Clinical relevance of sensitization to peanut and lupine

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Klinische relevantie van sensibilisatie voor pinda en lupine
(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Abbreviations

BLAST	Basic Local Alignment Search Tool
CCD	Cross-reactive carbohydrate determinants
CPE	Crude Peanut Extract
DBPCFC	Double-blind placebo-controlled food challenge
ED	Eliciting dose
ELISA	Enzyme-Linked Immuno Sorbent Assay
IgE	Immunoglobulin E
kDa	Kilodalton
LC-MS/MS	Liquid chromatography-mass spectrometry-mass spectrometry
LTP	Lipid transfer protein
MALDI-TOF MS/MS	Matrix Assisted Laser Desorption /Ionization- Time of Flight Mass Spectrometry
NMR	^{1}H Nuclear Magnetic Resonance spectroscopy
NOAEL	No Observed Adverse Effect Level
n	Number
OAS	Oral Allergy Syndrome
PBS	Phosphate Buffered Saline
PCDA	Principal Component Discriminant Analysis
Ppm	Parts per million
SPT	Skin Prick Test

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General introduction

Food allergy: sensitization and clinical symptoms

Food allergy is an adverse immunologic reaction to food that may be due to either an immunoglobolin E (IgE)- or a non IgE-mediated immune mechanism.[1-3] To most physicians, the term food allergy is synonymous with reactions that involve IgE.
It is important to distinguish between sensitization and allergy. Sensitization represents the presence of allergen-specific IgE, but does not automatically mean clinically active disease (allergy).[2,4]
Food allergy is an important health problem within the general population and its prevalence appears to have increased.[5-7] About 2-4% of the adult population of Western countries surveyed suffer from food allergy.[8,9] The prevalence among children is generally accepted to be higher, about 5-8%.[10] Many foods are capable of causing allergic reactions, but traditionally eight foods were considered as the most frequent food allergens and account for 90% of food-allergic reactions.[11] These foods are cow's milk, with a prevalence in young children of 2.5%, egg (1.3%), peanuts (0.8%), wheat (approximately 0.4%), soy (approximately 0.4%), tree nuts (0.2%), fish (0.1%), and shellfish (0.1%).[12] Unlike allergy to foods such as milk, egg, soy and wheat, allergic reactions to peanut, tree nut, and sea food are rarely outgrown.[13] Adults are therefore more likely to have allergies to shellfish (2%), peanut (0.6%), tree nuts (0.5%) and fish (0.4%). In addition, allergies to fruits and vegetables are common (approximately 5%).[12]
Symptoms to food can vary from mild to severe and may even result in fatalities.[14,15] The most common food allergy-related manifestation is the "oral allergy syndrome" (OAS).[16] The term OAS is classically described as isolated oral symptoms caused by exposure of the oral and pharyngeal mucusa to heat- and pepsine-labile proteins in fresh fruits and vegetables that cross-react with airborne allergens.[17] Since these foods can also cause severe symptoms with or without oral symptoms,[18,19] the term pollen-food syndrome is considered to be more appropriate than OAS.[4,20] Food allergy can (simultaneously) affect many target organs like the skin and mucous membranes (urticaria, angio-edema, rhinitis, conjunctivitis), the gastro-intestinal tract (abdominal pain, diarrhoea, vomiting), the respiratory tract (asthma, hoarseness), and the cardiovascular system (anaphylactic shock).
The severity of a reaction depends on a variety of factors, including the type of allergen, the amount of ingested allergen, the rate of digestion and uptake of allergen in the gastro-intestinal tract, food processing, as well as on cofactors such as the health status of the individual (asthma), physical exercise, intake of alcohol or drugs such as ß-blocking agents or non-steroidal anti-inflammatory drugs.[21] Allergic reactions to peanut and tree nuts have accounted most frequently for fatal reactions, respectively in 63% and 31% of the fatalities to food.[14,15] More recently, it has been shown that

lupine, a member of the legume family like peanut, also has the potential to cause severe reactions.[22-24] Lupine is a plant cultivated around the world and its use for human consumption has been permitted since the 1990s. The seeds can be eaten as snacks, but more frequently lupine protein or flour, because of its high protein and fibre content, is used as an ingredient in various foods such as bread, pasta, cookies and sauces in order to improve their nutritional value.[25] Lupine should be considered in cases of unexplained allergic reactions to certain baked goods.

Peanut allergy: an important health problem

In Western countries, peanut allergy affects about 0.2-1.5% of children[26,27] and 0.4-0.6% of adults.[12,26] Symptoms to peanut can already occur at a dose of 100 μg peanut protein (1 mg peanut protein corresponds with 1/150 peanut).[28] Using a statistical model based on published data it was demonstrated that the doses eliciting a reaction of one in a million in susceptible patients were an order of magnitude lower for peanut flour compared with other foods.[29] Lupine was not included in this study, because no data on eliciting doses were available.
As the current advice to food-allergic consumers is to avoid the food to which they are allergic, information about allergenic ingredients provided to food-allergic consumers on food labels is very important. In response to this, a new EU-food labelling law came into effect in 2005 which provides allergic consumers more comprehensive information and allows them to identify eleven common allergenic ingredients (and sulphite): cereals containing gluten, crustaceans, eggs, fish, peanuts, soy, milk including lactose, nuts, celery, mustard, and sesame.[30] However, this regulation only applies to ingredients that have intentionally been introduced in a food. There is still the potential for cross-contamination during transport and storage as well as in the factories by shared equipment and final processing, e.g., chocolate confections with different ingredients, which may lead to potentially hazardous levels of allergens in the food resulting in allergic reactions.[31] Especially with respect to peanut, this may represent a high risk for peanut-allergic patients, because of the low dose that can elicit an allergic reaction and the potentially severe symptoms.

Diagnosis of food allergy

The gold standard for the diagnosis of food allergy is the double-blind placebo-controlled food challenge (DBPCFC).[32] The food under investigation is given to the patient via a masked vehicle to blind its taste and texture, interspersed with placebo doses. Assignation for active food (verum) or placebo doses must be randomized by

personnel not involved in the challenge to ensure proper blinding. An international protocol was drawn up to facilitate the use of DBPCFC in daily practice and to allow for comparison of results in various centers from different parts of the world.[33] However, DBPCFC are expensive and laborious, and severe anaphylactic reactions can occur. Therefore, the diagnostic approach always starts with a thorough medical and dietary history, supported by both *in vivo* skin tests and *in vitro* serological assays.

Skin prick testing (SPT) provides a rapid method to demonstrate sensitization to defined food allergens *in vivo*. The negative predictive accuracy of this test is generally greater than 95%.[34] However, the test has in general a relatively low specificity of about 50%. The positive predictive value is only 20%-50%,[35] depending on food extracts, which differ in their content of individual allergens, and vary between manufacturers and even between batches.[36] To improve the diagnostic value of SPT, predictive wheal diameters for a positive outcome of food challenges have been determined for cow's milk, peanut, egg, and wheat in children, but inconsistent results have been obtained in different studies, possibly as a result of the different age distributions of the patients studied.[37-39] Because cutaneous reactivity varies with age,[40] different cut-off levels are needed for different age populations. Another way to improve the reliability and accuracy of the diagnostic material for SPT is standardization of the test materials by using recombinant or purified food allergens.[41]

In vitro tests for specific IgE (e.g. radioallergosorbent test (RAST) and ImmunoCAP) are also very helpful in evaluating IgE-mediated food allergy. Cut-off levels of specific IgE to cow's milk, egg, peanut and fish have been defined to predict the likelihood that sensitized children are allergic. These levels differed between the allergens studied.[42-44] For wheat and soy, positive predictive values were low, which limits their use. This shows that decision points have to be studied for each food allergen separately. Moreover, the age and the patient population under investigation must be taken into account, since different cut-off levels were found for the same allergens in different age groups.[44]

So, to date no *in vivo* or *in vitro* tests exist which show full correlation with clinical symptoms. Therefore, DBPCFC still represent the most reliable way to establish food allergy.

Peanut and lupine allergens

Proteins can be clustered together into superfamilies and families based on their structural and functional properties.[45,46] Most plant food allergens belong to the cupin and prolamin superfamilies and to the protein families of the seed storage proteins.

Eight peanut allergens are officially listed on the IUIS Allergen Nomenclature website (www.allergen.org) in September 2006, designated Ara h 1-Ara h 8. In addition, two

other peanut allergens, peanut oleosin[47] and peanut agglutinin[48], have been identified but have not reached the status of "official" allergens. Ara h 1 is a 63.5 kDa glycoprotein which belongs to the vicilin family of seed storage proteins and appears as an oligomer in peanut.[49-51] Ara h 2 has been identified as a member of the conglutin seed storage proteins and is found in peanut as 2 isoforms of 17 and 20 kDa.[52,53] Ara h 3 was firstly identified as 14 kDa allergen with high-sequence similarities to glycinins.[54] Ara h 3 is synthesized as a precursor protein that is cleaved into an N-terminal acidic subunit of approximately 40 kDa and a C-terminal basic subunit of approximately 25 kDa.[55] The 14 kDa Ara h 3 represents the N-terminal part of the acidic subunit of peanut glycinin.[55] The individual subunits are held together by disulfide bonds, and non-covalent interactions support a hexameric organisation of Ara h 3. Ara h 4 is considered to be the same allergen as Ara h 3.[55,56] Ara h 5 belongs to the profilins, which are recognized as highly cross-reactive plant allergens.[56] Ara h 6 (15 kDa) and to a lesser extent Ara h 7 (15 kDa) show similarities to Ara h 2, all of which belong to the conglutin family.[57,58] Ara h 8 is a 17 kDa pathogenesis related (PR)-10 protein.[59] These proteins have been shown to be expressed constitutively in roots, old leaves and fruits of many plant foods and their expression will be further induced in response to stress stimuli.[46]

Among these allergens Ara h 1, Ara h 2 and also Ara h 3 are usually considered the major allergens since they are recognized by 70-90% of peanut-sensitized patients.[60-63] However, Ara h 6 shows homology to Ara h 2[57,58,64] and recently was shown to be recognized in peanut-allergic patients to a similar extent as Ara h 2,[57] suggesting that Ara h 6 might also be considered a major peanut allergen.

The relative occurrence of the major allergens Ara h 1, Ara h 2, and Ara h 6 in the various main peanut cultivars was about the same, approximately 13%, 7% and about 7%, respectively, and the amount of Ara h 3 was somewhat higher.[57,65]

Ara h 1 (vicilin), Ara h 3 (glycinin) and Ara h 8 (PR-10) showed significant homologies with lupine proteins, based on computer-aided amino-acid sequence comparison.[66] However, no lupine allergen has been characterized in detail until now.

Factors influencing the allergenicity

Different factors can affect the recognition of an allergen. Thermal processing can influence the outcome of food allergy by altering the features of food allergens. This mechanism depends on the structure and chemical properties of the allergens as well as on the methods of food preparation. The food matrix[67] and functional alterations of separate allergens after processing[68] may have an effect on the overall allergenicity of the food. For example, the per capita consumption of peanuts in China and the United States is about the same,[69] but there is virtually no peanut allergy in China.

This may be explained by the fact that the Chinese eat predominantly boiled or fried peanuts and the Americans dry-roasted ones.[70] It was shown that the Chinese method of cooking and eating peanuts reduces the allergenicity as compared with roasting.[69] The decrease in allergenicity of boiled peanuts resulted mainly from a transfer of allergens into the water during cooking.[71] Published reports on the effects of roasting on the allergenicity are contradictory. However, it seems likely that at least for certain allergens of peanut the allergenicity is increased upon heat treatment.[72]

Concerning oils, it is generally accepted that allergic reactions to oils are a result of the amount of residual proteins after processing. Production of oils involves pressing the oilseed, followed by a series of processes to refine the oil to the desired degree.[73] It has been demonstrated that oils processed at high temperatures usually do not contain detectable amounts of protein, in contrast to cold-pressed oils in which allergens can be found.[74] In addition, crude oils generally contain proteins, while refined oils have usually lost their allergenicity.[75]

Digestion is also an important variable that can influence sensitization and elicitation of allergic reactions to food. Resistance to digestion is a property common to many food allergens and is classically considered to be mandatory for the gastro-intestinal route of sensitization. Ara h 2 has been demonstrated to be relatively stable in simulated gastric fluid.[76] Recently, it has been shown that Ara h 1 is rapidly broken down by gastro-duodenal digestion, but that the low molecular weight peptides retain their allergenicity.[77] This may provide an explanation as to why peanut allergens are such potent food allergens. In contrast, IgE-binding to birch pollen-related food allergens is easily destroyed by gastro-intestinal digestion,[78] which can explain the frequent occurrence of only oral symptoms when ingesting raw fruit. In addition, ingestion of cooked birch pollen-related food can generally be consumed without difficulty because thermal processing causes conformational changes of the allergens, resulting in diminished IgE-binding capacity.[79]

Regarding lupine, heat treatment appears to reduce the IgE-reactivity of some proteins,[80,81] but the clinical relevance of this observation is not known. To date, there is no information about the digestion behavior of lupine allergens.

Cross-reactivity among legumes

Multiple allergies to different legumes are frequently observed. These allergies are often considered as cross-reactions but are seldom precisely confirmed as such. The term cross-reactivity is described as the induction of an allergic reaction as a result of an allergen binding to an IgE antibody that has been produced through sensitization to another homologous allergen.[82] Determination of IgE does not always reflect clinically relevant sensitization.[83] This is shown by the fact that wide cross-reactivity

between legumes was observed by RAST-inhibition,[82] but that only 5% of patients with a positive SPT to at least one legume reacted to more than one legume.[84] This is supported by a study in 32 proven peanut-allergic children, of whom 10 had a positive SPT to soy, but only one had clinical symptoms to soy.[85]
Clinically relevant cross-reactivity between peanut, soy, lupine and pea has been reported,[23,85-87] which may be explained by sequence identity among legume allergens.
Co-sensitization to peanut and tree nuts (especially almond, Brazil nut and hazelnut) is also a common observation in peanut-allergic patients[88,89] and can result in clinically relevant symptoms.[90]
Immunochemical characterization of allergens is needed to define the degree and nature of cross-reactivity.

Therapies

Once the diagnosis food allergy is established, the only therapy proven so far remains complete elimination of the offending allergen, which is often not possible because of hidden allergens. It is very important to educate patients and their caregivers about food avoidance and the identification of food allergens via labelling, early recognition of symptoms, and the management of allergic reactions. All patients with previous severe reactions to a food, especially asthmatic patients, should receive self-injectable epinephrine with thorough instructions on how to use it.
Over the past several years, many reports have been published about potential new therapies for peanut allergy. Subcutaeous immunotherapy with peanut extract has been attempted and increased tolerance was achieved, but there was an unacceptably high rate of serious adverse reactions.[91,92] Immunotherapeutic agents using modified IgE-binding epitopes of major peanut allergens to achieve a safe peanut preparation are under investigation.[93-95] A multicenter, randomized, double-blind, placebo-controlled clinical trial showed that monthly injections of humanized recombinant anti-IgE increased the threshold sensitivity to peanut without treatment-related adverse events.[96] However, this IgE treatment does not cure peanut allergy and life-long injections are required.

Outline of this thesis

There is an urgent need for new and improved diagnostic approaches, since currently used diagnostic tests have limited sensitivity and specificity, and because DBPCFC is a burden to both the patient and the doctor, and is expensive to carry out. Knowledge and characterization of the allergens responsible for clinically relevant allergies will improve diagnostic accuracy.
With regard to peanut sensitization, there is a debate on the possible role of peanut oil-containing skin products. Therefore, in *chapter 2* the allergen content in different crude and refined peanut oils and in a number of currently used peanut oil-containing products was investigated. In *chapter 3* the frequency of sensitization to lupine and its clinical relevance determined by DBPCFC was studied in peanut-sensitized patients. In addition, the degree of sensitization to soy and pea were investigated and their clinical relevance was determined by reported symptoms to these foods. Since little is known about risk factors for lupine allergy, predictive factors were investigated. In *chapter 4* we studied whether lupine-allergic patients with or without a concomitant peanut allergy did recognize different allergens, which may be of diagnostic importance. Furthermore, the eliciting dose (ED) of lupine was investigated in both groups. Major peanut allergens have been identified and in *chapter 5* we investigated whether sensitization to the individual allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6 was correlated to the clinical reactivity of peanut allergy by history and by ED. In *chapter 6* a completely new manner of diagnosing peanut allergy was studied by comparing NMR-resolved patterns of metabolites in plasma and saliva samples of peanut-allergic and peanut-tolerant patients.
All findings and their significance are discussed in *chapter 7*.

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2

Peanut allergy: Sensitization by peanut oil-containing local therapeutics seems unlikely

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Recent findings have renewed the debate on the allergenicity of peanut oils by describing an association between the use of skin preparations containing peanut oil and peanut allergy in children.[1] Peanut oil use is widespread in pharmaceutical products, such as vitamins and skin ointments, but also in more specialized products, such as bone cement for orthopedic surgery. Obligated to local pharmacopeias, only peanut oil that meet the specified requirements (e.g., refining) is used in pharmaceuticals. Refined food oils could also contain a certain amount of unrefined peanut oil to improve taste and flavor.

We investigated the allergenicity of crude peanut oils, peanut oils at different steps of the refining process, refined peanut oils from supermarkets, pharmaceutical peanut oils and a number of current products containing peanut oil, like vitamins, by means of ELISA and immunoblotting (Table 1 and Fig 1). These methods are supplementary to each other.[2]

Crude oils contained significant amounts of protein, whereas in refined oils and products thereof, no protein was detectable (<0.3 ng/mL). The ELISA results were confirmed by measuring nitrogen as a marker for protein (data not shown). Because hydrophobic peanut proteins, such as oleosins,[3] might be extracted inefficiently during sample preparation for ELISA, we investigated representative peanut oil samples directly by using immunoblot technology with sera of patients with peanut allergy. It is clear from Fig 1 that crude peanut oil contains immunoreactive protein bands at molecular weights typical for peanut, whereas refined oils do not show any reactivity. The intensity of the protein bands of extracted peanut oil (Fig 1, lane 2) is an order of magnitude higher compared with that of the direct measurement (Fig 1, lane 1). This is in full agreement with the oil/buffer ratio of 10:1 applied during extraction. Therefore, it is unlikely that significant amounts of protein remain in the oil phase after the extraction.

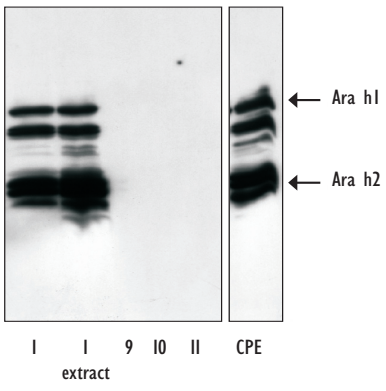

Figure 1
Immunoblot of pharmaceutical oils, with crude oil used as a control. Numbers correspond to those of Table 1. In the second lane an extract of number 1 (crude oil) was used as a control for the extraction process.
Arrows indicate the major peanut allergens Ara h1 (63 kD) and Ara h2 (doublet at 17-20 kD). CPE, crude peanut extract.

Table 1
Peanut protein concentration in peanut oil containing products

Product class	Description	Sample number	Protein (ng/mL) detected with ELISA
Crude Oils	Crude oil *	1	6470
	Crude oil *	2	90
	Cold pressed oil * #	3	2550
Samples from the refining process	Neutralized groundnut oil *	4	<0.3
	Bleached groundnut oil *	5	<0.3
	Deodorized groundnut oil *	6	<0.3
Refined food oils	Arachid oil	7	<0.3
	Groundnut oil	8	<0.3
Pharmaceutical ingredients	Refined arachid oil	9	<0.3
	Arachid oil	10	<0.3
	Arachid oil	11	<0.3
Pharmaceutical products	Vitamin K (1)	12	<0.3
	Vitamin K (2)	13	<0.3
	Vitamin K (3)	14	<0.3
	Vitamin K (4)	15	<0.3
	Bone cement	16	<0.3

* Not commercially available. Only supplied for this study.
# Prepared at our own pilot facilities for oil processing.

Lack et al[1] posited that the cutaneous route of exposure to the residual proteins of peanut oil may be a plausible determinant of allergic sensitization. Our data show that in refined peanut oils the protein levels are less than 0.3 ng/mL. Hsieh et al[4] reported that allergen exposure through the skin in BALB/c mice could serve as a sensitization pathway for food allergy. An amount of 100 μg of ovalbumin applied to a 1 by 1 cm patch for one week was needed to require a positive challenge. The concentration used (10 mg/mL) is more than a million-fold higher than the detection limit of our ELISA in this experiment.

We recognize the limitations of the animal model used and the fact that peanut is a more potent allergen compared with ovalbumin. Nevertheless, we believe that our results cast doubt on the proposition that pharmaceutical products containing refined peanut oil play a role in sensitization to peanut.

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3

Clinical relevance of sensitization to lupine in peanut-sensitized adults

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Submitted

Abstract

Background
The use of lupine in food has been increased during the last decade and allergic reactions to lupine have been reported, especially in peanut-allergic patients. The frequency and the degree of cross-reactivity to other legumes is unknown.

Objective
To investigate the frequency of sensitization to lupine, and in addition to pea and soy, and its clinical relevance, in peanut-sensitized patients. Furthermore, to determine the eliciting dose (ED) for lupine using double-blind placebo-controlled food challenges (DBPCFC).

Methods
Thirty-nine unselected peanut-sensitized patients were evaluated by skin prick tests (SPT) and ImmunoCAP to lupine, pea, and soy. Clinical reactivity was measured by DBPCFC for lupine, and by history for pea and soy.

Results
Eighty-two percent of the study population was sensitized to lupine, 55% to pea, and 87% to soy. Clinically relevant sensitization to lupine, pea, or soy occurred in respectively, 35%, 29% and 33%. None of the patients was aware of the use of lupine in food. The lowest ED for lupine inducing mild subjective symptoms was 0.5 mg. The No Observed Adverse Effect Level (NOAEL) was 0.1 mg lupine flour for subjective symptoms. No predictive factors for lupine allergy were found.

Conclusion
In peanut-sensitized patients, clinically relevant sensitization to either lupine or to pea or soy, occurs frequently. The minimal eliciting dose for lupine was 0.5 mg.

