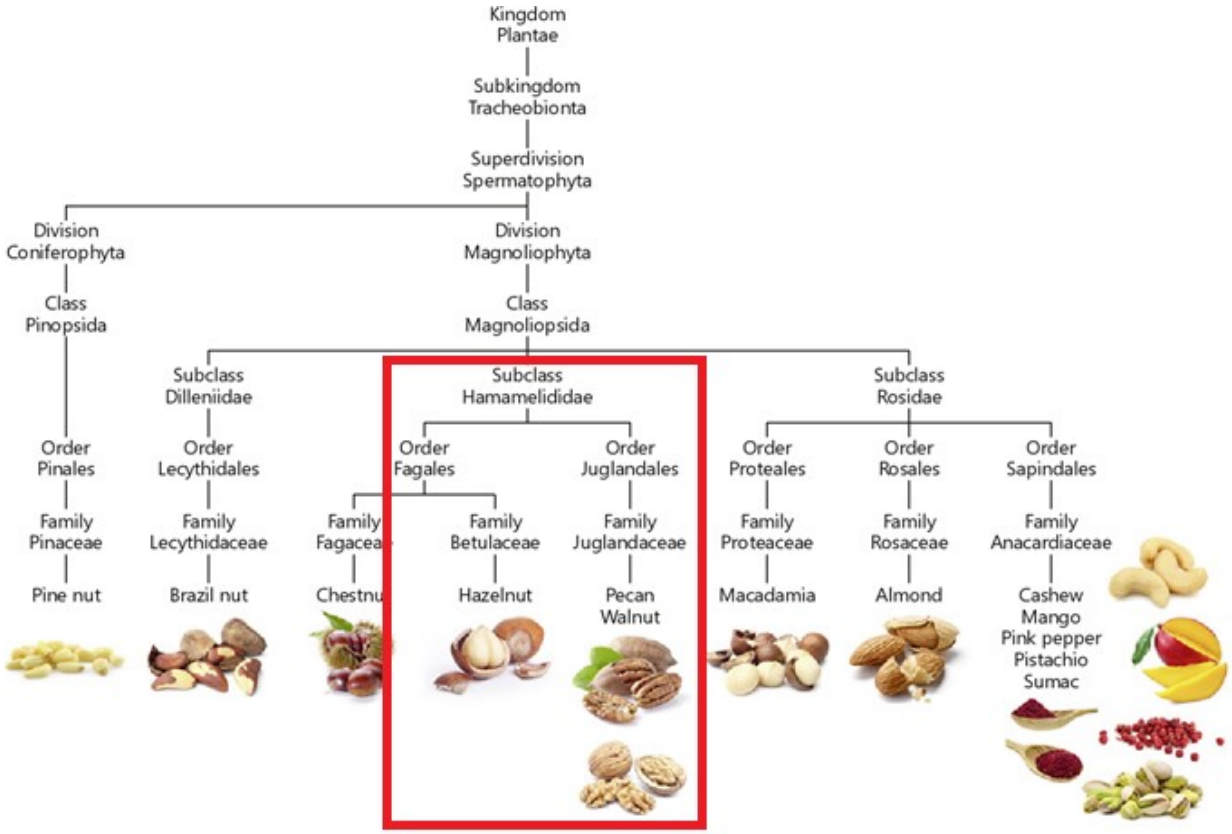
Retrospective and serological analysis of the potential cross-reactivity among hazelnut and walnut



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List of Abbreviations

- ***Cor a*:** Corylus avellana (Hazelnut)
- **ELISA:** Enzyme-Linked ImmunoSorbent Assay
- **HRP:** Horseradish Peroxidase
- **(s)IgE:** (Specific) Immunoglobulin E antibody
- ***Jug r*:** Juglans regia (Walnut)
- **LTP:** Lipid Transfer Protein
- **OD:** Optical Density
- **PBS:** Phosphate Buffered Saline
- **SPT:** Skin Prick Test
- **TMB:** Tetramethylbenzidine

Abstract

BACKGROUND: Tree nut allergies are a worldwide cause of anaphylactic events. Many allergic patients are sensitized to more than one tree nut. Independent sensitization to multiple tree nuts (co-sensitization) is common, whereas reactivity of IgE antibodies to homologous proteins in different allergens (cross-reactivity) is less well defined. Proteins are very homologous between cashew and pistachio, as well as walnut and pecan, leading to already demonstrated and clinically relevant cross-reactivity. Allergen components in hazelnut and walnut also share homology, however the extent of cross-reactivity among these tree nuts remains understudied.

OBJECTIVE: To survey the clinical and serologic cross-reactivity among hazelnut and walnut.

METHODS: A retrospective study with 29 patients sensitized to both hazelnut and walnut (positive SPT and positive allergen specific IgE levels) was conducted to determine the clinical relevance between the two nut allergies. Besides that, inhibition Enzyme-Linked ImmunoSorbent Assays (ELISA) were conducted to assess the degree of cross-reactivity among hazelnut and walnut.

RESULTS: The retrospective study reflected the broad sensibilization pattern and the clinical relevance of pollen allergy to hazelnut and walnut cross-reactivity in 29 patients. 52% of the patients had experienced anaphylaxis following hazelnut or walnut ingestion and 93% carries an adrenalin-autoinjector for their multi-food allergies, indicating the severity of multi-food allergies. Inhibition ELISA demonstrated dose-dependent inhibition of hazelnut-specific IgEs after pre-incubation of patient serum with walnut extract.

CONCLUSIONS: The tree nuts hazelnut and walnut, from the subclass *Hamamelididae*, did serologically cross-react with each other in inhibition ELISA. Allergic individuals might be sensitized to both nuts due to this cross-reactivity, which should be considered in allergy tests and personal diet recommendations. More assays, including inhibition ELISAs in both directions and immunoblot inhibition assays, should be conducted with a larger sample size to provide more evidence of this cross-reactivity. Besides that, assays with the hazelnut-walnut allergen component couples from separate protein families (2S albumins, vicilins, legumins, Lipid Transfer Proteins (LTP), profilins and PR-10) could clarify the allergen components associated with this cross-reactivity.

