuals with a hazelnut allergy as shown in chapter 6. The severity of the hazelnut allergy was not predictive for the presence and severity of the peanut allergy. Almost half of the children and adults with a sensitization to hazelnut seed storage proteins were also sensitized to their homologous proteins in peanut. ImmunoCAP inhibition experiments show that the peanut allergy was not the result of IgE cross-reactivity to hazelnut seed storage proteins. These data may imply that IgE to Cor a 14 and Ara h 2 are useful markers of primary sensitization to hazelnut and peanut respectively.

Chapter 7 describes the T cell responses to major hazelnut and peanut allergens. A high molecular weight molecule in hazelnut, possibly Cor a 11 was the strongest inducer of T cell proliferation of hazelnut specific T cell lines. At T cell level, cross-reactivity between hazelnut and peanut major allergens was observed. This suggests that the cross-reactivity may be induced at T cell level and is not always accompanied by cross-reactive IgE antibodies. The observed cross-reactivity at T cell level may in part explain the frequently observed concomitant hazelnut and peanut allergy.

In conclusion, our data show clinical aspects of a hazelnut allergy in children and adults. New diagnostic tests may improve the diagnosis of a severe hazelnut allergy, which may result in a reduction of the number of DBPCFCs and unnecessary elimination diets. The underlying mechanism leading to a concomitant hazelnut and peanut allergy was further studied, which may provide a basis for the development of preventive and therapeutic strategies.

SAMENVATTING

De hazelnoot behoort vanwege de prijs en smaak tot de meest geconsumeerde noten. Allergische klachten na de ingestie van hazelnoot komen echter regelmatig voor. Hazelnootallergie is in Nederland de meest voorkomende voedselallergie bij volwassenen, maar komt ook vaak voor bij kinderen. Een hazelnootallergie kan beperkt blijven tot milde klachten, zoals jeuk in de mond en keel, maar kan ook ernstige klachten veroorzaken en zelfs tot een anafylactische reactie leiden. Hoofdstuk 3 toont de verschillen in ernst van een hazelnootallergie bij kinderen en bij volwassenen.

Volwassenen met een hazelnootallergie hebben vaak milde klachten, zoals jeuk in de mond of keel en daarnaast een berkenpollenallergie. Kinderen met een hazelnootallergie hebben over het algemeen ernstiger klachten, zoals angio-oedeem, urticaria en benauwdheid. Bijna alle kinderen met een hazelnootallergie hebben ook constitutioneel eczeem. Een voedselallergie heeft vanwege het risico op onverwachte allergische reacties een grote impact op het dagelijks leven. Dit beïnvloedt niet alleen het dagelijks leven van allergische kinderen en hun ouders, maar ook dat van de omgeving, zoals familie en klasgenoten. Het is daarom belangrijk om de diagnose zorgvuldig te stellen en geschikte dieetbeperkingen en noodmedicatie te adviseren.

Bij volwassenen met een berkenpollenallergie, bij wie de klachten beperkt blijven tot jeuk of zwelling in de mond na het eten van hazelnoot, kan de diagnose vaak gesteld worden op basis van de anamnese. Aanvullende testen hebben bij hen geen meerwaarde voor de diagnose. In andere gevallen zijn aanvullende testen, waaronder een hazelnootprovocatie (liefst placebogecontroleerd), vaak nodig om de juiste diagnose te stellen.