Clinical implications
Lupine allergy is present in about a third of peanut-sensitized patients, but patients are unaware of its use in food.

Introduction

Lupine (*Lupinus albus*), a member of the legume family, has been recognized as a novel food since 1996. The bran and flour of this legume have been supplied to food manufacturing, where it contributes to fiber and protein content, and some textural properties, particularly in bakery products.[1] Lupine can be cultivated in all climates, making it an attractive crop.

The addition of lupine flour to foods was first permitted in countries like France and the UK.[2] The occurrence of lupine in foods has increased notably in many European countries during the last decade. Reasons for this development are the import of bakery products from France and the use of lupine as replacement for potentially genetically modified soy. The first published report of an allergic reaction to lupine appeared in 1994,[1] followed by 12 reports until 2006 based on a Pubmed search. Since thermal processing appears to have no effect on the allergenicity of lupine,[3-5] and lupine is not on the EU list of 12 commonly allergenic foods that must statutorily be labelled if present in packaged foods,[6] the presence of lupine in processed food represents a potential risk for allergic consumers.

Lupine allergy may arise by cross-reactivity in people who are already allergic to another member of the legume family, in particular peanut,[1,2,4,7] or by primary sensitization.[8-10]

Serological cross-reactivity between members of the legume family occurs frequently, but is not always reflected by clinically relevant allergies.[11-13] In 1994, Hefle et al investigated cross-reactivity between peanut and lupine. They showed that 5 of 7 (71%) peanut-allergic patients had a positive skin prick test (SPT) to lupine.[1] The clinical relevance of this sensitization was not investigated. Moneret-Vautrin et al showed that 44% of 24 peanut-allergic patients revealed a positive SPT response to both peanut and lupine, and 5 of 6 patients tested reacted to lupine determined by double-blind placebo-controlled food challenge (DBPCFC).[7] This suggests that the use of lupine is a risk especially for peanut-allergic patients.

In this study, we investigated the frequency of sensitization to lupine and its clinical relevance, and also to pea and soy, in peanut-sensitized patients. Furthermore, we determined the eliciting dose (ED) for lupine by DBPCFC.

Materials and methods

Patients

Ninety-two adult peanut-sensitized patients with or without symptoms to peanut, who visited the outpatient clinic of the Department of Dermatology/Allergology of the University Medical Center Utrecht between 2003 and 2006, were approached to participate via an invitation letter. The selection criteria were a positive SPT to peanut extract (ALK-Abelló, Nieuwegein, The Netherlands) with the area of the peanut wheal at least half of the area of the positive control (SPT ≥2+), and/or specific IgE to peanut ≥0.7 kU/L (ImmunoCAP, Phadia, Uppsala, Sweden).

Pregnancy, significant concurrent disease, unstable asthma and oral medication with corticosteroids or ß-blocking agents were exclusion criteria.

Thirty-nine patients (42%) met these criteria, agreed to participate, and gave written informed consent before enrolment in the study. Detailed histories of allergies to peanut, green pea and soy, and atopy were obtained by using a standardized questionnaire.

Sixteen patients were unwilling to participate because of lack of interest (n=10) or inability to discontinue antihistamines (n=6). Thirty-seven patients (40%) did not respond.

This study was reviewed and approved by the Medical Ethical Committee of the University Medical Center Utrecht.

Skin prick tests and specific IgE measurements

All 39 patients included in the study were subsequently evaluated by SPT with commercial extracts of peanut, green pea, soy, grass pollen and birch pollen (ALK-Abelló, Nieuwegein, The Netherlands) and with lupine extract prepared in the following way: Lupine flour (20 g) was suspended in 200 mL phosphate buffered saline (PBS, pH 7.4) containing 0.1% phenol, and was stirred overnight at 4 °C. After clarifying the suspension by centrifugation (3000 rpm, 30 minutes) and filtration, the supernatant solution was mixed with an equal volume of glycerol. The resulting solution in PBS-glycerol (50% v/v) was subsequently sterilized by filtration (0.22 μm pore size). The protein content of this extract was determined to be 10.2 mg/mL using the Bradford method.[14]

Histamine dihydrochloride (10 mg/mL) and the glycerol diluent of the SPT extracts served as positive and negative controls, respectively (ALK-Abelló, Nieuwegein, The Netherlands). The SPT were performed and recorded as described by Dreborg.[15] SPT responses were expressed as the ratio of the wheal reaction in millimetres squared, evaluated by computer scanning[16] divided by the wheal reaction of the positive control.[17] SPT ratios were regarded positive when the ratios were ≥0.25, i.e. when the

wheal areas were at least 25% of the wheals induced by the positive control. Since one patient had dermographism, the SPT results of this patient were not included in the analysis of SPT data.
Specific IgE levels to peanut, lupine, green pea, soy, grass pollen and birch pollen, were determined in all patients by the ImmunoCAP (Phadia, Uppsala, Sweden). An IgE level of 0.35 kU/L was taken as a positive result.

Clincal reactivity

Clinical reactivity to lupine was investigated by DBPCFC in 30 of the 39 peanut-sensitized patients according to the threshold consensus protocol[18] with some modifications.[19] Nine patients were unwilling to participate in this part of the study because of lack of time (n=4), anxiety (n=2) or lack of interest (n=3). The lupine flour used was a commercially available mild white lupine flour (protein content 36.2% by Leco 2000 method, *Lupinus albus*), obtained from Magenta Sales in England produced by CANA (Martigne-Ferchaud, France). The amounts of lupine flour were 0.01 mg, 0.1 mg, 0.5 mg, 1 mg, 10 mg, 100 mg, 300 mg, 1 g and 3 g. To mask the lupine doses, mashed potatoes were added. The hospital pharmacy prepared the challenge materials. Four similarly prepared placebo doses were randomly interspersed between the increasing lupine doses. The challenge was discontinued when objective symptoms occurred or when subjective symptoms lasted for more than 45 minutes. The ED was determined as the lowest dose eliciting a convincing subjective allergic reaction.
All of the challenges were conducted in a hospital setting, with careful monitoring of the patients. Full emergency treatment was readily available.

Statistics

Correlations were analyzed with the nonparametric Spearman's rank test. Differences in proportions between groups were tested by 2-sided Fisher exact test.
Calculations were performed using SPSS (version 12, SPSS Inc., 2001, Chicago, USA). P values <0.05 were considered statistically significant.

Results

Patient characteristics

Thirty-nine patients with sensitization to peanut entered the study. The mean age was 33 years (range, 17-57). Nineteen patients suffered from atopic eczema (49%), 17 from concomitant asthma (44%), and 36 patients reported pollinosis symptoms (92%). Sensitization to grass pollen was present in 79% of the study population and to birch pollen in 85%.
SPT responses to peanut were positive in 87% of the patients (n=33), 92% had a

positive CAP (n=36), and 79% (n=30) had both a positive SPT and CAP.
Levels of IgE to peanut ranged from <0.35 kU/L to >100 kU/L. SPT responses to peanut ranged from a ratio of 0 to 18.4 compared to the positive control.
Twenty-nine of 39 patients (74%) reported symptoms to peanut by history. Three of these patients were previously challenged with peanut, which confirmed the diagnosis of peanut allergy.

Co-sensitization to lupine, pea and soy

To further characterize our study population, the extent of co-sensitization to different legumes was investigated. The majority (82%) of peanut-sensitized patients was also sensitized to lupine as shown by CAP and/or SPT, whereas 55% was sensitized to pea, and 87% to soy.
The patients were further divided into 2 groups, having symptoms to peanut or not, to determine if clinically relevant peanut sensitization could have an effect on the extent of co-sensitization to other legumes. Sensitization to legumes as measured by CAP and SPT in the whole peanut-sensitized group and in the two sub-groups is summarized in Table 1. In general, the sensitization grade to lupine, green pea and soy was higher as measured by SPT than by CAP. The sensitization grade to lupine, pea and soy as measured by CAP, and to lupine and pea as determined by SPT tended to be higher in patients with symptoms to peanut compared to patients without symptoms, but this difference was not statistically significant.
The overlap of sensitization to lupine, pea and soy with sensitization to peanut is demonstrated in Table 2. About half of peanut-sensitized patients (53%) was sensitized to all three legumes, whereas only two patients (5%) were mono-sensitized to peanut.
Since cross-reactivity is certainly a prominent feature of the IgE response, we studied if the level of IgE to peanut was correlated with the level of IgE to the other legumes. A significant correlation was observed between the IgE level to peanut and the IgE level to respectively lupine (correlation coefficient (r)=0.6, $p<0.01$), pea ($r=0.42$, $p<0.01$) and soy ($r=0.69$, $p<0.01$). There was also a significant correlation between the SPT reactivity to peanut and lupine ($r=0.36$, $p=0.03$), whereas the correlations between peanut and respectively pea ($r=0.31$, $p=0.05$) and soy ($r=0.3$, $p=0.06$) were almost significant.

Clinical reactivity to lupine and eliciting doses determined by DBPCFC

None of the patients reported symptoms to lupine by history, because they were unaware that lupine was used as food ingredient and can cause allergic symptoms. In 30 patients, clinical reactivity to lupine was assessed by DBPCFC, and nine of them (30%) had a positive DBPCFC. The first symptom reported during all but one of the challenges was itching in the oral cavity (oral symptoms, OS), which recurred after

subsequent higher doses (Table 3). Four patients additionally developed more serious, but still subjective, gastro-intestinal symptoms (nausea and abdominal pain). In two patients, this resulted in discontinuation of the challenge because it lasted for more than 45 minutes. Dyspnea without an objective FEV1 decrease was found in three patients. In one of them, this was the first symptom reported.
All patients tolerated a dose of 0.1 mg lupine flour with neither subjective nor objective symptoms, so the No Observed Adverse Effect Level (NOAEL) for our patient group in this study was 0.1 mg lupine flour based upon elicitation of subjective reactions. The minimal ED for subjective symptoms for the individual patients in this study varied from 0.5-3000 mg (Table 3), inducing mild to moderate symptoms. The minimal eliciting dose based on objective symptoms was 1000 mg and consisted of rhinitis (Table 3). Three patients failed to develop objective symptoms or subjective symptoms more serious than OS even at the highest administered dose of 3000 mg (Table 3).

Table 1
Sensitization to legumes in peanut-sensitized patients with and without symptoms to peanut (number of patients (%)).

	Peanut		Lupine		Green pea		Soy	
	CAP	SPT	CAP	SPT	CAP	SPT	CAP	SPT
Peanut-sensitized patients (n=39)	36/39 (92%)	33/38* (87%)	21/39 (54%)	26/37*° (70%)	14/39 (36%)	18/38* (47%)	18/39 (46%)	26/38* (68%)
Peanut-allergic patients (n=29)	27/29 (93%)	25/29 (86%)	18/29 (62%)	21/28° (75%)	11/29 (38%)	14/29 (48%)	15/29 (52%)	19/29 (66%)
Non-peanut-allergic patients (n=10)	9/10 (90%)	8/9* (89%)	3/10 (30%)	5/9* (56%)	3/10 (30%)	4/9* (44%)	3/10 (30%)	7/9* (78%)

Positive IgE: Specific IgE ≥0.35 kU/L determined by the ImmunoCAP, Phadia, Uppsala, Sweden.
Positive SPT response: ratio ≥0.25
* The SPT response of one peanut-sensitized patient without symptoms was not included, because of dermographism.
° One peanut-sensitized patient with symptoms was not tested by SPT with lupine.

Table 2
Number of peanut-sensitized patients with sensitization to other legumes.

Lupine	Pea	Soy	Number (percentage)
-	-	-	2 (5%)
+	-	-	3 (8%)
+	+	-	1 (3%)
+	-	+	7 (18%)
+	+	+	20 (53%)
-	-	+	5 (13%)
-	+	-	0 (0%)
-	+	+	0 (0%)

+ : sensitization present, -: no sensitization.
Sensitization: SPT ≥ 0.25 and/or specific IgE ≥ 0.35 kU/L

Table 3
Clinical reactivity to lupine flour during DBPCFC (n=9*)

Patient	DBPCFC Dose (mg)									ED(mg flour)
	0.01	0.1	0.5	1	10	100	300	1000	3000	
L02KP	-	-	-	-	OS	OS	OS	OS, r	-	10 mg
L11KP	-	-	-	-	-	-	-	-	OS	3000 mg
L18KP	-	-	-	-	-	-	-	OS	OS, n	1000 mg
L19KP	-	-	-	-	-	OS	OS	OS	OS	100 mg
L20KP	-	-	-	-	-	-	-	OS	OS	1000 mg
L34KP	-	-	-	-	d	d	d, n	d	n.t.	10 mg
L36KP	-	-	OS	OS	OS	OS, d > 1h	n.t.	n.t.	n.t.	0.5 mg
L37KP	-	-	-	OS, ap	OS, d, ap > 1h	n.t.	n.t.	n.t.	n.t.	1 mg
L39KP	-	-	-	OS	OS, n > 45 min	n.t.	n.t.	n.t.	n.t.	1 mg

*Nine of 30 patients had a positive DBPCFC with lupine.
ED: Eliciting dose, OS: Oral symptoms, r: rhinitis, n: nausea, d: dyspnea, ap: abdominal pain
n.t: not tested, -: no symptoms

Sensitization and clinical symptoms to lupine in relation to peanut characteristics

Since sensitization to different legumes in our peanut-sensitized patients frequently occurred, we evaluated the clinical relevance of these sensitizations separately (Table 4A).

Twenty-three of 30 (77%) lupine-challenged patients had a combined sensitization to peanut and lupine, in line with the co-sensitization frequency in the whole study population, demonstrating that lupine sensitization frequently occurs in peanut-sensitized patients. In 8 of these 23 lupine-sensitized patients (35%), the challenge with lupine flour was positive, illustrating that in 35% of peanut-sensitized patients with a combined lupine sensitization, ingestion of lupine could lead to clinical symptoms.

Twenty-one patients did not respond during the challenge, of which fifteen (71%) were sensitized to lupine, demonstrating a low specificity of determining clinically relevant allergy based on CAP and/or SPT.

Eight of nine lupine-allergic patients (89%) were lupine-sensitized, showing a high sensitivity. Only one of nine lupine-allergic patients was not sensitized to lupine as measured by SPT and/or CAP (Table 4A).

To study if symptoms to peanut had an effect on the frequency of symptoms to lupine, peanut-sensitized patients with and without symptoms were compared. The frequency of symptoms to lupine was not significantly higher in peanut-allergic patients (8/21) than in patients without symptoms to peanut (1/9) (p=0.13) (Table 4B).

Sensitization and clinical symptoms to pea and soy in relation to peanut characteristics

Twenty-one of 38 peanut-sensitized patients (55%) were also sensitized to pea. Six of these pea-sensitized patients (29%) had a positive history to pea, demonstrating that about 1 out of 3 patients with sensitization to pea had clinical symptoms (Table 4A). Regarding soy, 33 of 38 peanut-sensitized patients (87%) were also sensitized to soy. Eleven of these 33 patients (33%) reported symptoms to soy, whereas 3 of five non soy-sensitized patients (60%) also had a positive history to soy (Table 4A).

Together, these data demonstrate that in peanut-sensitized patients with sensitization to pea or soy, this sensitization will lead to clinical symptoms in about 30%, comparable to lupine.

Having symptoms to peanuts or not had no effect on the frequency of symptoms to pea and soy (p=0.35 and p=0.21, respectively) (Table 4B).

Table 4

A) Sensitization in relation to clinical symptoms to lupine, pea and soy in peanut-sensitized patients (number of patients). B) Clinical symptoms to lupine, pea and soy in relation to symptoms to peanut (number of patients).

A

	Sensitization to lupine		Sensitization to pea		Sensitization to soy	
	Yes	No	Yes	No	Yes	No
Symptoms*	8	1	6	1	11	3
No symptoms	15	6	15	16	22	2

Sensitization: SPT ≥ 0.25 and/or specific IgE ≥ 0.35 kU/L
* Symptoms to lupine were determined by DBPCFC, and to pea and soy by history

B

		Peanut-allergic	Non-peanut-allergic
Symptoms* to lupine	Yes	8	1
	No	13	8
Symptoms* to pea	Yes	6	1
	No	24	8
Symptoms* to soy	Yes	12	2
	No	18	7

* Symptoms to lupine were determined by DBPCFC, and to pea and soy by history

Predictive factors for lupine-allergy

Since an increase in the occurrence of allergic reactions to lupine has been suggested, and because many food-allergic patients are not aware of the use of lupine in food, it is relevant to estimate risk factors which are associated with lupine allergy. Therefore, our lupine-allergic peanut-sensitized patients were compared to non-lupine-allergic peanut-sensitized patients (Table 5). The distribution of sensitization to lupine, pea and soy was similar. In addition, there was no significant difference in the frequency of symptoms to peanut, pea and soy in lupine-allergic patients compared to non-lupine-allergic patients.

Independent predictive values of the IgE level and SPT response to lupine for lupine allergy could not be found by logistic regression analyses (data not shown), demonstrating that the sensitization level to lupine is not useful for suspecting an allergy to lupine within a peanut-sensitized population.

Table 5
Differences between peanut-sensitized patients with and without lupine allergy.

	Lupine-allergic (n=9)	Lupine tolerant (n=21)	p-value*
Lupine sensitization	8/9 (89%)	15/20 (75%)	p=0.29
Median CAP lupine	0.88 (0.50-2.85)	0 (0-0.76)	p=0.18
Median SPT lupine	0.58 (0.24-0.67)	0.38 (0.21-0.72)	p=0.31
Sensitization pea	6/9 (67%)	10/20 (50%)	p=0.23
Sensitization soy	7/9 (78%)	19/20 (95%)	p=0.20
Symptoms to peanut	8/9 (89%)	13/21 (62%)	p=0.13
Symptoms to pea	2/9 (22%)	4/21 (19%)	p=0.36
Symptoms to soy	4/9 (44%)	5/21 (24%)	p=0.18

*Fisher exact test

Discussion

Allergy to legumes may be mediated by primary sensitization via ingestion[8,9,20] or inhalation[8,21,22] or may be acquired after primary sensitization to another legume.[23,24] Although the mechanism of sensitization and the frequency of allergic reactions to lupine are unknown, most reactions have been reported in peanut-allergic patients.[1,2,4,7]

In this study, the patterns and frequencies of sensitization to lupine, pea and soy were analyzed in an unselected peanut-sensitized population. Moreover, clinical symptoms to these legumes were evaluated. We chose to focus not only on peanut-allergic patients but to include all peanut-sensitized patients, because peanut-sensitized patients without symptoms to peanut could have symptoms to other legumes as well. This is illustrated by the fact that one peanut-sensitized patient without symptoms to peanut did have symptoms to lupine during the DBPCFC.

In our population, co-sensitization to lupine (82%), pea (55%) and soy (87%) frequently occurred, in line with previous studies.[7,11,12] The majority of our patients (53%) was sensitized to all three legumes tested. It is not known whether this reflects co-sensitization towards distinct allergens or cross-reactivity. Since the level of peanut-specific IgE was significantly correlated to the levels of lupine-, pea- and soy-specific IgE, similar IgE-binding properties of these allergens (cross-reactivity) could be suggested. However, IgE-inhibition experiments are needed to investigate this, and to identify allergens within lupine responsible for cross-reactivity.

Clinically relevant co-sensitization to either lupine or to pea or to soy was present in one-third of the study population, making it a more common feature than previously reported.[13,25] However, those previous studies were performed in children. It might be that clinically relevant cross-reactivity grades are higher in adults compared to children due to dietary habits. The percentages of reactivity to pea and soy are probably somewhat overestimated. Clinical symptoms to lupine were confirmed by DBPCFC, but for pea and soy were only evaluated by history. Since soy is usually consumed as an ingredient and not as a single food, it is more difficult to attribute symptoms to this allergen in contrast to pea which is usually eaten as such. When peanut-sensitized patients with or without symptoms were analyzed separately, a higher extent of sensitization to lupine was observed in peanut-allergic patients, although this difference did not reach significance. Moreover, in peanut-allergic patients, the frequency of clinically relevant lupine sensitization was not significantly higher compared to non-peanut-allergic patients. This illustrates that peanut-sensitized patients without symptoms to peanut have similarly high risks for being lupine-allergic as peanut-allergic patients.

There is little information in the literature on the lowest dose that causes allergic reactions to lupine.[7] Our low-dose challenge data show that lupine-allergic patients start to react with mild subjective symptoms at doses from 0.5 mg lupine flour, with

objective symptoms beginning at higher doses including 3 patients who experienced only subjective responses even at the highest dose of 3000 mg. The lowest (cumulative) ED described previously was 265 mg lupine flour, inducing abdominal pain and asthma in peanut-allergic patients.[7] For peanut, we recently established the lowest dose that induced subjective symptoms using the same challenge protocol.[10] The ED for subjective symptoms started from 0.1 mg peanut flour, and for objective symptoms from 10 mg, in line with previous reports.[26,27] Comparing the results of both studies, it appeared that the ED for lupine is only 5-fold higher than for peanut. The symptom progression of peanut and lupine during the DBPCFC procedure with higher doses was similar, indicating that the allergenicity of lupine might be more similar to peanut that e.g. to soy, which usually induces mild symptoms. Further studies are needed in a larger group of lupine-allergic patients to confirm the ED findings in this study.[28]

Considering the potential severity of lupine allergy and the likelihood that reactions will occur in unsuspecting peanut-allergic consumers, factors were investigated to predict which patients were most likely to have lupine allergy. In our peanut-sensitized study population, no predictive factors could be identified by information that is commonly gathered in routine practice.

Food-allergic individuals are typically advised to employ specific avoidance diets to prevent reactions.[29] The success of avoidance diets implies that food-allergic patients would be aware of their allergy and the use of that ingredient in foods. Currently, the EU requires the declaration of commonly allergenic foods and ingredients derived from those foods on labels of packaged foods.[6] Peanut is included on the existing list of commonly allergenic foods in the EU. While the prevalence of lupine allergy is unknown, our results from this small study population suggest that one-third of peanut-allergic individuals could also be allergic to lupine. Since the prevalence of peanut allergy is approximately 0.5% of the overall population,[30] the prevalence of lupine allergy could be as high as 0.15%. This prevalence estimate is at least as high as that for several foods, namely mustard, sesame seed, and celery that are currently on the EU list.[31,32] Furthermore, lupine-allergic patients reacted at doses as low as 0.5 mg lupine flour so modest exposures could provoke reactions. While adding lupine to the EU list is arguably justified, mandating the declaration of lupine on food labels will be of limited benefit to individuals unaware of their lupine allergy. Our results demonstrate that peanut-sensitized individuals are neither aware of their potential lupine allergy nor the use of lupine in foods, which makes the recourse to this dilemma difficult. Clinically relevant sensitization to lupine occurs with a reasonably high frequency in the peanut-sensitized population. Moreover, we were unable to identify any diagnostic criteria other than use of lupine challenges to identify the fraction lupine-allergic patients.