Laymen's summary

Tree nut allergies are the worldwide most common food allergies. The most commonly reported tree nut allergies are: walnut, almond, hazelnut, pecan, cashew and pistachio. These tree nuts consist of multiple protein components (allergens) that can elicit an allergic reaction. Many patients that are allergic to one tree nut develop an allergy to another tree nut. This can either be caused by: 1) *co-sensitization*, when the human body produces multiple different Immunoglobulin E (IgE) antibodies to different types of allergens or 2) *cross-reactivity*, when one type of IgE antibody recognizes multiple different allergens, by well-conserved, similar looking parts in the protein sequence (homology) of these allergens (*figure 1*). When someone is allergic to a particular tree nut that cross-reacts with another tree nut, both tree nuts should be avoided from the patient's diet, since the patient's IgE antibodies can recognize both allergens. If there is no cross-reactivity with other nuts, these tree nuts can be safely consumed. For some tree nuts that share these similar looking allergens, such as walnut and pecan or cashew and pistachio, cross-reactivity has been scientifically proved. Other tree nuts, including hazelnut and walnut do also share similarity in multiple allergen components. However, cross-reactivity among these other tree nuts remains unclear, making avoidance of all tree nuts the safest approach. Having a tree nut allergy already significantly impacts quality of life, as it can entail anxiety for severe allergic responses and can limit social activities. Avoiding all tree nuts while there is no cross-reactivity can be an additional, unnecessary intervention besides all other social and mental hindrances that an allergic individual has to cope with. By mapping the clinical cross-reactivity to closely related tree nuts, the safety quality of life of allergic patients can be improved. This retrospective and serological study is one of the first studies that aimed to demonstrate the potential cross-reactivity among hazelnut and walnut. The retrospective study part reflected the severity of hazelnut and walnut allergies and the clinical relevance of other food or pollen allergies in cross-reactivity. The serological study part demonstrated that hazelnut-specific IgE antibodies in patient's serum could be inhibited by walnut extract, indicating clear cross-reactivity between these two tree nuts. Although the number of studies available in literature that examined the cross-reactivity between hazelnut and walnut is very low, all the results so far confirm cross-reactivity. Nevertheless, additional experiments should be executed to further prove the cross-reactivity between hazelnut and walnut. This information can be used to put together a more targeted and safer personal diet, which could significantly improve the quality of life and reduce the risk of severe or fatal allergic reactions of allergic individuals.

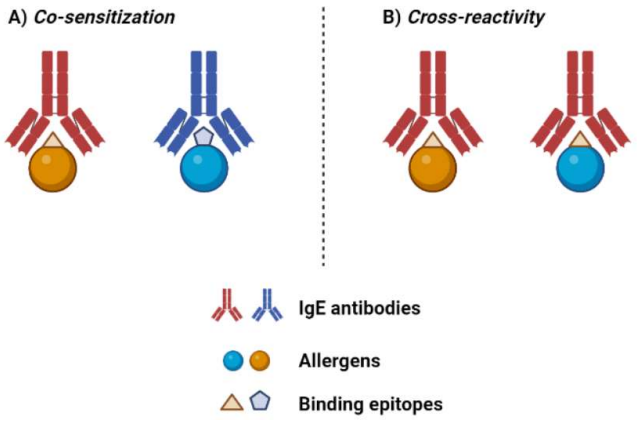

Figure 1: Schematic overview of co-sensitization vs. cross-reactivity. A) Antibody (red) recognizes the orange allergen with a specific binding site (triangle). The blue antibody recognizes a different blue allergen with a specific binding site (pentagon). B) The blue allergen has a well-conserved peptide sequence, and thus a similar (homologous) binding site as the orange antigen, enabling the red antibody to recognize and bind both of the allergens (cross-reactivity).

Introduction

Tree nut allergy prevalence ranges between 1% - 3% of the world population and these allergies are a major cause of (fatal) anaphylaxis [1]. The most common tree nut allergies are: walnuts, almonds, hazelnuts, pecans and cashews, from which almonds, hazelnut and cashews are worldwide most consumed [2,3]. After diagnosis of a tree nut allergy, 86% of allergic individuals develop and allergy to another tree nut by the age of 14 [4–6]. Thus, many allergic patients are sensitized to more than one tree nut, but it remains uncertain to what extent this is caused by either independent sensitization to multiple allergens (co-sensitization) or by reactivity of Immunoglobulin E (IgE) antibodies to homologous proteins or epitopes in different allergens (cross-reactivity) [7].

The majority of proteins involved in tree nuts allergy are the so-called seed storage proteins 2S albumins, vicilins (7S globulins) and legumins (11S globulins), Lipid Transfer Proteins (LTP), profilins and PR-10 [2,3,8,9]. From these, PR-10 and LTP allergens are known to be highly cross-reactive proteins [10]. Multiple different tree nuts have been shown to be cross-reactive to other tree nuts, fruits and legumes, caused by well-conserved allergens. Allergy to tree nuts can often be traced back to primary sensitization to birch pollen's major PR-10 allergen (Bet v 1) due to allergen homology [9]. Bet v 1 often induces cross-reactive IgEs that are able to react with these homologous allergens. A commonly held rule is that at least 70% sequence homology between allergens in these protein families is required for cross-reactivity [11]. However, a higher degree in amino acid or structure homology does not guarantee cross-reactivity among allergens.

Proteins are extremely stable and well-conserved between cashew and pistachio as well as walnut and pecan. Vicilin allergens in cashew (Ana o 1) and pistachio (Pis v 3) have 79% sequence homology and legumin allergens in walnut (Jug r 4) and pecan (Car i 4) have 95% sequence homology [9]. Cross-reactivity among these tree nuts has been clinically demonstrated. However, IgE-mediated cross-reactivity among most other tree nuts remains unclear, making avoidance of all tree nuts the safest approach. Having a tree nut allergy already significantly impacts quality of life, as it can entail anxiety for severe allergic responses and can limit social activities [12–14]. Avoiding all tree nuts while there is no cross-reactivity can be an additional, unnecessary intervention besides all other hindrances that an allergic individual has to cope with. By mapping the clinical cross-reactivity to closely related tree nuts, the safety and quality of life of allergic patients can be improved.

Tree nuts consist of multiple protein components (allergens), all of which can trigger an allergic reaction. The most important allergens in hazelnut are Cor a 1, 2, 8, 9, 11 and 14 and in walnut are Jug r 1, 2, 3, 4, 5, 6 and 7 [8]. Table 1 shows an overview of the identified, matching protein families between these hazelnut and walnut allergens [15–21].