Hoofdstuk 2 beschrijft de diagnostische waarde van hazelnootallergietesten bij kinderen. Weinig kinderen hebben een voorgeschiedenis van allergische klachten na het eten van hazelnoot, omdat de meeste kinderen al vanaf jonge leeftijd hazelnoot vermijden op basis van een sensibilisatie. De sensibilisatie is vaak bepaald in het kader van een screening bij kinderen met constitutioneel eczeem. Hierdoor zal de diagnose bij kinderen grotendeels gebaseerd zijn op diagnostische testen. De huidpriktest met hazelnootextract is een betere voorspeller voor een hazelnootallergie bij kinderen dan de bepaling van specifiek IgE voor hazelnoot uit het bloed. Door een huidpriktest met hazelnootextract uit te voeren, kunnen ongeveer 30% van de hazelnootprovocaties voorkomen worden. In veel gevallen zal echter toch een hazelnootprovocatie nodig zijn voor de juiste diagnose. Hoofdstuk 4 toont de waarde van IgE specifiek voor hazelnootallergenen bij de diagnostiek van een ernstige hazelnootallergie. De aanwezigheid van IgE voor de hazelnootallergenen Cor a 9 en Cor a 14 blijkt zeer specifiek te zijn voor een ernstige hazelnootallergie bij kinderen en volwassenen. Door de bepaling van IgE specifiek voor deze componenten werd bij bijna alle kinderen en de helft van de vol-

wassenen een ernstige hazelnootallergie opgespoord. Met name bij kinderen kunnen hierdoor vele hazelnootprovocaties voorkomen worden.

Hoofdstuk 7 toont de potentie van de verschillende hazelnootallergenen in de basofiele activatie-test, waarbij werd vergeleken met pinda-allergenen. Cor a 14 is het meest potente hazelnootallergeen, vergelijkbaar met Ara h 2 en Ara h 6 in pinda. Daarnaast laat hoofdstuk 4 zien dat enkele kinderen en de helft van de volwassenen met een ernstige hazelnootallergie alleen antistoffen hebben voor het berkenpollen-gerelateerde hazelnoot-allergeen Cor a 1. Deze bevinding suggereert dat Cor a 1 een rol kan spelen bij ernstige klachten door hazelnoot. De grote individuele variatie in de basofiele activatietest na stimulatie met Cor a 1 past ook bij deze bevinding.

Hoofdstuk 5 laat zien dat de hoeveelheid hazelnoot waarbij objectieve klachten optreden (drempels) vergelijkbaar is bij kinderen en volwassenen. Verschillende patiënt-karakteristieken zoals leeftijd, huidpriktest met hazelnootextract en de bepaling van IgE specifiek voor Cor a 9 en Cor a 14 waren van invloed op de gevoeligheid voor hazelnoot (drempelwaarde distributiecurve). De hieruit afgeleide dosering waarbij allergische klachten kunnen optreden kan gebruikt worden om de etikettering van producten te verbeteren. Op dit moment zijn mensen met een hazelnootallergie zeer beperkt in hun productkeuze, omdat steeds meer producten sporen van noten kunnen bevatten. Etikettering op basis van drempels kan de product keuze vergroten voor mensen met een voedselallergie.

Hazelnoot kan gegeten worden na verhitting, zoals vaak het geval is in voorverpakte producten, maar de inname van rauwe hazelnoot zonder verdere bewerking neemt toe in populariteit. Hoofdstuk 8 geeft een systematisch overzicht van de huidige literatuur over de invloed van verhitting op de allergeniciteit van verschillende noten, waaronder hazelnoot. Roosteren vermindert de allergeniciteit van een specifiek hazelnootallergeen (Cor a 1), gerelateerd aan berkenpollen. Antistoffen voor Cor a 1 komen vaak voor bij mensen met een hazelnoot- en berkenpollenallergie, door kruisreacties na primaire sensibilisatie voor Bet v 1 van berkenpollen. Klinische studies met voedselprovocaties lieten een afname in allergeniciteit van hazelnoot zien na roosteren bij mensen met een gecombineerde hazelnoot- en berkenpollenallergie. De allergische klachten verdwenen echter niet bij alle mensen na het roosteren van hazelnoot. Vermoedelijk zijn ook andere hazelnootallergenen betrokken. De hitte-labiliteit van het berkenpollen-gerelateerde hazelnoot allergeen *in vitro* en *in vivo* geeft aan dat rauwe hazelnoten meer allergeen zijn dan geroosterde hazelnoten bij mensen met een gecombineerde hazelnoot- en berkenpollenallergie. Deze informatie kan worden verwerkt in dieetadviezen. Verder kan verhitting ook het gebruikte testmateriaal bij IgE testen, huidpriktesten en voedsel-provocaties beïnvloeden.