Thus, education is important to build awareness because labeling strategies alone are unlikely to be sufficient.

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4

Lupine Allergy: not simply cross-reactivity with peanut or soy

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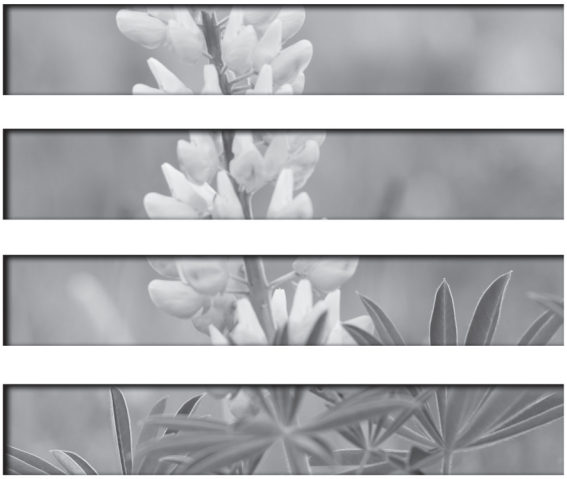

Abstract

Background

Reports of lupine allergy are increasing as the use in food products increases. Lupine allergy may be the consequence of cross-reactivity after sensitization by peanut or other legumes or *de novo* sensitization. Lupine allergens have not been completely characterized.

Objective

To identify allergens associated with lupine allergy, evaluate potential cross-reactivity with peanut and to determine eliciting doses for lupine allergy using double-blind placebo-controlled food challenges (DBPCFC).

Methods

Six patients with a history of allergic reactions to lupine flour were evaluated by skin prick tests (SPT), CAP and DBPCFC. Three of those were also allergic to peanut. Lupine allergens were characterized by IgE-immunoblotting and peptide sequencing.

Results

In all six patients the eliciting dose for lupine flour was 3 mg or less for subjective symptoms and 300 mg or more for objective symptoms. The low eliciting dose and moderate to severe historical symptoms indicates significant allergenicity of lupine flour. Two patients allergic to lupine but not peanut flour displayed IgE binding predominantly to approximately 66 kD proteins and weak binding to 14 and 24 kD proteins, whereas peanut-allergic patients with lupine allergy showed weak binding to lupine proteins of about 14 to 21 or 66 kD. Inhibition of binding was primarily species specific.

Conclusion

Lupine allergy can occur either separately or together with peanut allergy as demonstrated by three patients who are co-sensitized to peanut and lupine.

Clinical implications

Lupine flour is allergenic and potentially cross-reactive with peanut allergens, thus poses some risk if used as a replacement for soy flour.

Introduction

Lupine (*Lupinus albus*), like peanut and soy, is a member of the legume family, the second largest family of seed plants.[1] Lupine is cultivated all over the world, primarily for use as animal feed, but also to be plowed under as a soil enhancer.[1] Inclusion of lupine in wheat flour was first permitted in the UK in 1996 and in France at the end of 1997. Lupine flour has been introduced into food manufacturing, where it contributes protein content, fiber, and some textural properties. It has become increasingly important as a replacement for soy in many foods over the last decade, since the latter is often genetically modified and some food companies have sought alternative sources of food ingredients with properties similar to soy flour. Lupine flour was first suggested as a supplement for bread[2] and cookies[3] more than 20 years ago; it can be used in many food products, particularly those that are baked.

Since legumes, and in particular peanut and to a lesser extent soybean, are well known as allergens, it is not surprising that allergy to lupine has been reported. The first report was in 1994 and involved a girl with a known peanut allergy who experienced urticaria and angioedema after ingesting a spaghetti-like pasta fortified with sweet lupine seed flour.[1] Lupine allergy has been mainly reported in patients with allergies to other legumes, particularly peanut.[4-6] Sensitization may occur via the oral route but also via inhalation.[7-9] Isolated cases of lupine allergy have rarely been described.[9,10]

Serological cross-reactivity between different members of the legume family and lupine frequently occurs.[11-13] However, clinical cross-reactivity is quite rare, but has been identified with lupine and other legumes such as peanut and pea.[1,4-6] Vicilins, also named 7S globulins, are major storage proteins present in most legume seeds, which could possibly lead to cross-reactivity between legumes.[14,15]

Characterization of the allergen(s) might elucidate the role of cross-reactive and unique lupine allergens. This will facilitate the development of better diagnostic tools in legume allergy.[16] Since there is no consensus about the major allergens of lupine flour, we attempted to characterize some of the allergens responsible for lupine allergy in patients with and without concomitant peanut allergy by IgE-immunoblotting and amino acid sequencing.

Furthermore, we determined the eliciting dose (ED) in our group of patients by double-blind placebo-controlled food challenges (DBPCFC).

Materials and methods

Patients

Six adult patients with a suspected allergy to lupine who visited the outpatient department of Dermatology/Allergology of the University Medical Center Utrecht in 2003 were investigated in this study. Detailed histories of legume allergies and atopy were obtained. Symptoms by history were classified according to Mueller, a scoring system which was originally designed for the classification of allergic reactions to insect venom.[17] Symptoms involving the oral cavity ("oral allergy symptoms") were classified as Mueller grade 0, symptoms of the skin and mucous membranes (urticaria, angioedema, rhinitis, conjunctivitis) as grade 1, gastro-intestinal symptoms (diarrhea, vomiting, nausea, abdominal pain) as grade 2, respiratory symptoms (asthma, hoarseness) as grade 3 and cardiovascular symptoms such as a drop in blood pressure were classified as grade 4.

Ethical approval for the use of human subjects was obtained from the local ethics committee.

Sensitization

Skin prick tests (SPT) with commercial extracts of peanut, green pea and soy (ALK-Abelló, Nieuwegein, The Netherlands) and with research laboratory made lupine extract were performed. The lupine flour extract was prepared by suspending 20 g lupine flour in 200 ml PBS/0.1% phenol overnight at 4 °C. After clarifying the suspension by filtration and centrifugation, the supernatant solution was mixed with an equal volume of glycerol. The resulting solution in PBS-glycerol (50% v/v) was sterilized by filtration. The protein content of this extract was determined to be 10.2 mg/mL using the Bradford method.[18]

Histamine dihydrochloride (10 mg/mL) and the glycerol diluent of the SPT extracts served as positive and negative controls, respectively (ALK-Abelló, Nieuwegein, The Netherlands). The SPT were performed and recorded as described by Dreborg.[19] SPT were considered positive when the wheal reaction was 7 mm^2 (diameter 3 mm) and greater than the negative control. The SPT reactivity was expressed relative to the diameter of histamine: 3+ meant wheal diameter similar to the histamine wheal diameter, 2+ meant 50% and 1+ meant 25% of the histamine diameter. Specific IgE levels to peanut, lupine, green pea and soy were determined using the CAP system FEIA (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden). Sensitization to aeroallergens (mugwort, birch pollen and grass pollen) was also determined by SPT and CAP-FEIA.

Clinical evaluation by DBPCFC

Clinical reactivity to lupine was investigated by DBPCFC as described before.[20] The lupine flour used was a commercially available mild white lupine flour (protein content 36.2% by Leco 2000 method, *Lupinus albus*), obtained from Magenta Sales in England produced by CANA (Martigne-Ferchaud, France). In short, increasing doses of lupine flour (1 mg, 3 mg, 10 mg, 30 mg, 100 mg, 300 mg, 1000 mg and 3000 mg) hidden in mashed potatoes and randomly interspersed with four placebos, were administered to the patient. The challenge was discontinued when objective symptoms occurred or when subjective symptoms lasted for more than 45 minutes. The ED was determined as the lowest dose eliciting a convincing subjective allergic reaction. If symptoms occurred at the lowest dose tested, the ED could be lower than the dose that elicited the symptoms. The challenges were conducted in a hospital setting, with careful medical monitoring of the patients, and full emergency treatment readily available.

IgE-Immunoblotting

Green pea flour, white lupine flour, roasted Virginia peanut (ground) and raw Vinton soybean (ground) were extracted by mixing overnight at 4°C, 1:10 (w/v) in 0.01 M phosphate buffered saline (PBS), pH 7.4. Extracts were clarified by centrifugation (3020 x g for 30 minutes). Proteins were denatured using sample buffer containing 350 mM electrophoresis purity dithiothreitol (DTT) (Bio-Rad Laboratories, Inc., Hercules, California, USA) and heating at 95°C for 5 minutes. The proteins were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10-20% Tris-HCl pre-cast Ready Gels gradient gels (Bio-Rad Laboratories, Inc., Hercules, California, USA) based on the method of Laemmli[21] using the protocol outlined by the Bio-Rad Mini-Protean® II Dual Slab cell instruction manual. Each well was loaded with 50 µg protein as determined by the Lowry[22] method. In regular immunoblotting studies broad range electrophoresis molecular weight standards were used. In immunoblotting inhibition and 2D experiments Precision Plus molecular weight markers were used (Bio-Rad Laboratories, Inc., Hercules, California, USA).
Separated proteins were transferred to Immun-Blot®PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, California, USA), based on the method of Towbin[23] and the membranes then blocked with 0.01 M PBS pH 7.5 containing 0.2% bovine serum albumin (Fraction V, RIA grade, USB Corp., Cleveland, Ohio, USA) and 0.05% Tween® 20 (Bio-Rad Laboratories, Inc., Hercules, California, USA) for 2 hours. Blocked membranes were incubated overnight with serum (1:20 v/v in blocking buffer) from individual allergic patients or a normal control sera pool, and then washed five times with blocking buffer. The washed blots were probed with iodine-125 labeled anti-human IgE (Diagnostic Products Corp., Los Angeles, California, USA) diluted 1:20 in blocking buffer, overnight. Probed blots were washed five times with blocking

buffer, and exposed to X-OMAT™ LS film (Eastman Kodak Co., Rochester, New York, USA) for 48 hours at -80˚C. The film was developed to reveal the binding patterns. For immunoblotting inhibition experiments all conditions were the same with the following exceptions: Birch pollen extract was prepared as a 1:20 extract in distilled water, adjusting the pH to 8.0 with 0.1 M NaOH. The extract was defatted with Freon, clarified and dialyzed against distilled water. The protein level was 8.3 mg/mL using a bicinchoninic acid (BCA) assay kit from Pierce Biotechnology, Inc. Rockford, IL, USA. Protein samples used in the inhibition experiment were prepared with Laemmli buffer, but without reducing agent and were not heated before separation in SDS-PAGE. Proteins were transferred to PVDF as described above. Inhibitors (200µg protein in 1:20 dilutions of individual sera) were pre-incubated for two hour before addition to the blocked blots. The amount of peanut protein included in gels was reduced to 5 micrograms per lane while the protein in the other samples was 50 micrograms because of the differential binding. Individual blots for patients 1, 2, and 3 were incubated with peanut, lupine or birch pollen extracts as possible inhibitors. Blots for patients 4, 5, and 6 were incubated with green pea, lupine, birch or soy extracts as possible inhibitors.

Peptide sequencing

The lupine bands at approximately 50-66 kD were recognized in 2 of the 3 patients with lupine allergy (Figures 2 and 3), and were selected for further study by 2-D electrophoresis and peptide sequencing. Samples of an extract of raw lupine flour in PBS (pH 7.4) representing 150 micrograms of protein were separated by 2-D electrophoresis using Invitrogen Life Technologies (Carlsbad, CA) IPG non-linear 4-7 isoelectric focusing IPG strip for the first dimension, and Novex 4-20% Zoom SDS-PAGE gel for the second dimension. Identical gels were stained with Coomassie blue or blotted on PVDF membranes and incubated with individual patient sera (1:20 dilution in PBS with non-fat dry milk) from lupine-allergic patients no. 2, 4 or 5, followed by horseradish peroxidase labelled monoclonal-anti-IgE from Southern Biotech (Birmingham, AL). Bound IgE was detected using electrochemiluminescence with ECL (Pierce Biotechnology, Rockford, IL) and signals were detected using a Kodak 1D-Imaging system and software (Rochester, NY). Stained spots corresponding to IgE-binding proteins were excised from the gel and sent to Macromolecular Resources at Colorado State University, Fort Collins, CO, USA for sequence analysis by MALDI-TOF-MS/MS for isolated spots or LC-MS/MS of two partially separated protein spots. Identified peptide sequences based on mass analysis were searched against the NCBInr database (http://www.protein.sdu.dk/gpmaw/GPMAW/Databases/NCBInr/ncbinr.html) using the Mascot (version 2.1) database search engine to identify known lupine protein matches. Sequences of the identified lupine proteins were compared to all

NCBI sequences using the Basic Local Alignment Search Tool (BLAST) algorithm to identify apparent homologous proteins from peanut and other legumes.

Results

Patient characteristics

Six patients with a suspected lupine allergy were included in this study. Patient characteristics are summarized in Table 1. Of the six patients, 4 were sensitized to grass and birch pollen, 1 patient was sensitized to grass pollen, whereas 1 patient was not sensitized to grass and birch pollen. None were sensitized to mugwort. Three patients had in addition a convincing history of peanut allergy (no. 1-3), and three patients tolerated peanut without any restriction (no. 4-6). Levels of IgE to peanut were >100 kU/L in all three peanut allergic patients, and IgE to lupine ranged from 3.2 to 7.1 kU/L. They all were also sensitized to pea and soy and two of the three patients reported symptoms after ingestion of one or both legumes. Interestingly, patient no. 1 did not show IgE binding on immunoblots even though the serum IgE was positive by CAP (3.2 kU/L), which may be due differences in presentation of epitopes (e.g. different source materials or different substrates). Of the three non-peanut-allergic patients only one (no. 5) was mildly sensitized to peanut by CAP (IgE 1.5 kU/L), whereas another (no. 4) only had a positive SPT to peanut. Specific IgE to lupine varied from 1.3 to 67 kU/L. None of the three patients reported symptoms to pea or soy and only one was sensitized to these allergens by CAP (no. 5).
The foods that elicited allergic reactions and the lupine-related symptoms are shown in Table 2.

Table 1
Sensitization and clinical reactivity to legumes of lupine-allergic patients (n=6).

Patient	Sex	Age(y)	Atopic history°	Sensitization to aeroallergens°°	Lupine		Peanut		Pea		Soy		Symptoms to other legumes* (Mueller)
					CAP	SPT	CAP	SPT	CAP	SPT	CAP	SPT	
1	M	21	A, AD, AR	b, g	3,2	4+	>100	4+	3,0	2+	1,8	**n.t.	p(3)
2	M	26	AR	b, g	7,1	3+	>100	n.t.	1,3	0	4,9	2+	p(4), s(0)
3	F	27	A, AR	b, g	6,8	2+	>100	n.t.	4,3	2+	5,2	1+	p(3), s(2), pea(3)
4	F	42	-	-	10,3	3+	0	2+	0	1+	0	n.t.	-
5	M	48	AR	b, g	67	3+	1,5	0	1,2	2+	3,7	2+	-
6	F	32	A, AR	g	1,3	n.t.	0	0	0	0	0	1+	-

° A: asthma; AD: atopic dermatitis; AR: allergic rhinitis
°° b: birch pollen; g: grass pollen
* p: peanut; s: soy
** n.t. means not tested

Table 2
Clinical reactivity to lupine flour by history and during DBPCFC (n=6).

Patient	Eliciting food	Symptoms by history (Muller)	DBPCFC Dose (mg)								ED (mg flour)
			1	3	10	30	100	300	1000	3000	
1	A piece of bread	3	OAS	OAS	n	OAS	OAS	OAS, d ($FEV_1\downarrow$)	n.t.	n.t.	≤1 mg
2	A piece of bread, potato chips	3	OAS	OAS	OAS	OAS	OAS	OAS, n, ap	n.t.	n.t.	≤1 mg
3	Croquette, cookie	2	-	OAS	OAS	OAS	OAS, n	OAS, n, d	n.t.	n.t.	3 mg
4	A bite of a small round croquette	3	-	OAS	OAS	OAS	OAS	OAS	OAS, h	n.t	3 mg
5	A small round croquette	3	OAS	OAS	OAS	OAS	OAS, ap	OAS, rc, d	n.t.	n.t.	≤1 mg
6	A bite of a waffle	3	OAS	OAS	OAS, ap	n.t.	n.t.	n.t.	n.t.	n.t.	≤1 mg

OAS: oral allergy symptoms; n: nausea; d: dyspnea; n.t: not tested; ap: abdominal pain; h: hoarseness; rc: rhinoconjunctivitis

Clinical reactivity to lupine and eliciting doses

Positive DBPCFC with lupine flour confirmed the diagnosis of lupine allergy in all six patients (Table 2). The ED for subjective symptoms varied from ≤1-3 mg and were similar in both peanut-allergic and non-peanut allergic patients. All but one developed moderate to severe nausea or abdominal pain in addition to oral allergy symptoms at significantly higher doses (10-300 mg). Eliciting doses inducing objective symptoms could be established in three patients and varied from 300 to 1000 mg. Objective symptoms varied among individuals and included a 50% decrease of the forced expiratory volume in 1 second (FEV_1), hoarseness and rhinoconjunctivitis (Table 2).

IgE-Immunoblotting

Figure 1 shows the India ink blot of the transferred proteins. Figure 2 shows the autoradiogram results of IgE-immunoblotting in the three patients who have both peanut and lupine allergy. The difference in relative intensities of IgE binding to peanut and lupine protein bands is quite pronounced and parallels the relative CAP results in Table 1. Patient no. 1 appeared only to have IgE binding to peanut proteins, however, experienced OAS in DBPCFC to 1 mg lupine flour. Patient no. 2 (ED ≤1 mg lupine flour) displayed prominent IgE binding to peanut, with some binding to lupine bands at approximately 14 kD, and to soybean at 36 kD with minor bands at 21 and 14 kD. Patient no. 3, who had an ED of 3 mg lupine flour, also bound to peanut, as expected, but had very light IgE binding to a protein at 36 kD in green pea and very light binding to a series of bands in soy, with one minor band in lupine at approximately 24 kD. The results of immunoblotting, along with the CAP results,

show that *in vitro* IgE binding to lupine does not correlate well with the presence or severity of lupine allergy (Table 2). Figure 3 shows the IgE-binding profiles of patients who are lupine allergic without concomitant peanut allergy. No peanut IgE binding was evident from blots with sera from these patients. Patient no. 4 (ED of 3 mg lupine flour) had faint IgE binding to a green pea peptide at 31 kD, and relatively strong binding to a 66 kD band in lupine, and some minor binding to proteins of various molecular weights in soy. Patient no. 5 (lupine ED was ≤1 mg) had prominent binding to two lupine proteins at approximately 50 and 66 kD, but also displayed moderately strong binding to a soy band at 60 kD. Serum from patient no. 6 bound lightly to a green pea protein at 31 kD. This patient also had slight binding to lupine proteins at 21 kD and 66 kD, with very faint binding to soy proteins between 16-97 kD. Due to the clinical observations of co-reactivity to peanut and lupine, inhibition experiments were attempted to evaluate whether the allergic responses in those with allergies to both peanut and lupine might be due to cross-reactivity.

Inhibition of IgE binding to proteins on non-reduced SDS-PAGE blots was accomplished by pre-incubation of sera with 200 micrograms of specific protein extracts of peanut flour, lupine flour, birch pollen, soy flour and green pea flour as demonstrated in Figure 4. Pre-incubation of peanut-allergic sera with peanut protein prevented binding to peanut proteins on the blot (Figure 4-3N and 3P). Pre-incubation of lupine-allergic serum no. 5 with lupine protein inhibited binding to lupine protein (Figure 4-5N and 5L). However, the only two cases where clear evidence of cross-reactivity between peanut and lupine was found were the inhibition of binding of IgE from serum no. 1 to peanut band at approximately 150 kD (Figure 4-1N and 1L) and inhibition of the faint lupine doublet bands from serum no. 3 (molecular weight approximately 18-22 kD) when sera was pre-incubated with peanut protein (Figure 4-3N, 3P and 3L). It is important to note that the relative IgE band intensity differences between peanut-allergic patients binding to peanut and lupine made it difficult to evaluate the extent of cross-reactivity.

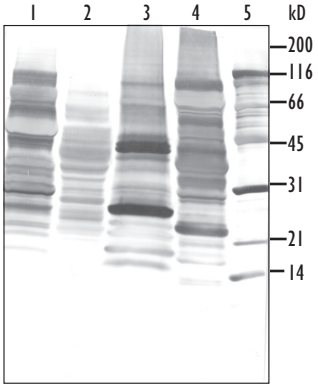

Figure 1.
India ink stain of PVDF blot. Equivalent loading of total protein from legume extracts as shown in lanes 1 to 5: green pea, lupine, roasted peanut and raw soybean, respectively. Lane 5 is the molecular weight standards.

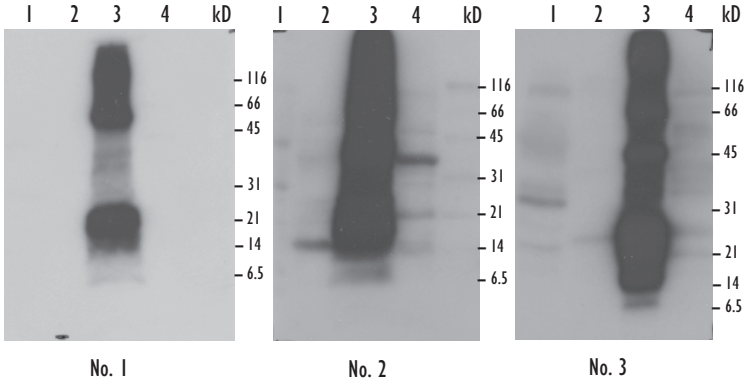

Figure 2.
Autoradiograms of IgE-immunoblots for patients with peanut allergy and lupine allergy. Extracts from specific legumes are represented in lanes 1 to 4: green pea, lupine, roasted peanut and raw soybean, respectively. Lane 5 is the molecular weight standards.