*Table 1. Allergens in hazelnut and walnut, their protein family and the sequence identity (homology) between allergens from matching protein families [8,9,22]. *Not Available in Literature, these sequences were aligned manually by using Expasy and FASTA sequences of the allergens [15–21]. Seed storage protein Jug r 2 (vicilin) is not shown since it has a <50% homology with hazelnut vicilin Cor a 11.*

Protein Family	Hazelnut (MW)	Walnut (MW)	Homology (%)
PR-10 (Bet v 1-like)	Cor a 1 (18 kDa)	Jug r 5 (14 kDa)	*69**
Profilin	Cor a 2 (14 kDa)	Jug r 7 (13 kDa)	*83**
2S Albumin	Cor a 14 (17 kDa)	Jug r 1 (15 kDa)	60
Vicilin (7S Globulin)	Cor a 11 (48 kDa)	Jug r 6 (47 kDa)	72
Legumin (11S Globulin)	Cor a 9 (40 kDa)	Jug r 4 (58 kDa)	73
LTP	Cor a 8 (10 kDa)	Jug r 3 (10 kDa)	*60**

Profilin allergens (*Cor a 2* and *Jug r 7*, 83%), vicilin allergens (*Cor a 11* and *Jug r 6*, 72%) and legumin allergens (*Cor a 9* and *Jug r 4*, 73%) in walnut and hazelnut have >70% sequence homology, which is a clear indication that there could be cross-reactivity among these allergens [9]. However, IgE-mediated cross-reactivity among these hazelnut and walnut remains unclear and understudied.

An Italian study with 36 allergic patients sensitized to either hazelnut or walnut, demonstrated co-sensitization to the other nut in 33% of the patients, suggesting potential co-recognition of the aforementioned homologous allergens [22]. So far, Villalta *et al.* (2019) performed an ImmunoCAP inhibition study that ascertained cross-reactivity specifically among hazelnut and walnut involving only 13 patients, using recombinant allergens of seed storage proteins rCor a 9, rCor a 14 and rJug r 1 [23]. They found that cross-reactivity among hazelnut and walnut seed storage proteins exists and occurs at the level of 2S albumins and 11S globulins. Besides that, Yoshida *et al*. (2019) studied the cross-reactivity among macadamia nut, walnut and hazelnut, including only 7 children with a confirmed macadamia allergy, however their main aim was to demonstrate the relation of hazelnut and walnut with macadamia nut [24]. Goetz *et al.* (2005) performed a serologic study, including inhibition ELISA, surveying the cross-reactivity among seven edible nuts [7]. They provided the first and only evidence of cross-reactivity among hazelnut and walnut in inhibition ELISA. However, they did not put much emphasis on the cross-reactivity among hazelnut and walnut and mainly focused on the tree nut combinations showing the strongest cross-reactivity (walnut and pecan and cashew and pistachio).

This makes hazelnut and walnut interesting allergens to assess for cross-reactivity. The profilin, vicilin and legumin proteins have 83, 72 and 73% homology, which gives them the highest chance for cross-reactivity following the 70% rule. Despite the fact that PR-10, 2S albumins and LTP in walnut and hazelnut only have homology below the 70% rule, these are also interesting to include in experiments. Seed storage proteins Jug r 1 and Cor a 14 have been identified as major component allergens with the ability to induce (primary) sensitization and the ability to inhibit each other in ImmunoCAP inhibition assays by Villalta *et al.* (2019) [22]. LTPs and PR-10s are normally considered to be highly cross-reactive proteins [17,18,23,25–27]. Further laboratory studies, assessing more allergic patients, are required to clarify this cross-reactivity among hazelnut and walnut.

Cross-reactivity can be demonstrated *in vitro* by so-called indirect and inhibition ELISA (Enzyme-Linked Immunosorbent Assay). In an indirect ELISA, an allergen is immobilized (coated) to the surface of a microplate and bound by a human IgEs specific for that allergen. The human IgE caught by the antigen then binds an enzyme-conjugated secondary anti-human IgE. By adding a substrate for this enzyme, a color develops and thereby the amount of bound IgE can be detected by measuring the absorbance (*figure 2*). In inhibition ELISA, the first antibody is pre-incubated with a different (non-specific) allergen, prior to incubation with the immobilized allergen. Depending on the affinity and specificity of the allergen-specific antibody for the other (pre-incubated) allergen, more or less free antibodies will be available to bind the immobilized allergen. This will cause a change in color development, which leads to a shift in absorbance. The difference between the absorbance results of the indirect ELISA and inhibition ELISA indicates the degree of cross-reactivity.

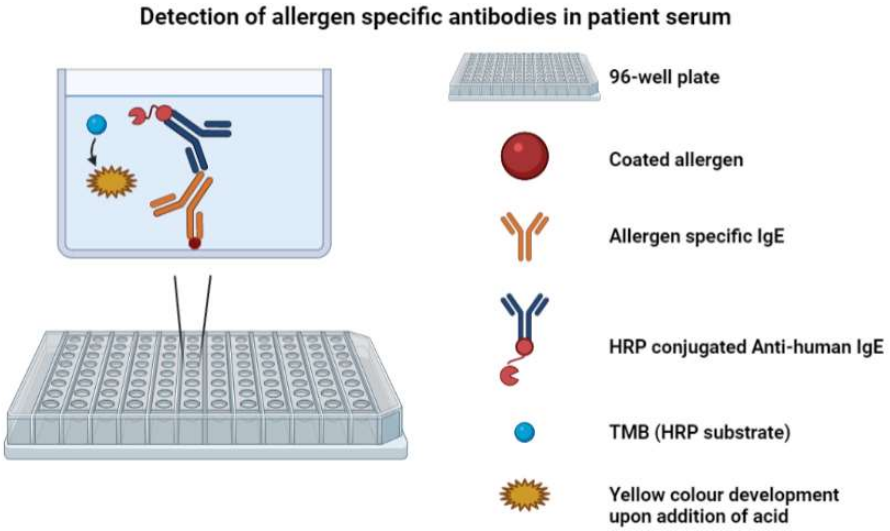

Figure 2: Basic principles of indirect ELISA. An allergen is immobilized on a microplate. Allergen-specific IgEs, from patient's serum, bind to this allergen. Secondary horse-radish peroxidase (HRP)-conjugated anti-human IgEs then bind to the caught human IgEs. After adding substrate for the HRP enzyme, intensity of the developed color, measured as absorbance at 450 nm, indicates the number of IgEs that were caught by the immobilized allergen. IgE = Immunoglobulin E, HRP = horse-radish peroxidase, TMB = tetramethylbenzidine. Created in BioRender.

This report describes a combination of a retrospective and serological study, aiming to assess the potential, but yet understudied cross-reactivity among hazelnut and walnut. In the retrospective study, laboratory results, anamnesis and SPT results from 29 patients (*males/females 17/12; mean age 16.8; range 2 – 67 years*) sensitized to both hazelnut and walnut, as determined by a positive SPT, were analyzed to clarify the scientific and clinical link between these two tree nut allergies. In the serological study, serum from 3 of these patients sensitized to both hazelnut and walnut was used in indirect ELISA and inhibition ELISA to demonstrate possible cross-reactivity.