Allergenen zonder een relatie met berkenpollen, die aanwezig zijn in verschillende noten zoals hazelnoot, cashew en walnoot zijn over het algemeen hittebestendig, waardoor verhitting weinig effect zal hebben op hun allergeniciteit.

Een allergie voor pinda, die behoort tot de peulvruchten en niet tot de noten, komt vaak voor bij mensen met een hazelnootallergie, zoals beschreven in hoofdstuk 6. De ernst van de hazelnootallergie lijkt echter geen goede voorspeller te zijn voor de ernst van de pinda-allergie. Bijna de helft van de kinderen en volwassenen met antistoffen voor Cor a 9 of Cor a 14 bleken ook antistoffen te hebben voor vergelijkbare eiwitten in pinda (Ara h 3 of Ara h 2). Met een twee-richting ImmunoCAP inhibitie werd geanalyseerd of deze co-sensibilisatie veroorzaakt werd door kruisreagerende antistoffen tussen hazelnoot en pinda. Hieruit bleek dat de pinda-allergie niet het gevolg was kruisreagerende antistoffen voor Cor a 9 of Cor a 14 in hazelnoot. Daardoor lijken de antistoffen voor Cor a 14 en Ara h 2 goede markers te zijn voor een primaire sensibilisatie voor hazelnoot of pinda.

In hoofdstuk 7 worden de T cel-responsen op de verschillende hazelnoot- en pinda-allergenen onderzocht. Een hoog moleculair eiwit in hazelnoot, mogelijk Cor a 11, gaf de sterkste T cel-proliferatie van de hazelnoot-specifieke T cel-lijnen. Verder werden er op T cel-niveau kruisreacties gezien tussen hazelnoot- en pinda-allergenen. Deze bevinding suggereert dat de kruisreacties tussen hazelnoot en pinda op T cel-niveau worden veroorzaakt en niet altijd leiden tot kruisreagerende antistoffen. De kruisreacties op T cel-niveau zouden een verklaring kunnen zijn voor het vaak samen voorkomen van een hazelnoot- en pinda-allergie.

Concluderend toont dit proefschrift de klinische aspecten van een hazelnootallergie bij kinderen en volwassenen. Nieuwe allergietesten kunnen het aantonen van een ernstige hazelnootallergie verbeteren, waardoor het aantal voedselprovocaties en onnodige diëten verminderd kan worden. Het onderliggende mechanisme van het gezamenlijk voorkomen van een hazelnoot- en pinda-allergie werd verder onderzocht. Deze bevindingen kunnen een bijdrage leveren aan de ontwikkeling van nieuwe preventieve en therapeutische behandelopties.

Chapter 11

Dankwoord (Acknowledgement)

DANKWOORD (ACKNOWLEDGEMENT)

Het proefschrift is af! Tijd om te beginnen aan het meest gelezen onderdeel van het proefschrift, het dankwoord. Graag wil ik iedereen bedanken die mee heeft geholpen aan dit proefschrift, allereerst de patienten die deelnamen aan het onderzoek en de collega's van de verschillende instituten die me allemaal geholpen hebben. Maar niet minder onbelangrijk de mensen die helemaal niet betrokken zijn geweest bij het onderzoek, die zorgden voor de leuke dingen hieromheen.