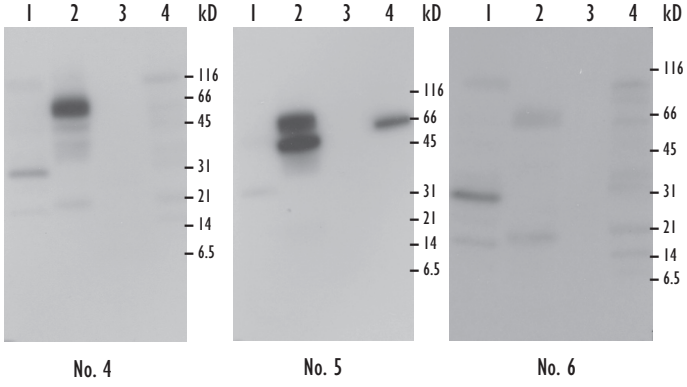

Figure 3.
Autoradiograms of IgE-immunoblots for patients with lupine allergy, but who tolerate peanut. Extracts from legumes are represented in lanes 1 to 4: green pea, lupine, roasted peanut and raw soybean, respectively. Lane 5 is the molecular weight standards.

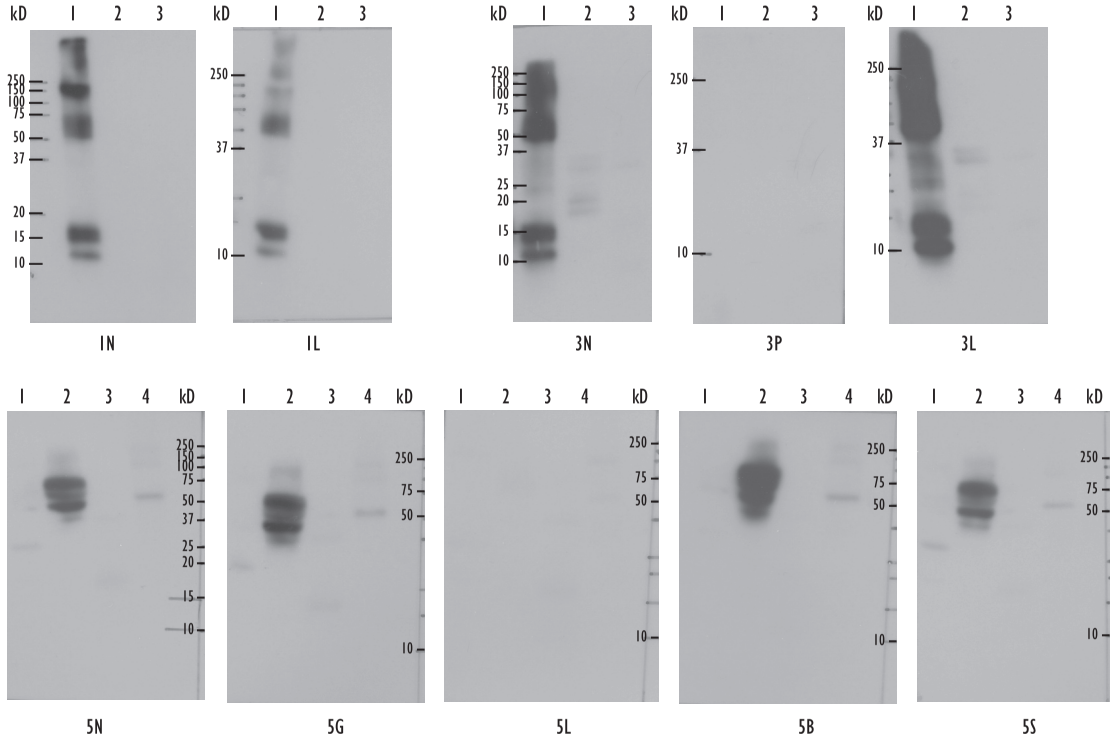

Figure 4.
Autoradiograms of IgE-Immunoblot Inhibition. For upper panels 1N, 1L, 3N, 3P and 3L; molecular weight standards are shown (kD), extracts represent protein loading of 5 µg roasted peanut, 50 µg lupine, and 50 µg birch pollen (lanes 1 to 3 respectively). Patient serum and treatments: N) patient no.1 with no inhibitor; 1L) patient no.1 with lupine inhibitor; 3N) patient no.3 with no inhibitor; 3P) patient no.3 with roasted peanut inhibitor; 3L) patient no.3 with lupine inhibitor. For lower panels 5N, 5G, 5L, 5B, and 5S; molecular weight standards are shown to the right (kD), extracts represent equal protein loading of green pea, lupine, birch pollen and raw soybean (lanes 1 to 4 respectively). Patient serum and treatments: 5N) patient no.5 with no inhibitor; 5G) patient no.5 with green pea inhibitor; 5L) patient no.5 with lupine inhibitor; 5B) patient no.5 with birch pollen inhibitor and 5S) patient no.5 with raw soybean inhibitor.

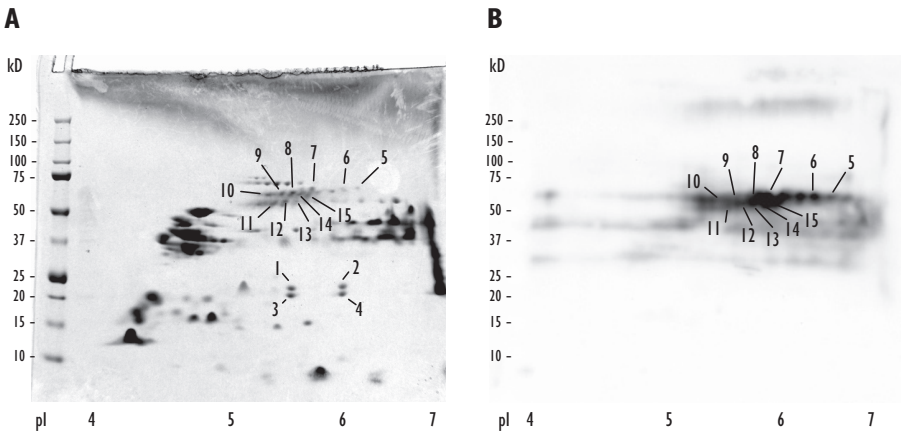

Figure 5.
Two dimensional gel protein stain and IgE binding. A) Coomassie blue stained gel of lupine extract separated by isoelectricfocusing in a pH 4-7 Invitrogen IPG strip in the first dimension followed by SDS-PAGE of separated proteins from the IPG strip in the second dimension. B) Proteins of an identically run gel were transferred to PVDF membrane, blocked and incubated with 1:20 diluted patient serum no. 5, then HRP-labeled anti-IgE, followed by detection with ECL and image capture on a Kodak 1D imaging system. Spots numbered 1 to 15 were isolated and partial peptide sequences determined with spots 5 to 15 representing IgE bound proteins as seen in B).

Peptide sequencing of a lupine allergy-associated IgE-binding protein

The 2D-gel patterns and immunoblots demonstrated multiple IgE-binding proteins in two close regions (50 to 66 kD) that represent a series of IgE-binding spots using sera from two patients as seen in Figure 5B. Similar patterns were visible from blots incubated with patient no. 4 (not shown) and no. 5 (Figure 5B), although the relative intensity of binding from patient no. 5 was markedly higher than patient no. 4. While these spots would appear as proteins of one or two molecular weights in 1D-gels, at least 11 clear spots were recognized between two patients. The putative identities of spots labeled 5 through 15 were determined by peptide sequencing and are listed in Table 3. Preliminary identification of the proteins bound by IgE from patients no. 4 and no. 5 was accomplished by LC-MS/MS and Maldi-TOF-MS/MS conducted on trypsin digested peptides from spots that were identified as binding IgE. The eleven spots were matched to either of two closely related lupine proteins that were previously identified from cDNA sequences with more than 30% coverage based on peptide fragments compared to the full-length proteins. Some of the protein spots more closely matched a beta-conglutin precursor (gi|46451223), a 533 amino acid protein with a nominal predicted mass of 62 kD and a calculated pI of 6.43. Other spots more closely matched a "vicilin-like" protein (gi|89994190), a 531 amino acid protein with a predicted nominal molecular weight of 62 kD and a calculated pI of

6.08. These two proteins were identified from *Lupinus albus*. The two proteins are 94% identical based on BLAST alignments. Both are likely homologues of a number of legume seed storage proteins, some of which are known allergens. The closest identity match is to soybean beta-conglycinin alpha-prime with approximately 52% identity in a full-length alignment. The peanut allergen Ara h 1 is approximately 47% identical to the lupine proteins. It is not clear if the multiple protein spots identified in this study represent products of multiple genes, proteins from differentially spliced RNAs or different post-translationally modified products of only two proteins. Interestingly, even though the sequence identities are so similar, the sera from the two lupine-allergic patients with IgE binding to the related proteins recognized spots with apparently different affinities or abundance of IgE based on visual comparison of spot intensities (not shown). Even more interesting was the apparent lack of cross-reactivity of these proteins and the homologues of other legumes based on failure of soybean or pea to inhibit binding to these proteins for serum no. 5 (Figure 4-5S and 5G) and lack of

Table 3
Peptide sequencing of IgE-binding lupine proteins*

Lupine protein†	Lupine Protein - best match		Serum no. Bound IgE	Matched Sequence coverage (%)
	Accession number	Description		
1	gi|62816184	Legumin-like	2	10%
2	gi|85361412	Legumin-like	2	18%
3	gi|62816184	Legumin-like	none	10%
4	gi|85361412	Legumin-like	none	18%
5	gi|46451223	Beta-conglutin precursor	4, 5	35
6	gi|46451223	Beta-conglutin precursor	4, 5	35
7	gi|46451223	Beta-conglutin precursor	4, 5	35
8	gi|89994190	Vicilin-like protein	4, 5	36
9	gi|89994190	Vicilin-like protein	4, 5	42
10	gi|89994190	Vicilin-like protein	4, 5	35
11	gi|89994190	Vicilin-like protein	4, 5	34
12	gi|89994190	Vicilin-like protein	4, 5	34
13	gi|89994190	Vicilin-like protein	4, 5	30
14	gi|89994190	Vicilin-like protein	4, 5	39
15	gi|46451223	Beta-conglutin precursor	4, 5	37

* Peptide sequences were identified by MALDI-TOF MS/MS or LC-MS/MS of stained protein spots isolated from gels. Mass comparisons and peptide identities were performed using Mascot (version 2.1) to search the NDBInr database, by Macromolecular Resources at Colorado State University.
† Protein number refers to marked spots on the Coomassie stained gel, Figure 5.

IgE binding of serum no. 5 to any peanut proteins (Figure 3). Serum from patient no. 2 bound to two different proteins, labeled as 1 and 2 in Figure 5, of approximately 23 kD. These were closely associated with protein spots labeled as 3 and 4 (Figure 5A). These four spots were analyzed by peptide sequencing, but had limited (approximately 10-18%) coverage of two lupine proteins (Table 3) and the results are tentative and IgE-binding to spots 1 and 2 was rather weak. The short peptide sequences that were identified matched those found for the non-IgE binding spots directly (3 and 4). Additional proteins were seen to bind some IgE from allergic patients, but the most prominent were identified in Table 3.

Discussion

Allergy to legumes is well known, peanut being the most prominent. Besides peanut, soy is often mentioned as the next important allergenic legume. Recently, lupine flour has been introduced as a food alternative to soy flour. It is now becoming more and more apparent that the usage of lupine is not without risk, given the increase in reported lupine-induced allergic reactions.[1,4-6] In addition, the UK-based Institute of Food Science & Technology has recommended that lupine flour should be added to the list of 12 potentially allergic ingredients and that methods for the detection of lupine proteins in processed foods should be developed.[24]

The lowest ED reported so far in literature is 200 mg for lupine, inducing asthma and abdominal pain.[4] Our data show that in four out of six lupine-allergic patients the ED was 1 mg or less for subjective symptoms (OAS). Objective symptoms began at 300 mg. These doses are similar to those identified as ED in peanut[25] and is an indication of the significant allergenicity of lupine flour. This is further shown by the fact that five out of six patients had moderate to severe clinical reactions to lupine in their history (Table 2).

So far, it has become clear that lupine allergy is often related to peanut allergy.[1,4,6] However, our data clearly show that lupine allergy can occur as a separate entity, even without any history of clinical reactivity to other legumes, as was found in three patients (no. 4-6). In 1994 Hefle et al reported that the IgE-binding proteins of lupine extract appeared to have an approximate molecular weight of 21 kD and 35 to 55 kD as determined by SDS-PAGE followed by immunoblotting with sera of peanut-sensitized patients.[1] Moneret-Vautrin et al[4] showed that the most reactive band in lupine flour proteins had a molecular mass of 43 kD for their peanut-allergic patients and identified it as a major lupine allergen. One could conjecture that the 43 kD band was a dimer of the 21 kD peptide noted by Hefle et al[1]. Moneret-Vautrin et al[4] also identified bands at 13, 38, and 65 kD that were not cross-reactive, so may be lupine-only allergens. In another study, an immunoblot with serum of a non-peanut-allergic

patient with allergic symptoms to airborne lupine flour showed binding to proteins with molecular weights of 34, 59 and 71 kD as the most important IgE-binding proteins, and 17 and 24 kD as additional bands.[8] In addition, immunoblot analysis performed with the serum of a peanut-sensitized and airborne lupine flour-allergic child revealed an IgE-binding band with an approximate molecular weight of 45 kD.[9] This indicates that a lot of proteins with different molecular weights have been described as possible allergens.

In this study patients with a combined lupine and peanut allergy showed weak IgE binding to protein bands of lupine at 14 kD, 24 kD, 30 kD or 66 kD. In contrast lupine allergic patients without peanut allergy showed IgE binding to proteins of lupine at approximately 50 and 66 kD and a slight binding at 21-22 kD. This indicates that there are remarkable differences in allergen recognition between these two groups of patients.

Peptide sequencing of the two closely associated proteins by MALDI-TOF MS/MS and LC-MS/MS showed that multiple spots represent two highly similar proteins that are approximately 47% identitical to Ara h 1, a vicilin and 52% identity to beta-conglycinins (or vicilin-like proteins) of soybean. Presumably the shared 47% identity with Ara h 1 does not provide enough structural similarity to allow significant shared IgE binding since clinical reactivity to peanut was absent and specific IgE and SPT reactivity low or absent. The predicted 61-62 kD proteins could be similar to the 65 kD band found by Moneret Vautrin[4]; in fact in our 1-D SDS-PAGE we observed an estimated molecular weight of between 50 and 66 kD. It is typically difficult to accurately predict the molecular weight of proteins based solely on migration distances in either one- or two-dimensional gels of various percent acrylamide. This is further complicated by uncertainties of accuracy of various molecular weight standards. This study also demonstrates the complexity of proteins that bind IgE and presumably cause allergic reactions. Further studies are needed to determine the structural differences in proteins identified as binding IgE. In addition a larger population of lupine-allergic patients will be needed to completely describe the allergens in lupine.

Together, the data indicate that lupine allergy might equal peanut allergy in its severity and therefore lupine might be a less attractive replacement for soy. Moreover, lupine allergy is more complicated than previously thought, since both cross-reactive but particularly unique allergens are involved.

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5

Does skin prick test reactivity to purified allergens correlate with clinical severity of peanut allergy?

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Abstract

Background
Recognition of specific peanut allergens or the diversity of IgE binding to peanut allergens may play a role in the elicitation of severe allergic reactions.

Objective
To investigate whether sensitization to individual allergens Ara h 1, Ara h 2, Ara h 3 and Ara h 6 is correlated with clinical severity.

Methods
The reactivity of purified peanut allergens was measured by skin prick test (SPT) and by IgE-immunoblot in 30 patients. The results were related to the clinical reactivity by history, and in 25 of them to the eliciting dose (ED).

Results
The majority of patients recognized Ara h 2 and Ara h 6. Patients with severe symptoms had a higher SPT response to Ara h 2 and Ara h 6 at low concentrations (0.1 μg/mL) and to Ara h 1 and Ara h 3 at higher concentrations (100 μg/mL), compared to patients with mild symptoms. They also recognized a greater number of allergens and showed a higher cumulative SPT response compared to patients with mild symptoms. No significant differences were observed between patients with a low or high ED.

Conclusions
Ara h 2 and Ara h 6 appeared to be more potent than Ara h 1 and Ara h 3. Both SPT reactivity to low concentrations of Ara h 2 and Ara h 6 and to higher concentrations of Ara h 1 and Ara h 3 were shown to be indicative of severe symptoms.

Introduction

Peanut allergy is a significant health problem because of its high and rising prevalence, its persistence, and the life-threatening nature of reaction.[1,2] In developed countries, it affects about 0.4-0.6% of children and 0.3-0.7% of adults.[3,4] Doses as low as 100 µg of peanut protein have been shown to elicit subjective allergic reactions.[5] Peanut allergy tends to be more severe in nature than other food allergies and accounts for 63% of the fatalities due to anaphylactic reactions to food.[6,7]

Several proteins have been identified as peanut allergens, characterized and subsequently designated Ara h 1 to Ara h 8. Historically, it was believed that Ara h 1 and Ara h 2 were the most relevant peanut allergens,[8-11] but a role for Ara h 3 and Ara h 6 could not be excluded. Ara h 1 and Ara h 2 are classified as major allergens, recognized by 70-90% of sensitized subjects.[9,11,12] Ara h 1 belongs to the vicilin family, and Ara h 2 to the conglutin family, which is related to the 2S albumin superfamily of seed storage proteins.[13] The peanut glycinin Ara h 3 is regarded as a minor allergen, but recently it was found that a group of peanut allergic Italian children were specifically sensitized to the basic subunit of Ara h 3.[14] Ara h 6 shows homology to Ara h 2.[10,15,16] In a recent study from our group it was shown that peanut-allergic patients recognize Ara h 6 both *in vitro* and *in vivo* to a similar extent as to that of Ara h 2,[10] indicating that Ara h 6 should be considered a major peanut allergen as well.

For some allergenic plant foods it has been shown that sensitization to a specific allergen is associated with severe allergic reactions. For example, sensitization to non-pollen-related hazelnut allergen Cor a 8 (lipid transfer protein, LTP) can be related to severe reactions to hazelnut,[17,18] whereas sensitization to the Bet v 1-related Cor a 1 is almost exclusively associated with oral allergy symptoms. For peanut, the association between sensitization to certain peanut allergens and the severity of reactions seems less clear. Lewis et al[19] showed that diversity of IgE binding to Ara h 1, Ara h 2 and Ara h 3 as determined by Western blotting was more important than the recognition of individual proteins. Shreffler et al[20,21] reported that patients with IgE antibody binding to multiple epitopes of Ara h 1, Ara h 2 and Ara h 3 tend to have more severe allergic reactions compared to those with IgE specific to a relatively few epitopes. Together, these data suggest that IgE binding to a greater number of allergens plays a more important role than specific allergen recognition does. However, preliminary data from our group indicate that specific recognition may play a role as well.[9]

To gain insight into the reactivity of the individual major allergens, the current work focuses on the reactivity of purified Ara h 1, Ara h 2, Ara h 3 and Ara h 6 *in vivo* by skin prick test (SPT) using tenfold dilutions, and *in vitro* by IgE-immunoblot. To investigate whether sensitization to (one of) these allergens can predict clinically severe reactions to peanut, the results were related to the clinical reactivity by history

and by eliciting dose (ED) determined by double-blind placebo-controlled food challenge (DBPCFC).

Materials and Methods

Study population

Thirty-one adult patients from the outpatient clinic of the Department of Dermatology/Allergology of the University Medical Center Utrecht were selected. Inclusion criteria were a history of allergic reactions to peanut in the patient's medical record, in addition to a positive SPT to peanut extract (ALK-Abelló, Nieuwegein, The Netherlands) with the area of the peanut wheal at least half of the area of the positive control (SPT ≥2+), and/or specific IgE to peanut ≥0.7 kU/L (CAP-FEIA, Pharmacia & Upjohn Diagnostics, Uppsala, Sweden).
Pregnancy, significant concurrent disease, instable asthma and oral medication with corticosteroids or ß-blocking agents were exclusion criteria.
This study was reviewed and approved by the Medical Ethical Committee of the University Medical Center Utrecht. All patients gave written informed consent before enrolment in the study.

Clinical evaluation

The subsequent step comprised a careful and complete medical history, SPT and blood sampling. One patient (P01KP) was excluded, because her reported allergic reaction did not appear to be caused by peanut. Symptoms by history were classified according to Muller, a scoring system which was originally designed for the classification of allergic reactions to insect venom.[22] Symptoms of the oral cavity were classified as Muller grade 0, symptoms of the skin and mucous membranes (urticaria, angioedema, rhinitis, conjunctivitis) as grade 1, gastrointestinal symptoms (diarrhea, vomiting, nausea, abdominal pain) as grade 2, respiratory symptoms (asthma, hoarseness) as grade 3, and cardiovascular symptoms as grade 4.
Specific IgE levels to peanut, and also to green pea, soy, grass pollen and birch pollen, were determined by the CAP system FEIA (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden).
Clinical reactivity to peanut was investigated by DBPCFC in 25 of the 30 patients according the threshold consensus protocol[23] with some modifications[24]. Five patients did not participate for various reasons: anxiety (n=2), too busy (n=2) or no interest (n=1).
The hospital pharmacy prepared the challenge materials. The amounts of peanut flour were 0.01 mg, 0.1 mg, 0.5 mg, 1 mg, 10 mg, 100 mg, 300 mg, 1 g and 3 g. The peanut flour used was partially defatted, light roasted (protein content 50%, fat 12%),

provided by J. Nordlee, Food Allergy Research and Resource Program, University of Nebraska, Lincoln, NE, USA. To mask the peanut doses, wheat instant cereal-cinnamon mix and apple sauce were added. Four similarly prepared placebo doses were randomly interspersed between the increasing peanut doses.
The challenge was discontinued when objective symptoms occurred, or when convincing subjective symptoms had occurred for at least three times or lasted for more than 45 minutes. The eliciting dose (ED) was determined as the lowest dose of peanut flour inducing convincing subjective symptoms. If the DBPCFC was negative, the patient subsequently underwent an open provocation with 1 g, 3 g and then 10 g of roasted peanuts.
All the challenges were conducted in a hospital setting, with careful monitoring of the patients. Full emergency treatment was readily available.