Materials and Methods

Retrospective study

Population

Children and adults with a physician diagnosed hazelnut and walnut allergy (IgE mediated nut-specific symptoms combined with positive SPT and positive sIgE values) were retrospectively included from a database at the department of allergology & clinical immunology of the Erasmus MC, Rotterdam. Inclusion period was 2018 – 2021.

Skin prick tests were performed in patients suspected of hazelnut and / or walnut allergy, as determined by previous allergic episodes after food ingestion and / or positive sIgE against the nut allergens. SPT were performed with homemade nut extracts, as previously described by de Jong *et al.* [28,29]. In three years, a total of 342 SPT were performed with hazelnut and 383 with walnut. For hazelnut, the SPT were performed with 5% extract in PBS (311 of 342 tests, 91%) or 10% extracts in PBS (31 of 342 tests, 9%). For walnut, the SPT were performed with 10% extract in PBS (213 of 383 tests, 56%), pure extract (120 of 383 tests, 31%) or prick-to-prick (50 of 383 tests, 13%). During the test period, 241 out of 342 (70%) hazelnut SPT were positive and 227 out of 383 (59%) walnut SPT were positive. From the hazelnut and walnut positive populations, 29 patients had a combined positive hazelnut and walnut SPT.

Serological study

Patient's serum (N = 3)

Three of the twenty-nine patients were included in the *in vitro* experiments to demonstrate possible cross-reactivity. Allergen-specific IgE level measurements were performed with the ImmunoCAP™ ISAC monoplex platform (Thermo Fisher Scientific, Sweden) or Allergy Explorer (ALEX) multiplex platform (Macro Array Diagnostics, Netherlands). Allergen-specific IgE levels were considered positive when ≥0.30 ISU (ISAC) or ≥0.35 kU/L (ALEX). ISAC and ALEX only measure the following specific allergen components for hazelnut and walnut: Cor a 1.0401, Cor a 8, Cor a 9, Cor a 14, Jug r 1 and Jug r 3. Only study specific sIgE results will be presented in the case descriptions. The clinical features of the three selected patients are as follows:

Patient 1

The first case is a 22 year old male with a history of hives, skin itching, oral allergy syndrome, GI symptoms, vomiting, angioedema of the lips and shortness of breath after the ingestion of hazelnut. At the age of 21 years, he experienced an anaphylactic reaction following the consumption of hummus with hazelnut. The patients has been diagnosed with tree pollen allergy, asthma, eczema and rhino conjunctivitis. He also suffered from angioedema of the lips after watermelon consumption. The patient also had a positive SPT and positive sIgE results for walnut. He follows a diet strictly avoiding all tree nuts and water melon, however he tolerates peanuts and almonds. He carries an adrenaline auto-injector as emergency medication and uses antihistamines, nasal spray and creams for his pollen allergy, asthma and eczema. *Allergen-specific Ige levels (ISAC); Bet v 1 (PR-10): 93 ISU, Cor a 1.0401 (PR-10): 31 ISU, Cor a 8 (LTP): 54 ISU, Cor a 9 (11S globulin): 2.4 ISU and Cor a 14 (2S albumin): >100 ISU, Jug r 1 (2S albumin): >100 ISU and Jug r 3 (LTP): 17 ISU.*

Patient 2

The second case is a 27 year old female with a history of throat itching, GI symptoms, nausea and vomiting after the ingestion of walnut. At the age of 26 years, she experienced an anaphylactic reaction with skin redness and itching, shortness of breath and trouble with speaking following the consumption of bread containing (traces of) walnut, which has been treated with adrenaline, prednisone, tavegyl and nebulization in the emergency room. The patient has physician diagnosed allergies for grass pollen, dogs, cats and house dust mite and has asthma. The patient also had positive SPT and positive sIgE results for hazelnut. She follows a diet strictly avoiding (traces of) all tree nuts and peanuts, dairy products, gluten and egg. She carries two adrenaline auto-injectors as emergency medication and uses nasal spray for her pollen allergy. *Allergen-specific Ige levels (ISAC); Bet v 1 (PR-10): 0 ISU, Cor a 1.0401 (PR-10): 0 ISU, Cor a 8 (LTP): 0 ISU, Cor a 9 (11S globulin): 9.8 ISU, Cor a 14 (2S albumin): 21 ISU, Jug r 1 (2S albumin): 65 ISU and Jug r 3 (LTP): 0 ISU.*

Patient 3

The third case is a 24 year old female with a history of repeated anaphylactic reactions caused by her known allergy for peanuts and tree nuts. At the age of 18 years, she experienced a severe allergic reaction after the consumption of bread containing walnut, experiencing the following symptoms: facial redness and itching, swollen throat and vomiting, treated with ranitidine, tavegyl, hydrocortisone and adrenaline injection. The patient also had a positive SPT and positive sIgE results for peanut, pistachio, almond, cashew, hazelnut and pecan and Brazil nut. She follows a diet strictly avoiding (traces of) all tree nuts and peanuts. She carries an adrenaline auto-injector as emergency medication. *Allergen-specific Ige levels (ALEX); Bet v 1 (PR-10): 3.26 kU/L, Cor a 1.0401 (PR-10): 2.13 kU/L, Cor a 9 (11S globulin): 8.54 kU/L and Cor a 14 (2S albumin): 6.26 kU/L and Walnut Jug r 1 and Jug r 3: >100 kU/L (sIgE levels of Cor a 8 are not available in electronic patient file).*

Homemade nut extract preparation

Hazelnut and walnut extracts were prepared as described before [28]. Briefly, fresh, unroasted and unsalted walnuts and hazelnuts were mechanically homogenized, after which they were defatted by ether extraction in a Soxhlet. Consequently, the defatted nuts were air dried and the powder was stored at -20°C. For ELISA, the nut extracts were ground with a mortar, dissolved in PBS pH 7.8 (0.5 mg / mL) and stored at -20°C until further use in ELISA experiments.

Indirect ELISA optimization

Indirect ELISA was performed as previously described, with minor modifications [30,31]. Briefly, 96-well microplates (ThermoFisher Scientific, USA) were coated with 100 µL homemade nut extract of varying concentration (*X* µg / mL) of hazelnut or walnut in coating buffer (PBS, pH 7.8) at 4°C overnight. The plates were washed with 250 µL washing buffer (0.05% Tween 20 in PBS, pH 7.4 (PBS-T)) and blocked with 200 µL blocking buffer (1% BSA in PBS, pH 7.4) 1 hour at room temperature (RT). After washing, 100 µL pre-diluted patient serum was added to each well and incubated for 2 hours at RT, while gently shaking. The plates were then washed with PBS-T and incubated with 50 µL mouse anti-human IgE Fc-horse-radish peroxidase (HRP) (SouthernBiotech, USA) (1:1500 v/v in 1% BSA in PBS, pH 7.4) for 2 hours at RT, while gently shaking. Unbound anti-human IgEs were removed from the wells by washing with PBS-T, followed by visualization of the enzymatic activity by incubation with 100 µL tetramethylbenzidine (TMB)/H_2O_2 for 30 minutes at RT. The reactions were stopped by the addition of 100 µL 1M H_2SO_4. Absorption was measured at 450 nm. Results were expressed as optical density (OD). An OD of 0.5 is considered as cut-off value for positive results. In the indirect ELISAs, values significantly above this cut-off value (>1.5) were considered positive and scientifically useable results, since these higher values will yield a broader analysis range in the inhibition ELISAs, which enables accurate calculations of the inhibition percentage. All assays were performed in duplicate.