Els en Suzanne, ik heb ervan genoten om samen met jullie mijn promotie-traject te kunnen doorlopen. Het voelde ook echt als samen met jullie. Jullie betrokkenheid en inzet voor het project waren ontzettend groot. Hierdoor hebben we ook dit mooie resultaat kunnen bereiken. Voor mij zijn jullie echt een goed team. Jullie zijn allebei heel verschillend, maar vullen elkaar daardoor goed aan. De etentjes in combinatie met data bespreken en brainstormen van afgelopen jaar vond ik echt geweldig. Dit voelde voor mij echt zoals wetenschap hoort te zijn en ik hoop dat we dit nog steeds kunnen blijven voortzetten.

Carla, aan het eind van mijn promotie werd jouw rol steeds groter. Ik heb zeer genoten van de besprekingen. Aangezien ik meestal nog zo diep in de details zat, vond ik de vertaalslag naar de kliniek lastig om te maken. Door de besprekingen met jou lukte het om meer afstand te nemen van de data en toch de vertaalslag naar de kliniek te maken.

André, jouw rol was groot in mijn promotie-traject. Bij de meeste manuscripten ben je actief betrokken geweest en steeds wist je me scherp te houden met kritische vragen.

Yolanda, jij zorgde ervoor dat ik met veel plezier in het WKZ kwam. Door de besprekingen met jou, leerde ik hoe ik de voedsel provocaties bij kinderen kon interpreteren.

Petra, ik vond het ontzettend leuk dat ik regelmatig mee heb gekeken en geholpen bij de voedsel provocaties in het WKZ. Verder heb je me enorm geholpen bij de inclusie van de kinderen.

Stans, Helma en Barbara, jullie hebben wegwijs gemaakt en goed geholpen met experimenten in het lab, waardoor ik me er goed thuis voelde en er met veel plezier experimenten heb gedaan.

Ronald, Laurian, Jaap en Serge, jullie zijn nauw betrokken geweest bij mijn promotie-traject. De besprekingen leverden steeds interessante en leerzame discussies op. Ronald, jij zorgde steeds voor een belangrijke bijdrage aan de manuscripten, omdat je goed weet waar reviewers over kunnen vallen. Serge, bedankt voor alle ImmunoCAPs en inhibities die je voor me hebt gedaan. Laurian, in vele Europese steden hebben we samen opgetrokken en je was altijd makkelijk te bereiken voor allerlei vragen. Jaap, dankzij jouw humor lukte het om beter te relativeren in lastige periodes.

Jonas and Lars, it was wonderful to work with you. Your scientific input was great, and always combined with a great sense of humor.

Geert, Marty en Carina, dankzij jullie is het gelukt om een mooi stuk te maken met de drempelwaarden van de hazelnoot.

Rob, Laura, Thuy-my, Ans, Kitty, Sandra, Jos, Marieke, Floor, Jorien, Henrike, Mignon, Maarten, Janneke, Ilse, Dirk-Jan, Edward, Jantine en Miranda, bedankt voor de vele gezellige koffie-pauzes en lunches. En natuurlijk niet te vergeten de vele gezellige borrels, labuitjes, bbq's bij Edward en natuurlijk de congressen die we samen bezochten. Met als toppunt Istanbul, waar ik samen met Rob en Sandra, nog een paar daagjes langer bleef. Wat er nu werkelijk zo leuk was aan Istanbul weet ik eigenlijk ook niet meer goed, maar wel dat het woord altijd resulteert in een schatterlach bij Sandra.

Rob, het was super om jou als buurman te hebben, gezellig en ook handig aangezien we veel vergelijkbare analyses hebben gedaan: jij voor de pinda en ik voor de hazelnoot. Super dat je me wilt bijstaan als paranimf.

Collega's van het lab van de longziekten, ik vond het altijd erg leuk om met jullie samen te werken en natuurlijk niet te vergeten de leuke labuitjes en memorabele stapavonden.

Mirjam, Chantal en Harmieke bedankt voor de statistische ondersteuning bij mijn analyses en Kees bedankt voor alle queries die je hebt gedaan. Anouska bedankt voor de input vanuit de diëtetiek op mijn project, verder heb ik het erg genoten van de lekkere provocatierecepten, waar je regelmatig proefpersonen voor zocht.