Purification of Ara h 1, Ara h 2, Ara h 3 and Ara h 6

Previously developed purification protocols were used for the preparation of Ara h 1[25], Ara h 2[10], Ara h 3[26] and Ara h 6[10] with resulting purities of >95% as judged by SDS-PAGE electrophoresis and Coomassie Brilliant Blue staining. Purified peanut allergens were stored and sterilized as described earlier.[9]

Skin Prick Tests

All 30 patients included in the study were evaluated by SPT with commercial extracts of peanut, green pea, soy, grass pollen and birch pollen (ALK-Abelló, Nieuwegein, The Netherlands) using 1 mm-tip lancets (ALK-Abelló, Nieuwegein, The Netherlands) on the flexor aspect of the forearm following the recommendations of the EAACI.[27] Histamine dihydrochloride (10 mg/mL) and saline served as positive and negative controls, respectively. In addition, patients were tested with purified Ara h 1, Ara h 2, Ara h 3 and Ara h 6. For optimal accuracy, SPT were performed in serial tenfold dilutions, ranging from 100 μg/mL to 0.01 μg/mL. Serial dilutions were prepared using a diluent containing 50% glycerol (v/v), 0.9 % NaCl (w/v), 0.4% phenol (w/v) and 0.3% human serum albumin (HSA) (w/v) in PBS.
Antihistamines were discontinued 1 week prior to the skin tests, and no topical corticosteroids were allowed on the day of skin prick testing on the flexor aspect of the arm.
The SPT reactivity was measured after 15 minutes. SPT responses were expressed as the ratio of the wheal reaction in millimetres squared, evaluated by computer scanning[28], divided by the wheal reaction of the positive control.[29] SPT ratios were regarded positive when the ratios were ≥0.25, i.e. when the wheal area was at least 25% of the wheal induced by the positive control. All SPT were carried out by the same investigator. Ten non-peanut-allergic patients were used as negative controls for SPT with purified allergens. SPT responses in these control subjects were all negative.

SDS-PAGE and IgE-immunoblotting

SDS-PAGE and IgE-immunoblotting was performed using 15% acrylamide gels as described earlier.[9] Patients' reactivity towards the purified peanut allergens was analyzed using IgE-immunoblotting, generally as described earlier.[9] Patient serum was diluted 50 times, and IgE bound to the membrane was detected with a peroxidase-conjugated goat-anti-human IgE (Kirkegaard and Perry Limited, Gaithersburg, MD, USA). Non-specific binding of the anti-IgE antibody conjugate was negligible as demonstrated by immunoblotting with non-peanut-sensitized human serum.

Statistics

All analyses of data were performed with nonparametric tests. The Mann-Whitney U test was used for comparison between groups. Correlations were analysed with Spearman's rank, or Gamma test when the data contain many tied observations (Figure 4A). Calculations were performed using SPSS (version 12, SPSS Inc., 2001, Chicago, USA). P values <0.05 were considered statistically significant.

Results

Patient characteristics

Thirty patients (20 female and 10 male) with a convincing history of peanut allergy and sensitization to peanut entered the study. The mean age was 28 years (range, 16 to 70). Eight patients (27 %) reported mild symptoms as their most severe symptoms by history (Muller grade 0 and 1), 7 patients (23 %) reported moderate symptoms (grade 2), and 15 patients (50%) reported severe symptoms (grade 3 and 4). Patient characteristics are summarized in Table 1.

Fifteen patients (50%) suffered from concomitant asthma and 24 (80%) patients from atopic eczema, whereas 27 (90%) patients reported pollinosis symptoms. Sensitization to grass pollen was present in 97% of the study population and to birch pollen in 90%. Twenty-three patients (77%) were sensitized to soy and 20 patients (67%) to green pea.

Levels of IgE to peanut ranged from 0.4 to >100 kU/L. Peanut specific IgE in patients with severe symptoms (median 18.4 kU/L) was higher than in the patient groups with mild (median 4.5 kU/L) and moderate symptoms (median 2.1 kU/L) ($p=0.01$ and $p=0.02$, respectively), but there was no significant difference between patients with mild and moderate symptoms ($p=0.82$).

Table 1
Clinical characteristics and reactivity to Ara h 1, Ara h 2, Ara h 3 and Ara h 6 by SPT and IgE-immunoblot.

Patient no	Sex	Age	Mul-ler*	CAP peanut (kU/L)	SPT peanut (ratio)	Subjective ED (mg)	Objective ED (mg)	Most severe symptoms during challenge at a dose (mg)°	Time interval (years) §	SPT (ratio)				IgE-immunoblot			
										Ara h 1	Ara h 2	Ara h 3	Ara h 6	Ara h 1	Ara h 2	Ara h 3	Ara h 6
P05KP	F	22	0	18	1.3	10	-	ap at 10	>5	0.25	1.80	0.34	1.98	+	++	+	++
P17KP	M	21	0	0.4	0.9	10	-	oas	<1	0.05	1.68	0.16	2.85	+	+	+	-
P21KP	F	21	0	0.4	1.4	10	-	oas	>5	0.14	0.75	0.03	1.53	+	+	+	-
P28KP	F	24	0	1.9	1.0	nt	-	nt	<1	0.00	0.00	0.00	0.22	-	-	-	-
P02KP	M	21	1	4.7	1.9	300	-	oas	1-5	0.15	1.59	0.00	0.39	-	++	-	+
P09KP	M	23	1	14	8.0	nt	-	nt	>5	0.42	1.98	0.00	3.71	+	++	-	++
P12KP	M	20	1	4.3	1.8	0.1	-	oas	<1	0.15	0.98	0.00	1.26	+	+	+	+
P20KP	F	35	1	16	1.4	1	300	rc at 300	>5	0.00	0.18	0.00	0.77	-	-	+	-
P07KP	M	32	2	1.8	2.4	100	-	n at 300 and ap at 1000	1-5	0.14	1.13	0.16	1.66	-	+	-	+
P08KP	F	24	2	9.2	2.8	nt	-	nt	<1	0.27	1.57	0.55	1.59	+	++	+	++
P13KP	F	39	2	2.1	4.6	nt	-	nt	1-5	0.49	1.02	0.30	1.27	-	+	+	+
P15KP	F	27	2	33	6.8	10	-	n at 300	1-5	0.31	4.42	0.49	4.17	-	++	+	++
P19KP	F	29	2	1.7	2.9	10	-	ap and n and d at 1000	1-5	0.39	1.75	0.12	3.75	-	+	-	+
P25KP	F	20	2	38	1.7	no	-	-	1-5	0.00	0.00	0.09	0.37	-	+	+	-
P29KP	F	56	2	0.8	0.6	no	-	-	1-5	0.05	0.00	0.00	0.00	-	+	-	-
P04KP	F	19	3	3.1	1.9	100	-	oas	>5	0.00	0.28	0.25	0.86	+	-	-	-
P06KP	F	37	3	12	3.8	10	10	dia at 10 and 100	>5	0.71	0.93	1.49	0.77	+	+	++	+
P14KP	F	24	3	9.2	0.6	10	-	ap and n at 300	<1	0.18	2.57	0.21	0.61	-	+	-	+
P16KP	F	23	3	>100	5.9	0.1	-	d at 10	1-5	1.79	4.13	0.74	3.55	+++	>+++	>+++	>+++
P18KP	F	30	3	41	1.3	nt	-	nt	1-5	0.16	1.29	0.13	0.24	-	+	-	-
P22KP	M	33	3	>100	6.0	1	-	ap at 100 and d at 1000	>5	1.42	0.63	0.77	1.94	>+++	>+++	>+++	>+++
P24KP	M	18	3	11	2.6	0.5	3000	ap and n at 10 and v at 3000	<1	0.21	0.74	0.08	0.48	+	+	+	+
P26KP	F	29	3	4.2	8.8	100	-	ap and n at 100	<1	0.56	2.37	0.58	2.65	+	+	-	+
P30KP	M	27	3	46	2.0	1	-	d and ap at 10	1-5	0.35	4.20	0.59	1.67	++	++	++	++

P31KP	F	70	3	7.9	0.7	no	-	-	1-5	0.10	0.15	0.10	0.22	-	+	-	+
P03KP	M	16	4	>100	1.9	100	1000	d at 1000	>5	1.07	2.21	1.31	1.73	+++	>+++	>+++	>+++
P10KP	M	25	4	44	6.4	0.1	300	n and v at 300	>5	1.36	0.81	1.32	3.07	+	+++	++	+++
P11KP	F	22	4	85	6.2	0.1	-	n at 0.1	1-5	2.19	3.25	0.55	3.05	++	> +++	+++	>+++
P23KP	F	24	4	18	4.2	10	1000	d and h at 1000	<1	1.54	1.53	0.54	2.56	+	++	+	++
P27KP	F	28	4	18	14.8	0.1	-	oas	<1	0.87	1.91	0.75	2.64	-	+	-	+

* Patients are categorized according to ascending Muller score (most severe symptoms by history): 0, symptoms of the oral cavity; 1, symptoms of the skin and mucous membranes; 2, gastro-intestinal symptoms; 3, respiratory symptoms; 4, cardiovascular symptoms.
° d, dyspnea; ap, abdominal pain; dia, diarrhea; n, nausea; v, vomiting; oas, oral allergy symptoms; rc, rhino conjunctivitis; h, hoarseness.
§ Time between most severe historical reaction and SPT.
no: no ED detectable
nt: not tested

Eliciting doses (ED) and No Observed Adverse Effect Level (NOAEL) as determined by DBPCFC

Positive DBPCFC confirmed the diagnosis of peanut allergy in 22/25 subjects (88%). Three patients did not respond during the DBPCFC, of which two (P25KP and P29KP) underwent an open provocation that was negative. These two patients had experienced their last clinical reaction to peanut less than one year before start of this study. So, although still sensitized to peanut, these patients were not longer peanut-allergic. The third patient (P31KP) did not proceed to the open challenge, because of intercurrent disease. Her last reported allergic reaction to peanut was six years before the DBPCFC.

All patients tolerated a dose of 0.01 mg peanut flour, so the No Observed Adverse Effect Level (NOAEL) for our patient group in this study was 0.01 mg.

The ED for subjective reactions varied from 0.1 mg up to 300 mg peanut, and for objective symptoms from 10 to 3000 mg. The ED for subjective symptoms was significantly lower than the ED for objective symptoms (p=0.001). Objective symptoms consisted of dyspnea (n=2), diarrhea (n=1), vomiting (n=2) and rhino conjunctivitis (n=1).

To study whether the subjective ED was associated with peanut-specific IgE (CAP-FEIA), patients were divided in a group with a high ED (consisting of the 3 highest ED of 10-300 mg) (n=13) and with a low ED (consisting of the 3 lowest ED of 0.1-1 mg) (n=9). The concentration of specific IgE in patients with a low ED (median 44 kU/L; interquartile range 16.4-85) was significantly higher than in patients with a high ED (median 4.7 kU/L; interquartile range 1.8-17.7) (p=0.018).

Reactivity to Ara h 1, Ara h 2, Ara h 3 and Ara h 6 by SPT and IgE-immunoblot

All thirty patients were subjected to titrated SPT with Ara h 1, Ara h 2, Ara h 3 and Ara h 6. There was a clear dose-response relationship for all allergens tested (Figure 1). Ara h 2 and Ara h 6 had a higher SPT reactivity than Ara h 1 and Ara h 3. Concentrations of 0.1 μg/mL resulted in positive reactions (ratio ≥0.25) for Ara h 2 and Ara h 6. Ara h 1 reactivity was positive starting at 10 μg/mL, whereas the reactivity to Ara h 3 began at 100 μg/mL. Ara h 2 showed comparable reactivity to Ara h 6, suggesting a similar potency of these allergens *in vivo*. The SPT results for Ara 1, Ara h 2, Ara h 3 and Ara h 6 in the highest concentration tested (100 μg/mL) for individual patients are shown in Table 1. The responses to this test concentration were similar in size to the response to the commercial peanut extract (Table 1).

The majority of patients with a positive SPT was sensitized to Ara h 2 (25/30, 83%) and Ara h 6 (26/30, 87%). Sixteen patients (53%) were sensitized to Ara h 1 and fifteen patients (50%) to Ara h 3. All patients with a positive SPT to Ara h 1 and/or Ara h 3, were also sensitized to Ara h 2 and/or Ara h 6. Three patients did not

recognize any purified allergen (P28KP, P29KP, P31KP).
Sera of all 30 patients were used for IgE-immunoblot analysis. IgE binding to purified allergens were scored in five categories from – (negative) to >+++ (strongly positive). Figure 2 shows representative examples of IgE-immunoblot experiments illustrating different intensities of IgE binding. The scoring results are summarized in Table 1, and largely confirm the SPT results. The majority of the patients showed IgE binding to Ara h 2 and Ara h 6. This IgE binding in the majority of the cases was more intense than binding to Ara h 1 and Ara h 3. There was a significant correlation between recognition in IgE-immunoblotting and SPT reactivity on the level of individual allergens, i.e. allergens that were most reactive in SPT were also most reactive in IgE-immunoblotting (correlation coefficient (γ) Ara h 1, Ara h 2, Ara h 3, Ara h 6: 0.552, 0.523, 0.54, 0.547, respectively ($p<0.001$ for all allergens)).

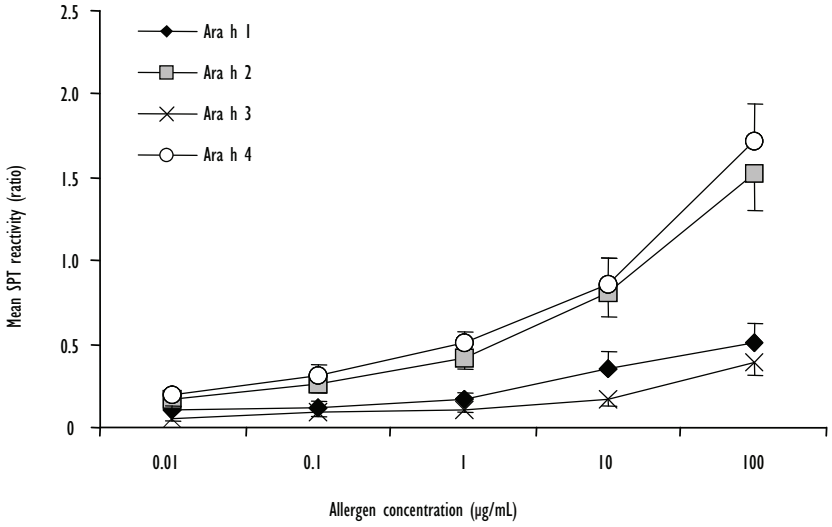

Figure 1
Dose response relationship of SPT reactivity to Ara h 1, Ara h 2, Ara h 3 and Ara h 6 (mean values ± SEM, n=30).

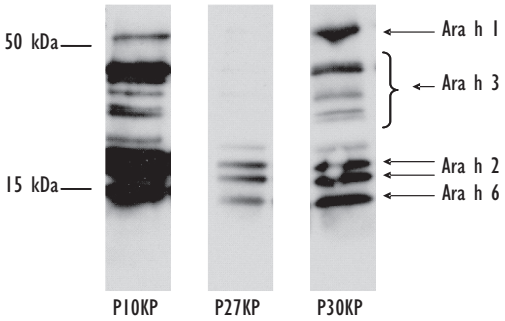

Figure 2
Examples of IgE-immunoblotting experiments with crude peanut extract (CPE). Patient sera (numbers indicated below blot lane) were incubated on blot membranes with SDS-PAGE separated CPE. Markers are shown left, and arrows indicate the positions of the individual peanut allergens.

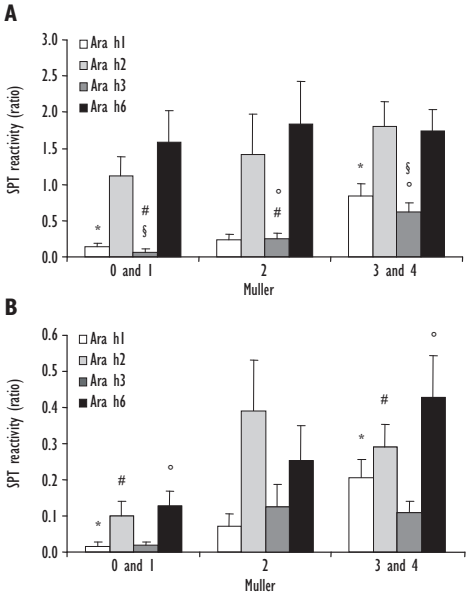

Figure 3
Sensitization to purified peanut allergens in different concentrations in relation to the most severe symptoms by history according to the Muller classification: (A) 100 µg/mL, (B) 0.1 µg/mL. *, #, §, and o: comparing two bars with the same symbols, p <0.05

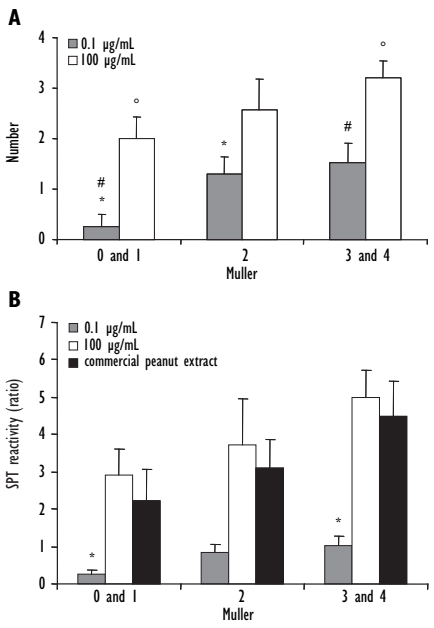

Figure 4
(A) Number of purified allergens recognized and (B) cumulative SPT score in relation to the reported symptoms by history according to the Muller classification.
*, #, and o: comparing two bars with the same symbols, p <0.05

Relationship between SPT reactivity to titrated purified allergens Ara h 1, Ara h 2, Ara h 3 and Ara h 6 and the severity of peanut allergy

To assess whether the SPT reactivity to individual allergens was predictive for the clinical presentation of peanut allergy evaluated by history, the patients were categorized in Muller groups as described before (Figure 3).

The SPT responses to purified allergen concentrations of 100 μg/mL (Figure 3A) were higher for Ara h 2 and Ara h 6 than for Ara h 1 and Ara h 3 among all patient groups. The SPT reactivity to both Ara h 2 and Ara h 6 was not significantly different between the three groups. Patients with more severe symptoms after peanut intake had significantly higher SPT reactivity to Ara h 3 compared to patients with mild and moderate symptoms ($p=0.001$ and $p=0.048$, respectively). The reactivity to Ara h 1 was higher in patients with severe symptoms compared to patients with mild symptoms ($p=0.006$). SPT responses of purified allergens at lower concentrations (10-0.1 μg/mL) revealed smaller SPT responses. At all concentrations tested, the highest SPT responses were observed to Ara h 2 and Ara h 6 in the three patient groups. At 10 μg/mL, a significantly higher response to Ara h 1 and Ara h 3 was again observed in the group with severe symptoms compared to the group with mild symptoms ($p=0.041$ and $p=0.049$, respectively; data not shown). At lower concentrations, patients with severe symptoms had a significantly higher SPT reactivity to Ara h 1 (1 μg/mL (data not shown) and 0.1 μg/mL), Ara h 2 (0.1 μg/mL), and Ara h 6 (1 μg/mL (data not shown) and 0.1 μg/mL) compared to patients with mild symptoms (Figure 3B).

To assess whether clinical reactivity to peanut as demonstrated by ED was associated with the SPT reactivity, patients were categorized in two groups according to ED, as described before. At all concentrations used, there was no significant difference in SPT reactivity to Ara h 1, Ara h 2, Ara h 3 and Ara h 6 between the two groups. Only a trend to higher SPT responses in the group with a low ED was observed (data not shown).

Number of purified allergens recognized in SPT

Since the difference in symptom severity might be related to the total number of allergens recognized, we investigated whether patients with a history of more severe symptoms recognized a greater number of allergens than patients with mild symptoms. For the allergens tested at 100 μg/mL, a significantly greater number of allergens was recognized in the severe group compared to the patient group with mild symptoms ($p= 0.029$), whereas for 0.1 μg/mL, this difference was significant between the mild and moderate group ($p=0.033$) and between the mild and the severe group ($p=0.021$) (Figure 4A). No significant difference was found in the number of allergens recognized between the groups with a low or high ED for both concentrations tested (data not shown).

Cumulative SPT reactivity to Ara h 1, Ara h 2, Ara h 3 and Ara h 6.
A relationship has been suggested between the level of specific IgE (SPT and CAP-FEIA) and clinical reactivity. Therefore, the sum of the SPT reactivities to Ara h 1, Ara h 2, Ara h 3 and Ara h 6 in relation to symptoms by history, and to ED was investigated (Figure 4B).
The cumulative SPT response of the purified allergens at a concentration of 0.1 μg/mL showed a significant difference between patients with mild and severe symptoms (p=0.002). Patients with more severe symptoms showed a trend to higher cumulative SPT response at a concentration of 100 μg/mL and also to a higher SPT reactivity to commercial peanut extract (p=0.155 and p=0.166, respectively).
Regarding the ED, no association between the cumulative SPT response and ED was observed (data not shown).