Inhibition ELISA (hazelnut coating)

In order to assess the potential cross-reactivity among hazelnut and walnut, ELISA inhibition was performed as previously described, with minor modifications [32,33]: 100 µL of diluted serum was pre-incubated with 0, 0.1, 1, 10, 100 and 1000 µg walnut extract for 2 hours at RT, while gently shaking. Diluted serum was also pre-incubated with 0, 0.1, 1, 10, 100 and 1000 µg hazelnut extract as a positive control. The pre-incubated samples were then added to microplates that had been coated with hazelnut (10 µg / well). The ELISA procedure thereafter was the same as described in 'ELISA optimization'.

Thereafter, inhibition percentages were calculated by: $\frac{OD\ value\ wit\quad inhibition\ nut\ X\ \mu g}{OD\ value\ without\ inhibitio\quad nut} * 100\%$.

Results and discussion

Retrospective study

Clinical characteristics of all patients (N = 29) are shown in table 2. Among the patients, 59% were male and the mean age was 16.8 years (range 2 – 67 years). The median wheal surface of positive hazelnut SPT was 70.18 mm^2 and 40.88 mm^2 for walnut. Sixteen (55%) patients had a history of eczema and seven (24%) patients had a history of asthma. Twenty-seven (93%) patients had a confirmed food allergy other than walnut or hazelnut and twenty-nine (100%) patients had a family history of other (food) allergies.

Table 2: Clinical characteristics of patients with positive SPT for both hazelnut and walnut. Data given in: Number (%), Median (Q1 ; Q3).

	Hazelnut-walnut positive SPT (N = 29)
Sex	
Male	17 (59%)
Female	12 (41%)
Mean age, years	16.8, range 2 – 67 years
Children, <18 years	20 (69%)
Adults, >18 years	9 (31%)
History of eczema	16 (55%)
History of asthma	7 (24%)
Confirmed food allergies other than hazelnut or walnut	27 (93%)
Clinically confirmed allergic rhinitis	25 (86%)
Sensitization to tree pollen with symptoms	15 (52%)
Sensitization to grass pollen with symptoms	16 (55%)
Positive hazelnut SPT	
Number of participants	29 (100%)
Median wheal surface (mm^2)	70.18 (25.1 ; 100.6)
Hazelnut-specific IgE	
Available in database of participants	18 (62%)
Median level (kU/L)	21.05 (5.03 ; 26.00)
Positive walnut SPT	
Number of participants	29 (100%)
Median wheal surface (mm^2)	40.88 (15.1 ; 66.95)
Walnut-specific IgE	
Available in database of participants	10 (34%)
Median level (kU/L)	15.79 (2.71 ; 32.1)
Anaphylaxis symptoms after hazelnut or walnut ingestion	
Information available in database of participants	15 (52%)
Urticarial rash	15 (52%)
Facial edema	14 (48%)
Throat tightness or dyspnea	11 (38%)
Throat or mouth itching	9 (31%)
Vomiting	6 (21%)
Nausea	2 (7%)
Abdominal pain	1 (3%)
Dizziness	1 (3%)
Medication	
Carries adrenaline auto-injector	22 (76%)
Uses other anti-allergy medication	29 (100%)

Fifteen (52%) patients had an allergic episode following hazelnut or walnut ingestion. The most prevalent symptoms were urticarial rash (52%), facial edema (48%), dyspnea (38%), throat or mouth itching (31%) and vomiting (21%). For fourteen (48%) patients, the symptoms and history of allergic episodes were not mentioned in the electronic patient file. Nineteen (66%) patients followed a diet strictly avoiding both hazelnut and walnut. Dietary information of the other ten (34%) patients was not mentioned in the electronic patient file. Twenty-two (76%) patients had a history of (severe) anaphylaxis, caused by any food allergen, and thus carry an adrenalin auto-injector as emergency treatment. Twenty-nine (100%) patients were using other prescribed anti-allergy medication, such as nasal spray, antihistamine tablets or cream for eczema.

Fifteen (52%) patients were sensitized to tree pollen (Bet v 1, PR-10), and showed clinically relevant allergic rhinitis symptoms. Taking into account the role of Bet v 1 in cross-reactivity, it is most likely that the combined hazelnut and walnut allergy is largely caused by their birch pollen allergy. Thereby not neglecting the possible sensitization in this patient group to non-Bet v 1 dependent allergens in tree nuts (e.g. 2S albumins, 7S globulins and 11S globulins), which will have a very important role in the cause of their severe allergic symptoms (e.g. anaphylaxis). The other 48%, which have allergic rhinitis with symptoms due to sensitization to grass pollen, house dust mite and / or animals, do not have this Bet v 1-related cross-reactivity, thus hazelnut and walnut co-sensitization or cross-reactivity due to the 2S, 7S or 11S proteins is the most likely explanation is these cases. Serum of three of the patients will be used in inhibition ELISA to demonstrate possible cross-reactivity among hazelnut and walnut.

Serological study

Indirect ELISA optimization

As mentioned in the introduction, cross-reactivity can be demonstrated *in vitro* by inhibition ELISA. Before starting any preliminary inhibition assays, the ELISA system (e.g. coating of allergen extracts) must be optimized by performing indirect ELISA with varying concentrations of allergen extracts in coating buffer.

Test serum with known hazelnut and walnut sIgE values were used in different dilutions on well-plates with different coating concentrations. Coating buffer was either PBS buffer (pH 7.8) or carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). All ELISAs were performed in duplicate. Table 3 and 4 give an overview of the different conditions that were tested for hazelnut and walnut and the corresponding measured optical density (OD) values.

Table 3: results of the indirect ELISAs that led to the optimal conditions of hazelnut coating and serum dilution for the inhibition ELISAs.