Ik wil graag de studenten bedanken die mee hebben geholpen aan mijn onderzoek: Kirsten, Marieke, Anne en Joost.

Lieve vrienden, ik wil jullie allemaal bedanken voor jullie luisterend oor en tips tijdens frustrerende periodes, maar vooral jullie geduld en steun tijdens de laatste drukke fase van mijn promotie. Marloes met jou kon ik altijd goed praten over het onderzoek, vaak tijdens een kopje thee of het hardlopen, omdat je zelf ook een promotietraject doet. Bedankt dat je mijn paranimf wilt zijn! Eefke bedankt voor de vormgeving van het notenmemoryspel (voor de kinderen die deelnamen) en dit boekje. Judith bedankt voor het maken van het mooie figuur in een van de artikelen. Karen bedankt voor het kritisch lezen en verbeteren van mijn samenvatting. Nooit geweten dat in het Nederlands schrijven zo moeilijk is.

Loek, Valerie en Evelien, mijn allerliefste broertje en zusjes

Pap en mam, jullie hebben me altijd vrij gelaten om mijn eigen keuzes te maken, en me daarin steeds gesteund ook tijdens tegenslagen.

Chapter 12

Appendices

Curriculum vitae

List of publications

List of co-authors

Abbreviations

CURRICULUM VITAE

Laury werd geboren op 2 januari 1983 in Sittard. In 2001 haalde ze haar Gymnasium diploma op het Trevianum te Sittard. In datzelfde jaar begon ze met de opleiding geneeskunde bij de Radboud Universiteit in Nijmegen. In het laatste jaar van de opleiding (2007) koos ze voor het tropen co-schap, waardoor ze drie maanden stage liep in een plattelandsziekenhuis in Rubya, Tanzania. Verder heeft ze in haar zesde jaar de wetenschappelijke stage gedaan bij het lab infectieziekten van de experimentele interne geneeskunde onder begeleiding van Mihai Netea, Bart Ferwerda en Theo Plantinga. Na het behalen van haar arts-examen heeft ze eerst acht maanden (2008) bij de afdeling interne geneeskunde in het Canisius-Wilhelmina Ziekenhuis in Nijmegen gewerkt. Hierna heeft ze een half jaar bij de afdeling Dermatologie/allergologie gewerkt als arts-assistent niet opleiding. Hierna (2009) is ze begonnen als arts-onderzoeker bij de afdeling Dermatologie/allergologie, hetgeen heeft geleid tot dit proefschrift. In 2013 is ze begonnen met de opleiding tot dermatoloog in het UMC Utrecht met Vigfús Sigurdsson als opleider.

LIST OF PUBLICATIONS

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ABBREVIATIONS

AUC:	Area under the curve
BP:	birch pollen
CCD:	Cross-reactive carbohydrate determinants
CI:	Confidence interval
DBPCFC:	Double-blind placebo-controlled food challenge
ELISA:	Enzyme-linked immunosorbent assay
Fr:	Fraction
IEC:	Ion exchange chromatography
IMAC:	Immobilized metal ion affinity chromatography
ImmunoCAP:	ImmunoCAP FEIA (Fluorenzymeimmunoassay)
IQR:	Interquartile range
LAL:	Limulus Amebocyte Lysate
LPS:	Lipopolysaccharide
NPV:	Negative predictive value
nsLTP:	Non-specific lipid transfer protein
PBMC:	Peripheral blood mononuclear cell
PPV:	Positive predictive value
rCor a 1:	recombinant Corylus avellana 1
ROC curve:	Receiver operating characteristic curve
RPC:	Reversed phase chromatography
SI:	Stimulation index
sIgE:	Specific IgE
SPT:	Skin prick test
TCLs:	T cell lines