Discussion

The aim of our study was to investigate whether sensitization to the individual peanut allergens Ara h 1, Ara h 2, Ara h 3 and Ara h 6 was predictive for severe peanut allergy. Together, Ara h 1, Ara h 2, Ara h 3 and Ara h 6 represent about three-quarters of the total protein content in peanut[30] and it is believed that these allergens are the most important ones in the peanut.[8-10,14]
The majority of all patients in this study recognized Ara h 2 and Ara h 6, both by SPT and IgE-immunoblotting. Immunoblotting evaluates allergen-specific IgE antibodies in serum on basis of direct binding to allergens separated by their molecular weight, whereas SPT is based on cross-linking of IgE molecules on the mast cells' IgE receptors. Our SPT data confirm our earlier reported data that Ara h 2 was more potent than Ara h 1 and Ara h 3.[9] This is in accordance with the study of Palmer et al[8], who found that Ara h 2 was a much more potent allergen than Ara h 1 using IgE-immunoblotting and a functional assay. We now additionally show that Ara h 6, which has structural similarities with Ara h 2[10], also has equal *in vitro* and *in vivo* potency.
To investigate whether sensitization to purified allergens was correlated with severity of symptoms, patients were categorized in three groups: mild (Muller grade 0 and 1), moderate (Muller grade 2) and severe (Muller grade 3 and 4). The group with severe symptoms had a significantly higher SPT response to Ara h 1 and Ara h 3 at a concentration of 100 μg/mL compared to the groups with milder symptoms (Figure 3). Lowering the test concentrations, Ara h 2 and Ara h 6 revealed also a significantly higher response in the group with severe symptoms compared to the other groups. In all three patient groups, the SPT reactivity to Ara h 2 and Ara h 6 was higher than the SPT responses to Ara h 1 and Ara h 3, demonstrating a higher allergenic potency

of Ara h 2 and Ara h 6 *in vivo*. The preferential recognition of Ara h 2 and Ara h 6 is not likely due to the allergen content of peanut, since the amount of Ara h 1 and Ara h 3 has been reported to be higher than Ara 2 and Ara h 6.[10,30] In summary, these data suggest that in general sensitization to Ara h 2 and Ara h 6, and in addition recognition of Ara h 1 and Ara h 3, are indicative for severe symptoms. Recently, Hourihane et al[31] devised a scoring system that combined the dosage that elicited the reaction and the clinical symptoms reported. Although it is a good concept to account for dose in a symptom scoring system, it is more difficult for patients (and investigators) to estimate the dose in milligrams of peanut protein that elicited the reaction than to describe clinical symptoms. Therefore, the patients were categorized using the Muller score.

In general, currently used commercial SPT extracts use high concentrations of allergens to reach a high sensitivity. However, our data show that differences in the patients' sensitivity to allergens may not be resolved when applied in saturating concentrations, as observed for Ara h 2 and Ara h 6. This suggests that the SPT response to allergens in very high concentrations may become positive in all sensitized patients, including patients with very mild or even without symptoms.

Recently, it was suggested that diversity of IgE binding to peanut epitopes[20] or allergens [19] is more important than the recognition of a specific allergen as determined by peptide-array and IgE-immunoblotting, respectively. To further investigate this, we determined the number of allergens that resulted in a positive SPT response in each patient (Figure 4A). We found that patients with histories of more severe symptoms did recognize a significantly greater number of allergens, in line with previous studies.[19,20,32] Furthermore, this was illustrated by the fact that a significantly higher cumulative SPT response was observed in patients with severe symptoms compared to patients with milder symptoms (Figure 4B). However, this was only detected at lower test concentrations, showing the importance of diluted purified allergens for SPT.

The allergic reactions to peanut by history were confirmed by DBPCFC in the large majority (88%) of the patients. The NOAEL in our study population was 0.01 mg peanut flour, corresponding to about 3/100,000 of a peanut. This is to our knowledge the first time that a NOAEL is described in peanut-allergic adults, according to the threshold consensus protocol. The lowest ED in our study was 0.1 mg peanut flour and this corresponds well with other reports.[5,33] We reported previously that patients with a history of severe symptoms had a significantly lower ED than patients with mild reactions.[5] This observation could not, however, be reproduced in this study. Hourihane et al[31] has theorized that prediction of future risk in the community may not be appropriate from the isolated result of DBPCFC, based on a poor correlation between the community- and challenge-based reactions and the effect of the fat content in the food on the ED. In addition, we could not find significant differences in SPT responses to the individual allergens, number of allergens recognized and

the cumulative SPT response between patients with a low ED and a high ED. This suggests that the ED does not reflect the severity of peanut allergy.
In conclusion, this study illustrates the relevance of SPT with diluted purified peanut allergens showing that the reactivity to all four allergens tested is correlated to the severity of peanut allergy by history. The observed difference in potency of Ara h 2 and Ara h 6 on the one hand and Ara h 1 and Ara h 3 on the other needs to be confirmed in oral challenge testing in order to further investigate the role of the individual peanut allergens in eliciting clinical reactions.

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6

Identification of metabolic fingerprints associated with peanut allergy

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Submitted

Abstract

Background

The impact of peanut allergy is large. Accidental ingestion of peanut can lead to severe reactions. Currently used diagnostic tests such as SPT and determination of specific IgE have limited sensitivity and specificity. Metabolic fingerprints may provide biomarkers for diagnosing peanut allergy as metabolite levels reflect actual physiological conditions.

Objective

To investigate whether a metabolite fingerprint can be found in peanut-allergic patients that may be used for diagnostic purposes.

Methods

Plasma and saliva samples were obtained from 23 participants (12 peanut-allergic and 11 peanut-tolerant). Samples were measured with ^{1}H Nuclear Magnetic Resonance spectroscopy (NMR) with subsequent multivariate data analysis.

Results

Differences were observed between NMR spectra of peanut-allergic and peanut-tolerant subjects in plasma as well as saliva. We were able to determine a metabolic fingerprint associated with peanut allergy. Allergic patients already showed an aberrant metabolic profile prior to peanut ingestion, thus before the start of allergic reactions.

Conclusion

This study shows that a metabolic fingerprint may serve as a novel biomarker for peanut allergy.

Clinical implications

Metabolic fingerprinting offers prospects for a convenient and non-invasive manner of diagnosing peanut allergy.

Introduction

Peanut is one of the most common foods responsible for severe allergic reactions.[1] Careful clinical histories, *in vivo* skin prick tests (SPT) and *in vitro* serological assays (e.g. RAST and immunoCAP) are often used by clinical practitioners to assist in the diagnosis of peanut allergy. SPT provides a rapid method with a negative predictive accuracy generally greater than 95%[2]. However, the test has in general a relatively low specificity of about 50%. Moreover, the positive predictive value is only 20-50% [3], depending on the food extracts used, which differ in their content of individual allergens, vary between manufacturers, and even between batches.[4] *In vitro* tests for specific IgE are very helpful in evaluating IgE mediated food allergy, especially in children[5,6], but they also have a limited predictive value. Therefore, the double-blind placebo-controlled food challenge (DBPCFC) remains the gold standard for diagnosing peanut allergy.[7,8] The DBPCFC is however very expensive and laborious and not completely without risk to the patient. Therefore, new diagnostic tools that are less expensive and less burdensome for patients, such as for example component resolved diagnosis[9,10], are presently under investigation.

Many diseases result in biological derailment, which translates into disease-specific changes in metabolite and protein contents and their levels in biological fluids and tissues. For decades, clinical chemistry has been based on this principle. With the ongoing developments in the comprehensive analysis of biological systems, such as proteomics and metabolomics, it is now possible to screen biological fluids and tissues in a holistic approach discovering novel biomarkers for future use in clinical chemistry.

Metabolomics and metabolic fingerprinting research use analytical techniques like ^{1}H Nuclear Magnetic Resonance spectroscopy (NMR) or liquid chromatography-mass spectrometry (LC-MS) in combination with multivariate statistics in order to identify differences in levels of metabolites in biological fluids and tissues of patients and healthy controls.[11,12] Ultimately, this should result in metabolite fingerprints and biomarkers that are specific for certain diseases. For metabolic fingerprinting, NMR is an attractive approach because a wide range of metabolites can be measured simultaneously without extensive sample preparation. For instance, the use of NMR combined with multivariate statistics resulted in a metabolic fingerprint of urine that reflects the osteoarthritic changes both in guinea pigs and humans.[13,14] In metabolic fingerprinting, plasma is the body fluid that is often studied because blood is the biological fluid that delivers essential compounds to organs and is also used by the organs to eliminate waste products of reactions occurring in cells. Blood will thus reflect both homeostasis and biological derailment. A biological fluid like saliva is attractive as well as a diagnostic specimen, since it is a readily attainable biological

fluid that contains many hormones and antibodies.[15]
The study of alterations in metabolite levels by metabolic fingerprinting may be a good alternative for diagnosing peanut allergy.
We performed a pilot study to investigate whether differences can be observed in metabolites in plasma and saliva of patients with peanut allergy compared to peanut-tolerant subjects by NMR in combination with multivariate statistics. Saliva may contain mediators of allergy, since the immunological reaction to peanut may already start in the oral mucosa, the first organ to be in contact with food allergens.[16]

Materials and Methods

Study population

Twelve peanut-allergic patients (8 male and 4 female, mean age 25.4 years) were included in this study. The diagnosis of peanut allergy was confirmed in all patients by a positive DBPCFC.[17] The severity of symptoms by history was classified according to Mueller, a scoring system which was originally designed for the classification of allergic reactions to insect venom.[18] Symptoms of the oral cavity were classified as Mueller grade 0, symptoms of the skin and mucous membranes (urticaria, angioedema, rhinitis, conjunctivitis) as grade 1, gastrointestinal symptoms (diarrhea, vomiting, nausea, abdominal pain) as grade 2, respiratory symptoms (asthma, hoarseness) as grade 3, and cardiovascular symptoms as grade 4.
Eleven non-peanut-allergic persons (6 male and 5 female, mean age 27.3 years) who could tolerate peanut without any symptoms were included as controls.
This study was reviewed and approved by the Medical Ethical Committee of the University Medical Center Utrecht. All subjects gave written informed consent before enrollment in the study.

Sample collection

Peanut-allergic patients were allowed to have a light breakfast consisting of low-fat foods and a cup of tea up to two hours before the peanut challenge. Just before the administration of the first dose of peanut, blood was drawn and processed into plasma, and saliva was collected. When convincing symptoms occurred during the challenge at a certain dose, saliva was directly collected and stored. Blood was drawn one hour after the consumption of the last dose of peanut. The samples were stored at -80 °C.
The peanut-tolerant persons were also allowed to have a light breakfast up to two hours before the peanut administration. Blood and saliva were collected before peanut ingestion. Five minutes after ingestion saliva was collected and one hour later plasma was drawn.

NMR analysis of plasma and saliva samples

NMR is based on the principle that protons act like a magnet when placed in a magnetic field. The energy needed to flip a proton from a stable to a less stable state depends on the applied magnetic field and the environment of the proton. The energy needed is measured and the result is an NMR spectrum showing frequencies at which the various protons of a molecule absorb energy.[19] The advantage of NMR is that sample pretreatment is not necessary and that all compounds in a biological fluid can be measured simultaneously. However, biological fluids contain many metabolites. Consequently, NMR spectra are very complex for visual inspection. To overcome this problem, multivariate analysis is often used. Multivariate analysis is the application of sophisticated mathematical and statistical methods to the analysis of chemical data. With multivariate techniques, the complexity of data with many variables as acquired from e.g. NMR measurements is reduced. A large set of related variables, such as for instance hundreds of signals in numerous NMR spectra, is converted to a smaller set of uncorrelated variables. These variables express maximum variation in the original variables, the NMR signals. In this way, it is possible to express differences between NMR spectra, that is metabolite contents of biological fluids, between patients and healthy controls. The result is a score plot in which each complete NMR spectrum is visualized by a single point. In a factor spectrum, henceforth named metabolic fingerprint, the contribution of each variable to the differences observed in a score plot is visualized.

The NMR procedure we applied in this study is described in detail in previous articles.[13,14]

NMR data pre-processing and multivariate data analysis

Each NMR spectrum in the data file was segmented into regions of 0.04 parts per million (ppm) and the integral value for each segment was calculated. The region of the spectrum giving rise to water peaks (4.7-5.2 ppm) was removed. The NMR data reduction file was imported into Winlin (Version 2.3, TNO, The Netherlands). Data were mean-scaled so that small and large peaks contribute similarly to the final study result. Subsequently, principal component discriminant analysis (PCDA) was chosen as the multivariate data analysis technique. For this analysis, the NMR data sets were randomly divided into a training data set and a test data set. The multivariate models were built upon the training data set. Subsequently, the test data set was used to test the reliability of the training model. Health status (peanut-allergic versus peanut-tolerant) and sex were used as a priori information for carrying out PCDA. Models were built to visualize differences between NMR spectra of peanut-allergic and control cases and the scores of the samples were plotted. This procedure was followed for NMR data obtained before as well as after ingestion of peanut. The distinction between scores of peanut-allergic versus peanut-tolerant cases was correlated to the

original features (expressed in ppm) in the NMR spectra, which resulted in plasma and saliva metabolic fingerprints for peanut allergy. These metabolic fingerprints provided insight into the type of metabolites responsible for the difference in PCDA scores.

Statistics

The unpaired T-test was performed to evaluate the statistical significance of the difference between the scores of the groups (Excel 2003, Microsoft Corporation, USA). P values <0.05 were considered statistically significant.

Results

Study population

Twelve peanut-allergic patients proven by a positive DBPCFC were included in this study. The lowest dose of peanut flour that elicited a convincing subjective reaction (eliciting dose, ED) ranged from 0.1 mg to 300 mg peanut flour. Three peanut-allergic patients (25%) reported mild symptoms as most severe symptoms by history (Mueller grade 0 and 1), 2 (17%) reported moderate symptoms (grade 2), and 7 patients (58%) reported severe symptoms (grade 3 and 4). Ten patients (83%) also reported pollinosis symptoms, 6 patients (50%) suffered from concomitant asthma and 6 patients (50%) from atopic eczema.

Eleven peanut-tolerant persons entered this study of which two persons were sensitized to peanut, but tolerated the highest dose of 10 g peanuts during the challenge and therefore were considered not to be peanut-allergic. Three subjects (27%) reported pollinosis symptoms, of which 1 also suffered from concomitant asthma and atopic eczema.

The subject characteristics are summarized in Table 1.

Plasma metabolic fingerprint before peanut ingestion

Using multivariate statistics, differences between complete NMR spectra can be visualized in a simplified way in a score plot. In such a plot, a complete NMR spectrum of one subject is represented by a single point. In Figure 1A the NMR spectra of the study population before peanut ingestion are shown. The NMR spectra of the allergic patients were in a significantly different position on the score plot compared to the peanut-tolerant subjects ($p<0.01$), which means that the peanut-allergic patients had already demonstrated a significantly different NMR spectrum before peanut ingestion compared to peanut-tolerant persons. This difference was still significant after dividing the peanut-allergic patients and the peanut-tolerant subjects into a male and a female group (both, $p<0.01$). The NMR signals that cause the

Table 1
Subject characteristics

Group	Subject	Sex	Age	Atopy #	IgE grasspollen°	IgE birchpollen°	IgE peanut°	Severity (Mueller)*	Subjective ED (mg) §
Peanut-allergic	1	M	21	AR, AE	39	44	5.37	1	300
	2	M	32	AR	8.1	2.4	1.8	2	100
	3	M	25	AR, AA	34.2	5.8	49.5	4	0.1
	4	M	20	AR	36	68	5.07	1	0.1
	5	F	27	AR, AA, AE	>100	>100	12.9	2	10
	6	M	21	AR, AE	1.2	59	<0.35	0	10
	7	M	33	AR, AA, AE	>100	<0.35	>100	3	1
	8	F	24	AR, AA	<0.35	<0.35	18.3	4	10
	9	M	18	AR, AA, AE	>100	79	7.76	3	0.5
	10	F	29	AR, AE	<0.35	36	4.15	3	100
	11	F	28	-	1	<0.35	16.1	4	0.1
	12	M	27	AA	<0.35	<0.35	41	3	1
Peanut-tolerant	1	M	24	-	<0.35	<0.35	<0.35	-	-
	2	M	28	-	<0.35	<0.35	<0.35	-	-
	3	M	22	-	1.7	<0.35	<0.35	-	-
	4	M	22	-	<0.35	<0.35	<0.35	-	-
	5	M	25	-	<0.35	<0.35	<0.35	-	-
	6	M	23	-	<0.35	<0.35	<0.35	-	-
	7	F	27	-	<0.35	<0.35	<0.35	-	-
	8	F	23	-	<0.35	<0.35	<0.35	-	-
	9	F	28	AR	1.6	15	<0.35	-	-
	10	F	21	AR, AE, AA	55	>100	33.5	2	no
	11	F	57	AR	0.6	7.7	0.63	2	no

M, male; F, female;
AR, Allergic rhinitis; AE, allergic eczema; AA, allergic asthma
° ImmunoCAP
* Most severe symptoms by history scored according to Mueller.
§ ED, Eliciting dose
no, no ED detectable

differences between the peanut-allergic patients and the peanut-tolerant subjects are represented in a metabolic fingerprint (Figure 1B). Peaks in the positive direction indicate metabolites which were more abundant in the peanut-allergic patients and peaks in the negative direction indicate metabolites which were decreased. Elevated metabolites in the plasma of peanut-allergic patients that could be identified using our in-house database were lactate (ð 1.35 ppm), creatinine (ð 3.06 ppm) and glutamine (ð 2.06, 2.34, 3.75 ppm), whereas lipids (ð 0.79, 1.20, 1.98 and 5.25 ppm) and nicotinic acids (ð 7.53, 8.62 ppm) were decreased.

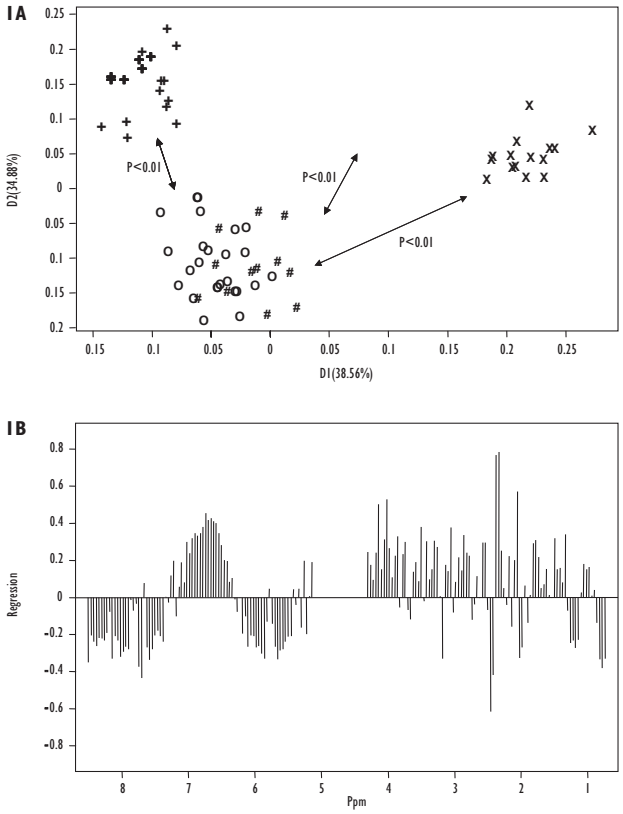

Figure 1
Differences between plasma metabolite contents of peanut-allergic patients and peanut-tolerant subjects before ingestion of peanut (+, male non-allergic; o, male allergic; x, female non-allergic; #, female allergic). (A) Score plot of NMR spectra of peanut-allergic subjects and peanut-tolerant controls. There is a difference between peanut-allergic and peanut-tolerant subjects ($p<0.01$). (B) Plasma metabolic fingerprint of peanut allergy before ingestion of peanut. Ppm, parts per million.

Plasma metabolic fingerprint after peanut ingestion

The NMR spectra after peanut ingestion were also studied. Figure 2A shows that the position of the NMR spectra on the score plot of the peanut-allergic patients was significantly different from the position of the peanut-tolerant subjects ($p<0.05$), demonstrating differences in NMR spectra of plasma between peanut-allergic and peanut-tolerant subjects after peanut ingestion. Taking into account the sex of the subjects, the NMR spectra of male peanut-allergic patients were still significantly different compared to the NMR spectra of male peanut-tolerant subjects ($p<0.01$) with a trend for the female group.

The metabolites responsible for the difference in position on the score plot of the peanut-allergic patients and the peanut-tolerant subjects are shown in the metabolic profile in Figure 2B. This profile shows similarities to the one obtained before ingestion of peanut, with increasing levels of lactate (ð 1.35 ppm), creatinine (ð 3.06 ppm) and glutamine (ð 2.06, 2.34, 3.75 ppm). In addition, an increase in tyrosine (ð 3.94, 6.91, 7.2 ppm) and tryptophan (ð 7.23, 7.6 ppm) was identified in peanut-allergic patients after peanut consumption compared to peanut-tolerant subjects.

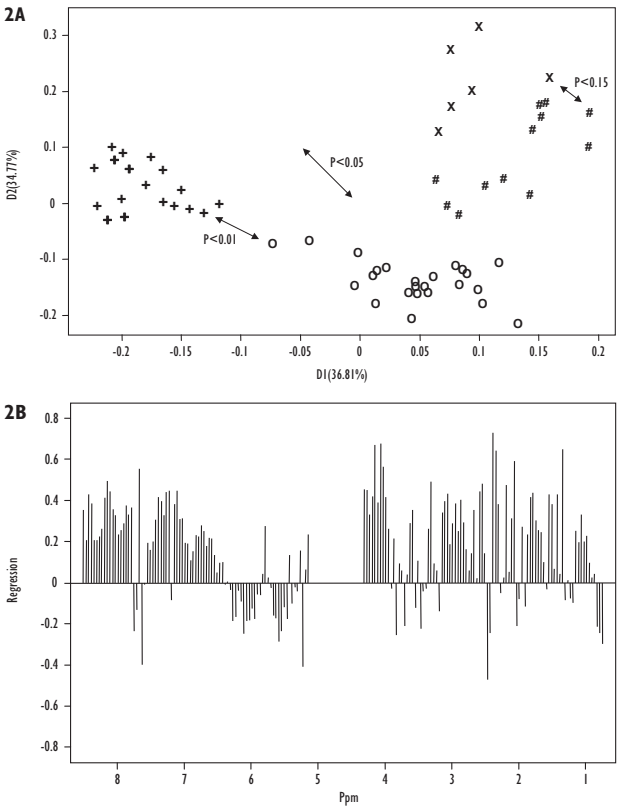

Figure 2
Differences between plasma metabolite contents of peanut-allergic and peanut-tolerant persons after ingestion of peanut (+, male non-allergic; o, male allergic; x, female non-allergic; #, female allergic). (A) Score plot of NMR spectra of peanut-allergic and peanut-tolerant subjects. There is a difference between peanut-allergic and peanut-tolerant persons ($p<0.05$). (B) Plasma metabolic fingerprint of peanut allergy after ingestion of peanut. Ppm, parts per million.