Cor a *X* IgE after dilution (kU/L)	**Hazelnut coating (µg)**	**Coating buffer**	**OD (450 nm)**
5 (Cor a 1.0401)	0.25	Carbonate	0.011
5 (Cor a 1.0401)	0.25	PBS	0.140
5 (Cor a 1.0401)	1	Carbonate	0.00
5 (Cor a 1.0401)	1	PBS	0.105
5 (Cor a 1.0401)	2	Carbonate	0.040
5 (Cor a 1.0401)	2	PBS	0.086
2.5 (Cor a 1.0401)	0.25	PBS	0.420
2.5 (Cor a 1.0401)	1	PBS	0.347
2.5 (Cor a 1.0401)	2	PBS	0.260
10 (Cor a 1.0401)	1	PBS	0.047
10 (Cor a 1.0401)	2	PBS	0.128
10 (Cor a 1.0401)	10	PBS	0.048
10, 20 (Cor a 9, 14)	1	PBS	1.336
10, 20 (Cor a 9, 14)	**10**	**PBS**	**2.843**

As shown in table 3, incubation of a hazelnut coated well plate with serum containing solely Cor a 1.0401 IgEs does not cause a color development and increase in OD. This could be caused by the poor presence of Cor a 1.0401 protein in the hazelnut extract. To draw conclusions about the extract, an SDS-PAGE gel has to be performed to analyze the whole extract. However, incubation with serum with Cor a 9 IgE level of 10 kU/L and Cor a 14 IgE level of 20 kU/L with 10 µg hazelnut coating gives an OD of 2.843, which is significantly above the aforementioned 1.5 threshold. All serum used in successive inhibition ELISAs will be diluted to yield these optimized Cor a 9 and Cor a 14 IgE levels, in combination with a coating of 10 µg hazelnut extract per well.

Since the PBS and carbonate coating buffers did not show a significant difference in the hazelnut coating optimization, PBS buffer will be used as coating buffer in all subsequent ELISAs, as this buffer is already in hand.

Table 4: results of the indirect ELISAs that led to the optimal conditions of walnut coating and serum dilution for the inhibition ELISAs.

Jug r 1 IgE after dilution (kU/L)	Walnut coating (µg)	OD (450 nm)
1	0.25	0.639
1	1	0.588
1	2	0.537
1.5	2	0.068
3	2	0.163
5	2	0.501
5	10	0.074
10	10	0.389
2.5	50	0.112
3	50	0.224
5	100	0.103
1	0.05	0.173
2	0.05	0.310
10	0.05	0.802
25	0.05	1.017
50	0.05	1.18
100	**0.05**	**1.598**

As shown in table 4, combinations of diluted serum with final concentration of 1 – 10 kU/L Jug r 1 IgEs with 0.25 – 10 µg walnut extract coating did give OD values between 0 and 0.6. As mentioned before, this is not high enough to draw well-founded conclusions from inhibition ELISAs, since the range from no inhibition to full inhibition is too small. An increase in coating amounts, in combination with multiple Jug r 1 concentrations in diluted serum, does not give higher OD values. On the other hand, a decrease to 50 nanogram (0.05 µg) walnut coating does cause an increase in OD after incubation with serum with increasing Jug r 1 concentrations. Our hypothesis of this issue will be discussed in the next section (*Inhibition ELISA)*.

For walnut, a new (commercially available) defatted extract should be tested in optimization ELISAs to assess whether the presence of fats is the problem and to increase the OD to similar values as the hazelnut indirect ELISA (±2.5). If this is not the case, and the current walnut extract still shows the best results, patients with high walnut IgE levels should be approached to get more serum. The optimized conditions of the hazelnut coating give useful OD results, thus these conditions can be used in the inhibition ELISAs.

Inhibition ELISA

As mentioned in the introduction, inhibition ELISA can be used to assess for cross-reactivity among allergens. Therefore, inhibition ELISAs with the optimized hazelnut coating were performed with serum of three patients from the study population. Serum was diluted to reach Cor a 9 levels near 10 kU/L and Cor a 14 levels near 20 kU/L, followed by pre-incubation with 0, 0.1, 1, 10, 100 and 1000 µg walnut extract (and hazelnut extract as positive control) prior to incubation on the coated well-plate. The results are shown in figure 3, all OD values are shown in Supplementary Information Table S1, S2 and S3.