Saliva metabolic fingerprint before peanut ingestion

Saliva would seem an attractive source for mediators of allergy, since the first contact with a food allergen takes place in the oral cavity. Therefore, saliva specimens were investigated with NMR as to aberrant levels of metabolites. The NMR spectra of saliva of peanut-allergic patients before peanut ingestion were in a significantly different position from peanut-tolerant controls ($p<0.05$; Figure 3A). When the males and females were analyzed separately, the difference between the peanut-allergic patients and the peanut-tolerant subjects remained significant for male subjects ($p<0.01$) but not for females ($p<0.4$).

The metabolites responsible for the differences between peanut-allergic patients and peanut-tolerant subjects are represented in the metabolic profile shown in Figure 3B. Identification of metabolites from this fingerprint was not possible with the in-house database, since this was dedicated only for analysis of plasma samples. Nevertheless, these data illustrate that differences between allergic patients and controls are also reflected in saliva.

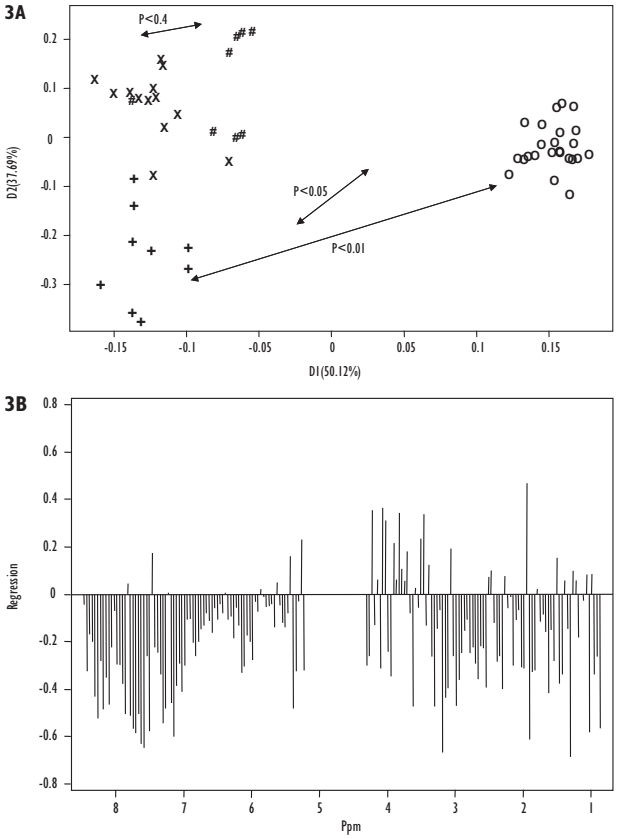

Figure 3
Differences between saliva metabolite contents of peanut-allergic patients and peanut-tolerant subjects before ingestion of peanut (+, male non-allergic; o, male allergic; x, female non-allergic; #, female allergic). (A) Score plot of NMR spectra of patients and peanut-tolerant subjects. There is a difference between peanut-allergic and peanut-tolerant subjects ($p<0.05$). (B) Saliva metabolic fingerprint of peanut allergy before ingestion of peanut. Ppm, parts per million.

Saliva metabolic fingerprint after peanut ingestion

Regarding the saliva samples after peanut ingestion, the NMR spectra from peanut-allergic patients and peanut-tolerant subjects were in a remarkably different position on the score plot (Figure 4A; $p<0.01$). Taking sex into account, the NMR spectra of male as well as female peanut-allergic patients were still significantly different compared to the NMR spectra of male and female peanut-tolerant subjects (both, $p<0.01$).

The metabolites that caused the difference in position on the score plot of the peanut-allergic and the peanut-tolerant subjects are represented in the metabolic profile in Figure 4B. The metabolites from the fingerprint could not be identified with the in-house database, but clear differences between peanut-allergic patients and tolerant persons are observed.

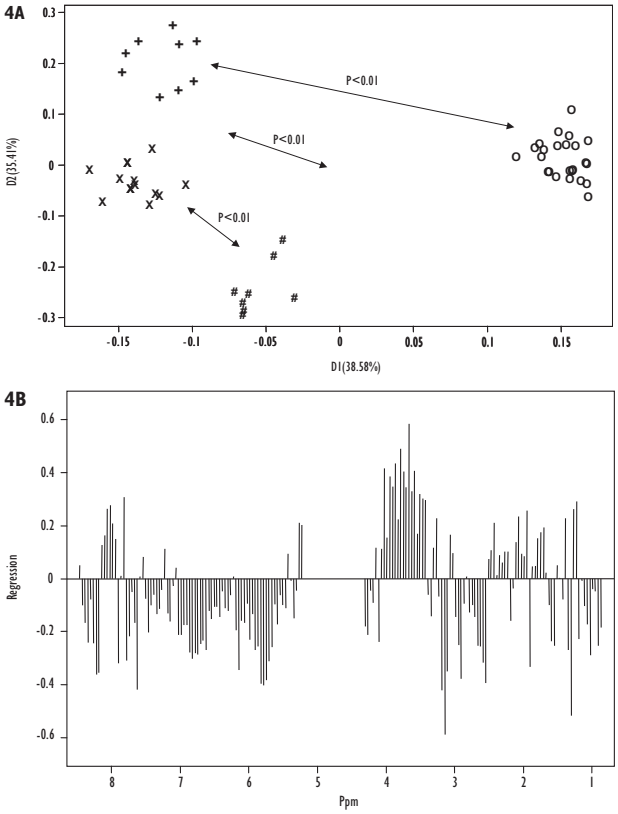

Figure 4

Differences between saliva metabolite contents of peanut-allergic patients and peanut-tolerant subjects after administration of peanut (+, male non-allergic; o, male allergic; x, female non-allergic; #, female allergic). (A) Score plot of NMR spectra of patients and peanut-tolerant persons. There is a difference between peanut-allergic and peanut-tolerant persons ($p<0.01$). (B) Saliva metabolic fingerprint of peanut allergy after ingestion of peanut. Ppm, parts per million.

Discussion

Metabolic fingerprinting can lead to the discovery of new biomarkers for diagnosis of diseases. In this pilot study we investigated whether we could find differences in plasma and saliva metabolites between patients with peanut allergy and peanut-tolerant subjects using NMR and subsequent multivariate data analysis.

We were able to visualize a difference in plasma metabolite levels of patients with peanut allergy compared to peanut-tolerant subjects, before as well as after ingestion of peanut (Figure 1A and 2A). Moreover, a clear difference was found in the saliva of peanut-allergic versus peanut-tolerant subjects which was most pronounced after peanut ingestion (Figure 3A and 4A). To investigate whether this finding was due to sex differences, males and females were considered separately. The NMR spectra of plasma and saliva before as well as after peanut ingestion in males were indeed still significantly different between peanut-allergic and peanut-tolerant subjects, whereas for females this difference was somewhat less clear and observed only in NMR spectra of plasma before peanut ingestion and in saliva after peanut ingestion. This suggests that metabolic profiles may be also influenced by hormonal fluctuations in women.

From the score plots of the NMR spectra, we were able to construct metabolic fingerprints that are associated with peanut allergy, but it was impossible to identify all the metabolites, because many of the observed NMR signals were not annotated in our database. For this purpose, LC-MS will be an indispensable tool in future. In this study we were able to identify elevated levels of lactate, creatinine and glutamine from the plasma fingerprint in the peanut-allergic patients before as well as after peanut ingestion (Figure 1B and 2B). This suggests that these are biomarkers for allergic status. Creatinine and lactate are commonly encountered metabolites in metabolomics studies which are often associated with production by gut microflora bacteria.[11] Glutamine is an important nutriment for the enterocytes that are abundantly present in the epithelial lining of the gastro-intestinal tract. It therefore plays a roll in the maintenance of gut mucosal integrity and function. Food allergy may result in a change in intestinal permeability through cytokine release in response to food allergens.[19,20] An elevation of glutamine levels in plasma may point to surplus production of glutamine in order to counteract increased permeability. On the other hand, cells such as lymphocytes and macrophages depend on glutamine as a primary fuel source.[21] As a result, the demand for glutamine increases when an immunological response is mounted. This may be reflected in an increase in plasma glutamine levels due to elevated production of glutamine by cells in response to the demand for glutamine.

Even before peanut consumption, lipid and nicotinic acid levels were also lowered in the peanut-allergic patients. Lipids, mainly polyunsaturated fatty acids, are known

to be involved in regulating immune function. It has been shown that levels of HDL cholesterol are positively associated with atopy, suggesting that fat consumption and metabolism play a role in the manifestation of allergy.[22] It remains unclear why lipid levels are lower in peanut-allergic patients.

After peanut ingestion an elevation in levels of tyrosine and tryptophan was observed in the peanut-allergic subjects, something that was not observed before consumption. Tyrosine is a precursor to the biologically active catecholamines dopamine, norepinephrine and epinephrine.[23] These compounds are produced by the body in response to stress. Ingestion of peanut will cause a stressful situation in peanut-allergic subjects, which may explain the elevation of tyrosine in plasma. The higher level of tryptophan in peanut-allergic patients might be due to indoleamine 2,3 dioxygenase (IDO), an enzyme that is involved in the catabolism of the amino acid tryptophan. Recently, it was suggested that IDO has a protective role in the prevention of progression from atopy to allergy.[24] So, the lower level of tryptophan in peanut-tolerant persons may be explained by a higher level of IDO.

A full explanation of the biology involved in peanut allergy is beyond the scope of our study. Studies in future may also comprise transcriptomics and proteomics to further unravel the metabolism involved in disease development and occurrence of peanut allergy.[25]

In conclusion, using NMR, differences between metabolite levels of patients with and without peanut allergy were detected as well as differences before and after DBPCFC in patients with peanut allergy. This indicates the presence of potential biomarkers for peanut allergy. The availability of tests based on a metabolic fingerprint from saliva or plasma will be of great benefit for patients because they present no risk to the patient.

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7

General discussion

Introduction

IgE-mediated food allergy is an important cause of hospital admissions for systemic allergic disorders and increased from 5 in 1990 to 26 per million of the population in 2003 in England.[1,2] Allergic reactions to food can even result in fatalities.[3,4] Peanut allergy tends to be more severe in nature than other food allergies and accounts for 63% of the fatalities due to anaphylactic reactions to food.[3,4] Lupine, like peanut, is a legume, and was introduced as a novel ingredient into food manufacturing during the 1990s. It has become increasingly important as a replacement for soy, since soy is often genetically modified and therefore less attractive for consumers. However, lupine has been shown to cause potentially severe allergic reactions.[5-7]

Since food allergy in general appears to have increased in prevalence,[2,8,9] its pathogenic mechanisms and predisposing factors are widely debated. Curative treatment of peanut and lupine allergy is not yet available, so patients have to cope with the burden of avoidance diets.[10]

This thesis focuses on the clinical relevance of sensitization to peanut and lupine in adults. The potential routes of sensitization, a key step in the pathogenesis of food allergy, will be discussed below, including the role of peanut oil-containing therapeutics. Moreover, the utility of measuring sensitization to individual peanut allergens in predicting the severity of clinical symptoms to peanut was investigated. With the aim of improving diagnostic tools, the potential of a new convenient and non-invasive manner of diagnosing peanut allergy by metabolomics was studied.

Routes of sensitization

The route of sensitization to food is still a matter of debate. Understanding of the mechanism of primary sensitization to allergens is important in elucidating the pathogenesis and for the development of preventive strategies. The skin, lungs and gastro-intestinal tract are the first lines of defence that are exposed to the external environment. Regarding food, the gastro-intestinal tract seems to be the most important route, since foods are ingested.

In the nineties, there was concern that the use of peanut oil-containing skin products could be a possible sensitizing factor for peanut allergy.[11] In *chapter 2* we showed that the protein content of pharmaceutical grade refined peanut oils and refined edible peanut oils was not detectable (<0.3 ng/mL) using different methods, suggesting that sensitization to peanut by peanut oil-containing skin products is unlikely. We recognize that our inability to detect peanut protein in refined peanut oils does not completely exclude the possibility of sensitization by doses lower than the detection

limit of our assays. As it is unethical to induce new sensitization in humans, this aspect was only studied in animals. For peanut, epicutaneous exposure to 100 μg of peanut protein (4 mg/mL) for 3 days on the ears of high IgE-responder BALB/c mice with disrupted skin was shown to have the capacity to induce a Th2 resonse.[12,13] However, apart from differences between the murine and human immune responses, one has to realize that murine skin is much thinner than human skin. Application of 100 μg of peanut protein for a few days on murine skin is a relatively high amount. In comparison, one drop of peanut oil with a protein content of less than 0.3 ng/mL, the detection limit of our assay, contains over a million-fold less protein than 100 μg. Nevertheless, in a case-control birth cohort study it has been suggested that peanut allergy could develop in response to topical exposure to peanut oil through inflamed skin in children.[14] More children were exposed to preparations containing peanut oil in the peanut-allergic group compared to the atopic and normal control groups, while the use of products not containing peanut oil was similar in all three groups. Since in this retrospective part of the study the reported severity of eczema was not statistically higher in the peanut-allergic group compared to the atopic control group, the association between the use of peanut oil-containing preparations and peanut allergy was not explained by the presence of eczema.[14] Therefore, the authors concluded that topical use of peanut oil-based preparations on inflamed skin might have been one of the causal factors in the development of peanut allergy.[14]

In the prospective part of the previously mentioned study a strong relation was observed between the severity of eczema and the prevalence of peanut allergy.[14] A possible explanation for this association is the presence of an epidermal barrier defect in patients with atopic eczema, which leads to an increased permeability of the skin. Theoretically, the barrier defect may allow entry of allergens, which can lead to sensitization. This is supported by the fact that in patients with eczema loss-of-function mutations in the gene encoding filaggrin, an epidermal skin barrier protein, were found.[15-18] However, filaggrin null mutations are only present in 10-40% of patients with eczema[17,18] and about 10% of the general population also carry one or more filaggrin null alleles.[16,18] These data, together with the fact that no peanut allergens were detectable in the topical preparations containing peanut oil, suggest that the severity of eczema and peanut allergy[14] are not causally related. The association may be explained by the atopic background.

Taken together, the arguments that primary sensitization to food allergens may occur through the skin are rather weak. A more probable route of sensitization to peanut is exposure of allergens via the gastro-intestinal tract.

The mucosal surface of the body is about 200 times larger than that of the skin[19] and is much more permeable, which makes primary sensitization through the gastro-intestinal tract more plausible. Peanut is introduced early into children's diets. For example, peanut butter on bread is given to children before the age of 1 year.[20] In addition,

peanut is often used as an ingredient in prepared foods and can also unintentionally occur in food because of cross-contamination during food processing. Moreover, unintentional transfer of trace amounts of peanut from the skin to the mouth can take place easily. It has been reported that inadvertent peanut exposure by contamination in environmental settings such as homes or schools frequently occurs.[21] Samples taken from hands which had been in contact with peanut butter revealed residual Ara h 1 (a major allergen) after washing with plain water or antibacterial hand sanitizer (0.2-12.4 μg and 0.2-2.6 μg, respectively),[21] which supports the possibility of accidental ingestion of peanut allergens. Although the amount and frequency of peanut ingestion needed to require sensitization is not known, this route of sensitization seems to be most important.

Apart from direct sensitization as is described above, indirect sensitization based on cross-reactive IgE-antibodies can also result in food allergy. Extensive serological cross-reactivity between peanut, pea, chick pea, and soy has been described in peanut-allergic children.[22] Clinically relevant co-sensitization only occurs in about 5% of legume-allergic children.[23,24] In *chapter 3* we showed that clinically relevant co-sensitization to either lupine, pea, or to soy was present in a third of peanut-allergic patients, demonstrating that this is more common than previously thought. However, it has not yet been established whether combined allergies to different members of the legume family were caused by cross-reactivity or by independent sensitization to these legumes in this study. Since previous studies were performed in children,[23,24] it may be that clinically relevant cross-reactivity rates of legumes are higher in adults compared to children due to dietary habits. This is supported by the fact that allergy to cow's milk and egg usually starts before the second year, whereas allergy to foods of vegetable origin (fruit, legumes), mainly begins after the second year, demonstrating a clear relationship with the moment of introduction of these foods into a child's diet.[25,26] This is further illustrated by the finding that sesame allergy is a common food allergy in Israeli infants and young children, as they ingest sesame in high amounts before their first birthday.[27] It has been suggested that the consumption of soy by infants may be a risk factor for developing peanut allergy by cross-sensitization through common epitopes.[14] However, a recent prospective study in cow's milk-allergic infants showed that the use of soy formulas during the first 4 years of life did not result in an increase of peanut allergy.[28]

It is not completely known what factors differentiate between clinically relevant and non-relevant cross-reactivity. Among possible explanations are IgE-affinity differences[29] and recognition of cross-reactive carbohydrate determinants (CCD).[30] Variations in the stability of allergens after food processing[31-34] and matrix effects of food components[35] are other factors that affect the clinical outcome.

Cross-reactivity between legumes is frequently reported. In *chapter 4* we studied lupine-allergic patients with and without concomitant peanut allergy. Patients with a

combined lupine and peanut allergy recognized other lupine allergens than did patients allergic to only lupine. It could be that lupine allergy in patients with a combined peanut allergy was a consequence of peanut allergy, because peanut was introduced to these patients earlier in life than lupine. However, we did not find evidence of cross-reactivity of the allergens involved. Lupine-only allergic patients did not report any symptoms to other foods, so in these patients primary sensitization by ingestion of lupine seems plausible.

Sensitization to lupine via inhalation has also been reported to give rise to allergic reactions to lupine in food.[36] However, these subjects worked at a lupine laboratory, which is not representative for airborne exposure to lupine allergens in general, making this route of sensitization very unlikely for a general population.

A third route of sensitization to legumes is via exposure to birch pollen through the respiratory tract. Bet v 1 homologues were found in various legumes: Ara h 8 in peanut[37], Gly m 4 in soy[38,39], and Vig r 1 in mungbean.[40] Birch pollen-related allergy to fresh fruit, vegetables, or nuts caused by IgE cross-reactivity of food allergens with the major birch pollen allergen Bet v 1 is a well known phenomenon.[30] In general, Bet v 1-related food allergy is mild and mostly limited to the oral cavity, because Bet v 1-related allergens appear to be pepsine-sensitive and heat-labile proteins. However, severe symptoms to food containing Bet v 1-related allergens have been described.[37,39,41] Ara h 8 was found to be unstable upon heating, and pepsine digestion completely removed its IgE reactivity.[37] These findings suggest that systemic symptoms in patients sensitized to Ara h 8 possibly occurred because of buccal mucosa absorption of intact allergens,[42] or that these allergic reactions were induced by more digestion-stable peanut allergens like Ara h 2 and Ara h 6.[43,44] Considering birch pollen sensitization as possible route for legume allergy, we found that most of our peanut-allergic patients studied in *chapter 5* were indeed sensitized to birch pollen. However, in the majority the onset of peanut allergy occurred before the onset of birch pollen allergy, suggesting that peanut allergy was developed before sensitization to birch pollen allergens.[25] Moreover, peanuts are usually consumed as roasted peanuts that no longer contain Ara h 8, which makes the clinical relevance of birch pollen-induced sensitization based on cross-reactivity between Bet v 1 and the heat-labile Bet v 1-related allergen Ara h 8 very unlikely.

Taken together, it is likely that both peanut and lupine sensitization occurs via the gastro-intestinal tract.

Peanut allergy testing: component resolved diagnosis or determination of biomarkers?

Component-resolved diagnosis (CRD) uses defined recombinant or purified allergens to dissect the individual patient's IgE reactivity profile with the aim of identifying significant associations between individual allergen components and clinically relevant aspects of the allergic disease.[45,46] The clinical significance of current diagnostic tests may improve by using purified allergens.

The major and minor allergens of peanut and most of their epitopes have been identified. Ara h 1, Ara h 2 and Ara h 3/4 have been generally accepted as major allergens of peanut, but a role for Ara h 6 should not be excluded.[47-50]

In *chapter 5* we showed that sensitization to a specific purified peanut allergen determined by skin prick test (SPT) was not related to the severity of symptoms. Most patients included in this study reacted to Ara h 2 and in addition to the structurally similar[50] allergen Ara h 6, suggesting that these allergens appear to be the most interesting ones with regard to diagnosing peanut allergy. It was previously shown that Ara h 2 was a more potent allergen than Ara h 1[51,52] and Ara h 3[51] using IgE-immunoblotting, basophil-histamine release and intracutaneous testing. In *chapter 5* we confirmed that Ara h 2 and Ara h 6 are more potent allergens than Ara h 1 and Ara h 3, because SPT responses to Ara h 2 and Ara h 6 became positive at lower test concentrations than Ara h 1 and Ara h 3. This may imply that allergen concentrations can affect the outcome of an SPT response, especially when allergens have different allergenic potencies. To estimate the relevance of the different allergens, comparisons of SPT reactivities were related to the allergen content in food. The relative presence of Ara h 1, Ara h 2, and Ara h 6 in various main peanut cultivars was about the same while the Ara h 3 content was somewhat higher,[50,53] which justifies comparing these allergens at the same test concentration.

For many allergenic plant foods, purified or recombinant allergens are becoming increasingly available and can soon be evaluated for use as diagnostic tools.[54] For example, the sensitivity of the ImmunoCAP test for soy has been increased from 45% to 95% by coupling the individual allergen r Gly m 4 (the Bet v 1-related allergen) to this serological test instead of whole soybean extract.[38] For some other foods, e.g. cherry, hazelnut, peach and apple, component-resolved diagnosis might be used to correlate recognition of a particular allergen with the severity of symptoms.[32,55-58] In the case of apple allergy, mild symptoms were related to sensitization to Mal d 1 (the Bet v 1-related apple allergen), whereas severe symptoms were related to Mal d 3.[32] Although for peanut the severity of symptoms could not be related to a specific allergen, in *chapter 5* it was shown that patients with polysensitization to peanut allergens had more severe symptoms than patients who recognized few allergens, in accordance

with previous studies.[59,60] Shreffler et al[61,62] reported that patients with IgE-antibodies binding to many epitopes of Ara h 1, Ara h 2 and Ara h 3 tend to have more severe allergic reactions compared with those who have IgE-antibodies binding to relatively few epitopes. Beyer et al[63] demonstrated differences in peanut epitope recognition pattern of Ara h 1, Ara h 2 and Ara h 3 between peanut-sensitized patients who are clinically tolerant and those who have symptomatic peanut allergy despite the presence of high or low peanut-specific IgE. The most striking difference was observed for Ara h 2 and Ara h 1, with significantly more recognition of immunodominant epitopes in symptomatic patients compared to asymptomatic patients. The association between the severity of symptoms and the number of allergens recognized may thus be explained by the presence of epitope diversity.