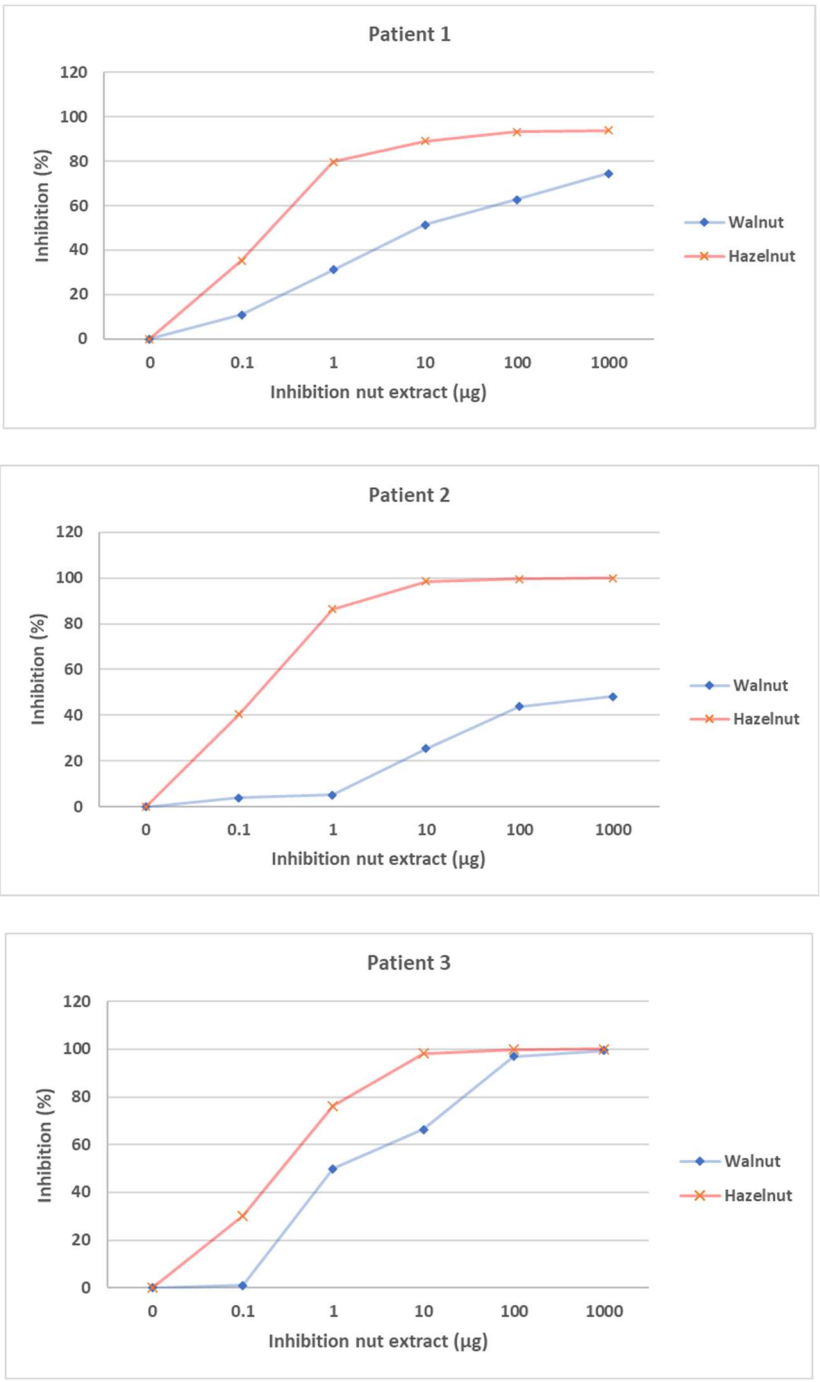

Figure 3: Dose-dependent inhibition of hazelnut-specific IgEs on ELISA using hazelnut and walnut extract. All wells were coated with 100 µL of 100 µg / mL hazelnut extract in PBS, pH 7.8. IgE inhibition in pre-diluted serum of patient 1, 2 and 3 was performed by pre-incubation of serum with 0; 0.1; 1; 10; 100 and 1000 µg of nut extract. All OD values are given in Supplementary Information Table S1, S2 and S3.

Figure 3 shows that hazelnut-specific IgE reactivity to the hazelnut extract could be inhibited in a dose-dependent manner when hazelnut and walnut extracts were pre-incubated with serum from patients 1, 2 and 3. Inhibition results from patient 1 and 3 do get close to 100% for both hazelnut and walnut inhibition, whereas walnut inhibition with serum from patient 2 only reaches 48%. This can be explained by the high contribution of PR-10 and LTP in cross-reactivity in patient 1 and 3. Both patient 1 and 3 have elevated IgE levels for PR-10 (Bet v 1 and Cor a 1.0401) and LTP (Cor a 8 and Jug r 3) proteins, which make a major contribution to the almost 100% inhibition of hazelnut-specific IgEs by walnut extract. In contrary, patient 2 does not have positive laboratory results for these PR-10 and LTP proteins, causing a significantly lower inhibition of hazelnut-specific IgEs by walnut extract, compared to the positive control inhibition with hazelnut extract. Therefore, the hazelnut and walnut sensitization in patient 2 is largely a primary sensitization to the major allergens (storage proteins) and is only for a small part caused by cross-reactivity with so far in this experiment not identified allergen components.

As shown in the hazelnut-coated inhibition results, the walnut extract does inhibit the hazelnut-specific IgEs. This indicates that there are walnut protein components present in the walnut extract. This suggests that the protein yield after ether extraction should not be the cause of the walnut coating issues, as mentioned in indirect ELISA optimization. Our hypothesis for this issue is that the walnut extract appears to be very fatty, even after ether extraction. Since fats cause problems in the coating process, an increase in coating concentrations gives a lower OD. In contrary, a well-diluted coating buffer with a final 50 nanogram coating does decrease the ratio of fat, enabling a better coating process. However, since this dilution also decreases the protein ratio, a very high concentration of Jug r 1 IgE (100 kU/L) is required (*Table 4*). A coating of 50 nanogram, combined with serum with 100 kU/L Jug r 1 IgEs does give an OD of 1.598, which is above the relevant, aforementioned 1.5 OD. The main issue with these optimized conditions is the low availability of patient's serum with such high walnut IgE levels. More indirect ELISA optimizations are needed to enable reliable and scientifically correct walnut-coated inhibition ELISAs with serum of patient 1, 2 and 3.

Ignoring these facts, to gain more information, a walnut-coated (2 µg) inhibition ELISA was performed with unoptimized coating conditions and serum from patient 1. The results are shown in figure 4.

Inh. (µg)	OD walnut inh.	OD hazelnut inh.
0	0.551	0.277
0.1	0.336	0.224
1	0.256	0.1265
10	0.234	0.116
100	0.141	0.1
1000	0.11	0.107

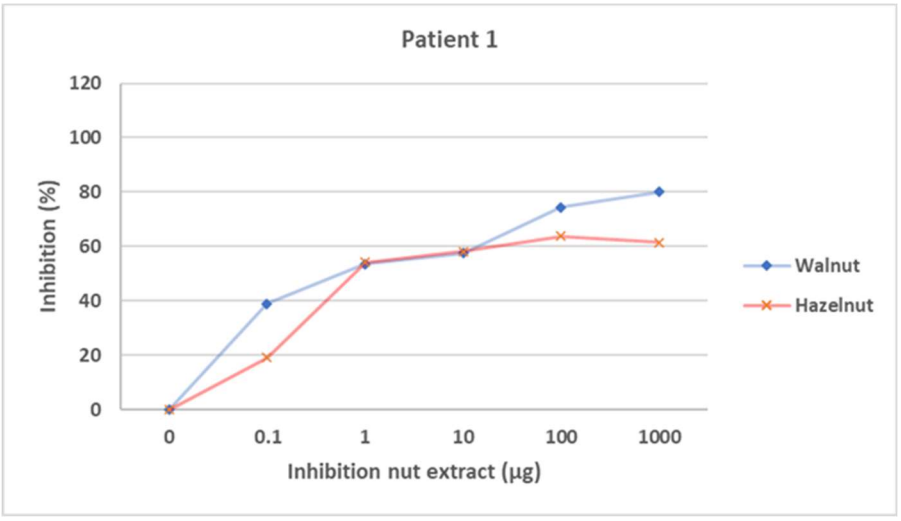

Figure 4: Dose-dependent inhibition of walnut-specific IgEs on ELISA using hazelnut and walnut extract. All wells were coated with 100 µL of 20 µg / mL walnut extract in PBS, pH 7.8. IgE inhibition in diluted serum of patient 1 (final Jug r 1 level of 5 kU/L) was performed by pre-incubation of serum with 0; 0.1; 1; 10; 100 and 1000 µg of nut extract. Inh. = Inhibition.

At first sight, these results do show a promising inhibition trend, similar to those shown in figure 2. However, the positive control inhibition should go towards 100% with increasing concentration of walnut inhibition (as shown in Figure 2). Besides that, the range from OD 0 – 0.277 and 0 - 0.551 is too small, causing a possible misrepresentation of the actual inhibition percentages due to being too close to each other and to zero. However, these results only reinforce the need for optimized walnut coating conditions, to demonstrate that this inhibition pattern is maintained at higher initial OD values.

Conclusion and Future Directions

This pilot study demonstrated the cross-reactivity among hazelnut and walnut in inhibition ELISA, and the possible clinically relevant link between hazelnut and walnut sensitization, symptoms and tree pollen sensitization. The results are in line with the previously mentioned study of Villalta *et al.* (2019) and Goetz *et al.* (2005), indicating clear cross-reactivity among hazelnut and walnut [7,34].

This study has some limitations. The retrospective study consisted of 29 patients, from which 15 had a clinically relevant Bet v 1-like sensitization. However, the serological study only consisted of three patients, which is a rather small sample size. The poor availability of suitable serum (e.g. sufficient sensitization and high enough sIgE levels) was the main problem for this small sample size. Another limitation was the walnut-coated (inhibition) ELISA. This (inhibition) ELISA should be optimized and performed with serum from the same patients, to see whether inhibition, and thus cross-reactivity, occurs on both sides. Finally, due to a lack of time, no immunoblot inhibition assays could be performed. These assays could demonstrate if serum inhibition with nut extract inhibits the binding of sIgEs to allergen proteins bound to a (nitrocellulose) membrane.

Nevertheless, this is as far as we could find in literature, one of the first studies performing inhibition ELISA with hazelnut and walnut, to demonstrate the potential cross-reactivity specifically among these two tree nuts. Although the sample (N=3) is rather small, all three cases showed clear dose-dependent inhibition of hazelnut-specific IgEs by walnut extract. The difference in inhibition can be easily explained by the remarkable difference in sensitization patterns between patients 1 and 3, in comparison with patient 2. Patient 2 has no birch pollen-related allergy, no hazelnut or walnut PR-10 and LTP sensitization and the extent of cross-reactivity is much lower than in the other patients. These results are an extension of and addition to the aforementioned study by Goetz *et al.* (2005), which was the only study that performed inhibition ELISA with tree nuts [7]. Their study demonstrated a dose-dependent inhibition of hazelnut-specific IgEs with walnut extract, leading to ±93% inhibition at the highest concentration of walnut extract (100 µg / mL), compared to 100% inhibition with hazelnut extract (positive control). This pilot study confirms these results and is one step closer towards mapping the cross-reactivity among hazelnut and walnut.

The retrospective study provides a small insight into the (global) problem and severity of tree nut and food allergies. Twenty-nine patients, with an average age below 18 years, had a wide range of sensitizations to multiple foods. Their hazelnut and walnut sensitization caused severe anaphylaxis in, as far as available in anamnesis in patient's database, at least fifteen (52%) patients. Twenty-two (76%) patients carry an adrenaline auto-injector, indicating the severity of these multi-food allergies. The young age at which these allergies develop and the fact that food allergies remain incurable, requires a diet strictly avoiding these allergens, which has a major, negative impact on the patient's Quality of Life.

The walnut-coated inhibition ELISA and inhibition immunoblots should be performed in the future to provide stronger evidence of cross-reactivity, ideally with serum of more and other patients from the study population. These assays should confirm and reinforce the inhibition ELISA results. Besides that, more assays should be performed to clarify the allergen components associated with the cross-reactivity. The important couples are PR 10 *Cor a 1* and *Jug r 5* (69% homology), profilins *Cor a* 2 and *Jug r* 7 (83% homology), 2S Albumins *Cor a 14* and *Jug r 1* (60% homology), 7S Globulins *Cor a 11* and *Jug r 6* (72% homology), 11S Globulins *Cor a 9* and *Jug r 4* (73% homology) and LTP *Cor a 8* and *Jug r 3* (60% homology) (table 1). For this, an ELISA well-plate should be coated with one component, while serum will be pre-incubated with the other component to see which of the protein pairs are the main contributors to this cross-reactivity.

As previously mentioned, hazelnut and walnut allergy are one of the most common tree nut allergies and often induce severe symptoms following food ingestion. Since these tree nut allergies often develop early in life, patients are already at risk of severe anaphylaxis at young age, since hazelnut and walnut are processed in many food sources, such as salads, sandwiches, snacks and desserts (hidden allergens). More attention should be paid to this group of hazelnut-walnut allergic patients. Allergic individuals might be sensitized to both nuts due to cross-reactivity and should therefore be tested for both allergies if they are sensitized to either hazelnut or walnut, and most importantly if they show clinically relevant symptoms following allergen ingestion. This information can be used to put together a more targeted and safer personal diet, which could significantly improve their Quality of Life and reduce the risk of severe or fatal allergic reactions of allergic individuals.

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This internship, together with my Master's literature thesis about peanut allergy immunotherapies, made me realize where my passion lies: the world of food allergies. After my Master's Drug Innovation, with two research internships, I am ready for something else. I am going to broaden my study knowledge for one year, and then I will definitely come back to the (research) world of food allergies. Science or industry, I hope to continue in this field and gain as much knowledge in the future as you have done.

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Supplementary Information

Table S1: Patient 1, OD values hazelnut-coated inhibition ELISA. The OD of a negative control ELISA measurement (only serum) has been subtracted from the OD values. All measurements were performed in duplo, the averages are shown.

Inhibition nut extract (µg)	***OD walnut inhibition***	***OD hazelnut inhibition***
0	*2.772*	*1.553*
0.1	*2.659*	*0.926*
1	*2.628*	*0.213*
10	*2.065*	*0.025*
100	*1.554*	*0.009*
1000	*1.436*	*0.001*

Table S2: Patient 2, OD values hazelnut-coated inhibition ELISA. The OD of a negative control ELISA measurement (only serum) has been subtracted from the OD values. All measurements were performed in duplo, the averages are shown.

Inhibition nut extract (µg)	***OD walnut inhibition***	***OD hazelnut inhibition***
0	*2.675*	*2.456*
0.1	*2.386*	*1.589*
1	*1.844*	*0.498*
10	*1.299*	*0.267*
100	*0.999*	*0.166*
1000	*0.684*	*0.150*

Table S3: Patient 3, OD values hazelnut-coated inhibition ELISA. The OD of a negative control ELISA measurement (only serum) has been subtracted from the OD values. All measurements were performed in duplo, the averages are shown.

Inhibition nut extract (µg)	***OD walnut inhibition***	***OD hazelnut inhibition***
0	*2.019*	*1.928*
0.1	*1.999*	*1.344*
1	*1.013*	*0.460*
10	*0.680*	*0.035*
100	*0.061*	*0.002*
1000	*0.013*	*0.001*