Regarding lupine, allergens of molecular weights ranging from 14 to 75 kDa have been described.[31,64-68] In *chapter 4* it was shown that patients with a combined lupine and peanut allergy recognized proteins with molecular masses of 14 kDa and 24 kDa, whereas in contrast, lupine-allergic patients without peanut allergy almost exclusively recognized a lupine protein of 66 kDa, identified as a beta-conglutin precursor. This indicates that there are remarkable differences in allergen recognition between lupine-only-allergic patients and lupine-allergic patients with a combined peanut allergy. When the relevant or major lupine allergens have been completely identified, it remains to be determined whether component-resolved diagnosis will aid in diagnosing lupine allergy. In addition, if specific roles can be attributed to individual lupine allergens, the recognition of major lupine allergens may be linked to the clinical severity of symptoms.

In *chapter 6* we showed that metabolic profiling using ^{1}H Nuclear Magnetic Resonance spectroscopy (NMR) and subsequent multivariate data analysis offers prospects for a completely new, convenient and non-invasive manner of diagnosing peanut allergy, since metabolite profiles which were associated with peanut allergy were discovered in plasma and saliva.

As differences between metabolite levels were observed in peanut-allergic patients before and after double-blind placebo-controlled food challenges (DBPCFC), NMR and subsequent multivariate data analysis may be well suited for a more objective manner of determining subjective symptoms during challenges. It has been recommended to continue DBPCFC until objective symptoms have occurred,[69] which can result in potentially severe clinical symptoms. Besides, DBPCFC are time-consuming and costly. Moreover, challenge materials to diagnose clinical symptoms to labile allergens in a double-blind placebo-controlled fashion are difficult to prepare, because of their rapid degradation. Therefore, NMR and subsequent multivariate data analysis would improve the reliability and accuracy of ascertaining clinical symptoms to food during challenges by avoiding the patient's subjective bias.

But first, identification of the biomarkers associated with peanut allergy and their

specificity in relation to different food allergens needs further study before this tool can be implemented in diagnosing food allergy. Moreover, evidence of reproducibility needs to be provided in a larger study population.

Impact of clinical reactivity to peanut and lupine

To date, despite its drawbacks, DBPCFC is still the gold standard for diagnosing food allergy.[70] In 2004, a consensus protocol was developed.[69] Using this protocol, data from various clinical investigators can more easily be compared. In *chapter 3, 4* and *5* we determined eliciting doses (ED) and no-observed-adverse-effect-levels (NOAEL) using this consensus protocol with some modifications.[71] Individual doses eliciting subjective symptoms varied in our studies from 0.1 to 300 mg peanut flour for peanut-allergic patients (corresponding with 1/3000 to 1 peanut) and from 0.5 mg to 3000 mg lupine flour for lupine-allergic patients (a certain cookie contains about 300 mg lupine flour, information from the hospital dietician). Considering objective symptoms, the ED that could be established in peanut-allergic patients varied from 10-3000 mg peanut flour and for lupine from 300-1000 mg lupine flour. These data demonstrate a great individual variability for both legumes tested within comparable ranges. Previous studies showed that subjective symptoms to peanut were elicited at doses as low as 100 µg peanut protein[72,73] (corresponding with about 0.2 mg[72] and 0.4 mg[73] peanut flour), whereas objective symptoms were elicited at doses of 2 to 50 mg[72] (corresponding with 4-100 mg peanut flour) and at doses of 10 to 30 mg peanut protein[73] (corresponding with 40-120 mg peanut flour). Although interpretation and comparison of these and our clinical data is complicated by the use of different protocols,[74] these studies show that low doses of peanut are already able to induce symptoms in peanut-allergic patients. This can further be illustrated by the fact that allergic reactions via saliva exchange can occur.[75,76] Regarding lupine, there is little information in the literature on the lowest dose of lupine that will induce clinical symptoms. So far, the lowest (cumulative) ED determined in 6 peanut-allergic patients by DBPCFC with increasing lupine flour doses starting from 5 mg, is 265 mg lupine flour, inducing abdominal pain and asthma.[65] In *chapter 3* and *4*, much lower ED for lupine were established, starting from 0.5 mg lupine flour. Further research is needed in a larger lupine-allergic population to confirm the ED results of our studies.[77]
Although one has to realize that that many factors, including alcohol use or the matrix in which the allergen is presented, may affect the elicitation of symptoms,[35,78] the knowledge of an individual ED can play a role in the prediction of future reactivity and in the establishment of safe and effective avoidance diets. For example, if the ED is relatively high, the level of concern about ingestion of unintentionally encountered food allergens would be lower than for patients with a low ED. Apart from the lowest

dose eliciting symptoms in allergic patients, more information is needed for risk assessment, like the amount of allergenic protein present in the ingredient, the level of usage of the ingredient in food and the nature of the ingredient.[69,79]

To provide allergic consumers with better information about food allergens in food, new food labelling legislation came into effect in the European Union in November of 2005. The new legislation requires labelling of 11 major allergens and sulphite that have intentionally been introduced in a food, regardless of the level of use: milk, egg, fish, crustacean shellfish, peanut, soybean, tree nuts, cereals containing gluten, celery, mustard, and sesame seeds.[80] Efforts are underway to consider the addition of lupine and molluscan shellfish (oysters, clams) (www.efsa.europe.eu). Since all lupine-allergic patients included in *chapter 3* and *4* were not aware of the use of lupine as an ingredient and did not even know they were lupine-allergic, this will be of limited benefit for food-allergic patients without education about the use of lupine in food. The food labelling regulation does not apply to residual amounts of allergens that could unintentionally occur because of cross-contamination during processing, although the consequences may be similar in terms of risk for allergic consumers.

A particular area of interest concerns the allergenicity of edible oils, which are frequently derived from commonly allergenic foods such as peanut and soybean. Refined soybean oil can be safely ingested by soy-allergic individuals,[81] whereas sesame or tree nut oil may contain protein residues depending on the way of processing and are more likely to be hazardous to consumers with allergies to these foods.[82,83]

It is accepted that the allergenicity of oils is related to the residual protein remaining after processing. In *chapter 1* we showed that in crude oils amounts of protein (approximately 100-6000 ng/mL) were detectable, while in edible refined and pharmaceutical refined oils and products made from them no protein was detectable (<0.3 ng/mL). These results were supported by measuring nitrogen as a marker for protein and by immunoblot experiments with serum of peanut-allergic patients. It is known that crude peanut oil, which can contain up to 300 μg protein per ml,[84] is sometimes added to the odourless and flavourless refined oil for its peanut flavour,[85] but also in these edible oils, no protein was detectable. Even the peanut-allergic patients with the lowest ED, those who react to 0.05 mg of peanut protein (*chapter 5*), would have to consume over a hundred litres of peanut oil containing 0.3 ng/mL in order to induce symptoms. So, refined peanut oil can be safely ingested by peanut-allergic patients. This is in accordance with a previous study in which none of 60 peanut-allergic patients reacted to a total dose of 16 ml refined peanut oil.[85]

Future research

An obstacle in the field of food allergy diagnosis is that with the exception of DBPCFC, no single *in vitro* or *in vivo* test is able to reliably predict the clinical reactivity of a patient. If the immune response is directed to particular allergens within the same food with different clinical relevance, novel diagnostic tools can be developed that better predict clinical reactivity.

The observation that sensitization to multiple peanut allergens is associated with the severity of symptoms to peanut (*chapter 5*) offers the possibility of developing reagents containing different numbers of allergens for SPT in order to predict severity. Since both the potency of allergens and the test concentration used appear to have an effect on the SPT reactivity, these topics needs to be further investigated before such an approach can be implemented in daily practice. Protein micro-array, a technology under development which requires just a few drops of blood, may be another tool to determine individual peanut sensitization profiles. The *in vivo* technique of SPT used in *chapter 5* provides information regarding direct activation of skin mast cells, thereby inducing histamine release. However, challenges in patients with a clinically proven allergy should be performed with individual allergens in order to assess their clinically relevant allergenicity.

The importance of lupine allergy becomes increasingly evident. It is necessary to improve the current diagnostic tests, since two thirds of lupine-sensitized patients were not lupine-allergic. Therefore, the major allergens involved in allergic reactions should be identified and characterized.

Another ongoing line of research which may aid the diagnosis of food allergy in a completely different way is that of NMR and subsequent multivariate data analysis, as was described in *chapter 6*. Because it is still in its infancy, this diagnostic test needs further study.

Over the past several years, a significant amount of research has been conducted into possible therapies for food allergy. Unfortunately, at this time the only solution is still to avoid the offending ingredient. Efforts are underway to develop potential therapies, e.g. T-cell epitope based peptide immunotherapy[86] or mutated protein immunotherapy[87]. Cross-reactive allergens have also been suggested as desensitizing agents in allergen-specific immunotherapy. These new approaches will benefit from an increased knowledge of the molecular and immunologic characteristics of food allergens.

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Summary

Food allergy is a frequently occurring type of hypersensitivity to food in which immunoglobulin type E (IgE) antibodies play a role. Allergic reactions to food have received increasing attention in recent years. Whether the actual prevalence or just the awareness of food allergy has risen remains unclear. A distinction should be made between sensitization (the presence of specific IgE without symptoms) and allergy (the presence of specific IgE with symptoms). Skin prick tests and/or blood tests are needed to determine whether an individual has become sensitized.

Allergic symptoms may consist of an itchy feeling in the mouth and throat (oral allergy syndrome), either isolated or combined with gastro-intestinal symptoms, respiratory symptoms, and in the most severe cases, a drop in blood pressure and shock, which may be fatal. Of all food allergens, peanut is the most frequent cause of serious allergic reactions to food. Peanut-allergic patients however, manifest wide variations in the severity of their symptoms. Some patients may already feel itch in their mouth and throat after ingestion of less than a thousandth of a peanut.

The skin prick test that is used to reveal specific IgE has a fairly low sensitivity, but if the test is negative, allergy to the allergen tested is very unlikely. The blood test also has a low sensitivity and moreover often gives false-positive test results. Currently, the double-blind placebo-controlled food challenge is still considered the most reliable test for diagnosing food allergy. This test is conducted by offering the suspected food to the patient in gradually increasing doses. As a control, a challenge is carried out with placebo portions not containing the allergen (placebo-controlled). Because neither the patient nor the doctor or nurse know which portions contain the allergen (double-blind), subjective influence is kept to a minimum. An allergy is diagnosed if symptoms arise after ingesting the portions containing the allergen but not after eating the placebo portions. Such a food challenge is burdensome to the patient and requires good facilities and experienced personnel. For this reason, researchers are actively seeking new diagnostic tests as well as methods to improve the diagnostic value of existing blood and skin prick tests.

The route of sensitization to food is still a matter of debate, but the most probable one is through the gastro-intestinal tract. Sensitization via inhalation can also lead to food allergy. A well-known example is that of birch pollen-allergic patients developing allergies to nuts, fresh fruits and some vegetables (cross-reaction). The skin has also been suggested as a possible route of sensitization. It has been proposed that peanut allergy might develop in response to topical exposure to peanut oil-containing skin products through inflamed skin in children. In *chapter 2* we investigated whether unrefined peanut oils and refined peanut oils at different stages of the refining process (neutralization, bleaching, deodorization) still contain peanut allergens. Moreover, samples of various peanut oils for sale in Dutch supermarkets were investigated. We also looked at peanut oils used in pharmaceutical products and at pre-packaged products containing peanut oil, such as vitamin K drops. Our results indicate that

only crude, unrefined peanut oils contain peanut allergen in very small quantities. As mandated by local pharmacopeias, only refined peanut oil is used in pharmaceutical preparations. Pharmaceutical products such as vitamin K drops, which are over-the-counter preparations, fall under the auspices of the Dutch Commodities Act and don't have to meet the specified requirements. Nevertheless, no allergens were discovered in these products either. We therefore think it is extremely unlikely that peanut allergy could develop in response to the use of skin products containing peanut oil.

In recent decades the Dutch diet has undergone changes. Foods are now imported from everywhere on the globe, and allergies to these 'new' foods can be expected. Examples of allergic reactions to food that relatively recently entered the Dutch market are kiwi and lupine. Lupine, like peanut and soy, is a legume and has been used in the food industry since the 1990's. Because lupine is a high-protein food that is inexpensive and easy to cultivate, it is frequently used as an alternative for soy. Soy, moreover, is sometimes genetically modified, which raises objections amongst consumers.

In *chapter 3* we determined the extent of sensitization to lupine (and pea and soy) in peanut-sensitized patients. We also investigated the proportion of sensitized patients who actually develop symptoms, so-called clinically relevant sensitization. We demonstrated that sensitization to lupine, pea and soy very frequently coexists with peanut allergy. Clinically relevant sensitization occurred in about 30% of patients. This is a high percentage, because this was previously reported in only about 5% of legume-allergic patients.

A striking finding in this study was that none of the 39 included patients had ever heard of lupine as an ingredient in food. Oral symptoms occurred in some patients already after ingesting tiny amounts of lupine flour (0.5 mg). To compare, one cookie contains about 300 mg of lupine flour. The lowest dose inducing an allergic reaction is called the eliciting dose. All patients studied in *chapter 3* tolerated a dose of 0.1 mg dose of lupine flour.

Because there is still no effective therapy for food allergy, food-allergic patients have to avoid offending foods. Knowledge of individual patients' eliciting doses would help physicians provide better dietary advice to these patients. However, caution is still called for as little is known about the stability of individual eliciting doses over time. Knowledge of eliciting doses is also of great importance in enabling the food industry and government oversight agencies to ensure the safety of foods for allergic patients in the future.

The new European food-labeling legislation assists patients in avoiding allergens by requiring labeling of the 11 most common food allergens, such as peanut and tree nuts, in food products. Food companies are not yet compelled to label lupine, but its inclusion in the list has already been recommended. One must realize, however, that labeling is only useful if a patient is aware of his food allergy. This bears out the importance of both accurate diagnostic methods and good patient education.

In *chapter 4* we studied whether lupine allergy in patients with and without concomitant peanut allergy is caused by recognition of various allergens in lupine. The IgE antibodies of patients with a combined lupine and peanut allergy recognized other lupine allergens than those of patients allergic to only lupine. The allergen with a molecular weight of 66 kDa that was recognized only by the lupine-allergic patients who were not allergic to peanut was further investigated and seemed to be a storage protein. In peanut allergy storage proteins such as Ara h 1, Ara h 2, Ara h 3/4 and Ara h 6 are known to cause allergic reactions. Inhibition experiments (immunoblot inhibitions) performed with the blood of lupine-allergic patients with a combined peanut allergy failed to demonstrate any appreciable cross-reactivity between lupine and peanut. This study showed that unique allergens were involved in lupine allergy. Lupine allergy can thus occur either separately or together with allergies to other legumes (co-allergy).

At present, skin prick tests are usually performed with food extracts. Some important allergens can be lost in this process, leading to false-negative results. If the relevant allergens in a food product are known, skin prick tests can be carried out with these purified or recombinant allergens instead of the whole extract, improving the reliability of the test of peanut. The most important allergens of peanut are known. In *chapter 5* we investigated whether sensitization to these peanut allergens (Ara h 1, Ara h 2, Ara h 3 and Ara h 6) is correlated to the severity of peanut allergy. Most patients recognized Ara h 2 and Ara h 6. Patients with severe symptoms to peanut had a higher skin prick test response to Ara h 2 and Ara h 6 at low concentrations, and to Ara h 1 and Ara h 3 at high concentrations as compared to patients with mild symptoms. Patients with severe symptoms also recognized more allergens than did patients with mild symptoms. No significant differences were observed between patients with respectively high and low eliciting doses to peanut. We conclude that skin prick tests with purified peanut allergens can be important for predicting the severity of peanut allergy, because the skin prick test reactivity to all four allergens tested is correlated to the severity of peanut allergy.

In *chapter 6* we investigated the value of using nuclear magnetic resonance (NMR) in combination with multivariate data analysis (MVDA) as a new diagnostic method for peanut allergy. NMR is a technique that enables identification and quantification of intermediate or end products of biological processes in body fluids. Previously, this technique revealed differences in the composition of urine between osteoarthritis patients and healthy controls. We showed that the blood and saliva of peanut-allergic patients, both before and after ingestion of peanut, have different biomarker profiles than peanut-tolerant subjects. In the future this non-invasive method may offer new prospects for diagnosing peanut allergy.

This thesis demonstrates that the use of purified allergens and techniques such as NMR has the potential to improve current diagnostic capabilities. It also shows that

lupine allergy is fairly common in The Netherlands. Characteristics of lupine allergy, such as the eliciting dose and the severity of symptoms, closely mimic those of peanut allergy, while patients are unaware of the presence of lupine in their food.
Lupine allergy can occur as a separate allergy or together with other legume allergies. The responsible allergens have been identified and showed remarkable differences. However, in both cases unique allergens were involved. Lupine allergy thus occurs as a co-allergy rather than a cross-reactive allergy.

Samenvatting

Voedselallergie is een veel voorkomende vorm van overgevoeligheid voor voedsel, waarbij immunoglobuline type E (IgE)-antistoffen een rol spelen. Allergische reacties op voedsel staan steeds meer in de belangstelling. Het is echter onduidelijk of dit komt door een toename van het voorkomen van voedselallergie of dat de bekendheid van voedselallergie is toegenomen. Het is belangrijk om onderscheid te maken tussen sensibilisatie (aanwezigheid van specifiek IgE zonder klachten) en allergie (aanwezigheid van specifiek IgE met klachten). Om na te gaan of iemand gesensibiliseerd is, wordt gebruik gemaakt van huidpriktesten en/of bloedonderzoek. Allergische klachten kunnen bestaan uit jeukklachten in de mond en keel (orale allergie syndroom), al of niet gecombineerd met maagdarmklachten, luchtwegklachten en in het meest ernstige geval met bloeddrukdaling en shock, wat zelfs kan resulteren in de dood. Van alle voedselallergenen is pinda het meest frequent de oorzaak van zeer ernstige allergische reacties op voeding. De ernst van de klachten verschilt overigens sterk tussen pinda-allergische patiënten. De meest gevoelige patiënten kunnen al jeuk in de mond en keel krijgen na inname van minder dan eenduizendste pinda.
De huidpriktest die gebruikt wordt voor het aantonen van specifiek IgE heeft een matige gevoeligheid (sensitiviteit), maar als de test negatief is dan is een allergie voor het desbetreffende allergeen zeer onwaarschijnlijk. De bloedtest heeft ook een matige gevoeligheid en resulteert bovendien vaak nog in een fout-positieve uitslag. Op dit moment is de dubbelblinde placebo-gecontroleerde voedselprovocatie nog steeds de enige test waarmee een voedselallergie betrouwbaar kan worden vastgesteld. Het voedingsmiddel waarvoor de patiënt mogelijk allergisch is, wordt daarbij door de patiënt in geleidelijk toenemende hoeveelheden ingenomen. Als controle vindt provocatie plaats met placebo porties die het allergeen niet bevatten (placebo-gecontroleerd). Omdat zowel de patiënt als de arts of verpleegkundige niet weten welke porties het allergeen bevatten (dubbelblind), wordt subjectieve beïnvloeding van deze test geminimaliseerd. Indien er duidelijke klachten op de porties die het allergeen bevatten optreden, maar niet op de placebo porties, is een allergie vastgesteld. Deze voedselprovocatie is een belasting voor de patiënt en vereist goede faciliteiten en ervaren personeel. Vandaar dat gezocht wordt naar nieuwe diagnostische tests en naar methoden om de diagnostische waarden van de bestaande bloedtesten en huidpriktesten te verbeteren.
Het is onduidelijk hoe mensen gesensibiliseerd raken voor voedselallergenen, maar de meest waarschijnlijke route is via de darm. Sensibilisatie via de luchtwegen kan ook leiden tot voedselallergieën. Een bekend voorbeeld hiervan is dat patiënten met een berkenpollen allergie een voedselallergie kunnen ontwikkelen voor noten, fruit en sommige groenten (kruisreactie). Ook wordt de huid gesuggereerd als mogelijke sensibilisatie route.
Het gebruik van pindaolie bevattende huidverzorgingsproducten op een ontstoken huid bij kinderen wordt genoemd als een van de factoren die zou kunnen bijdragen

aan het ontwikkelen van pinda-allergie. In *hoofdstuk 2* hebben wij onderzocht of ongeraffineerde pindaoliën en geraffineerde pindaoliën na diverse stappen van het raffinage proces (ontgumming, bleking, ontgeuring) nog pinda-allergeen bevatten. Tevens zijn monsters van diverse pindaoliën uit de supermarkt onderzocht. Daarnaast zijn pindaoliën die gebruikt worden in farmaceutische producten en kant-en-klare pindaolie bevattende producten zoals vitamine K druppels onderzocht. Het bleek dat alleen in de ruwe ongeraffineerde pindaoliën in zeer kleine hoeveelheden pinda-allergeen aantoonbaar was. Pindaolie in farmaceutische preparaten met keurmerk van de Europese Farmacopee is geraffineerd. Farmaceutische producten, zoals vitamine K druppels, die te koop zijn bij de drogist vallen onder de warenwet en hoeven niet aan de eisen van een geneesmiddel te voldoen. Ook in deze producten kon geen allergeen worden aangetoond. Daarom achten wij het zeer onwaarschijnlijk dat het gebruik van huidverzorgingsproducten met pindaolie leidt tot het ontstaan van pinda-allergie.
Door veranderingen in het Nederlandse voedingspatroon, bijvoorbeeld door import van voedingsmiddelen uit de hele wereld, kunnen allergieën voor 'nieuwe' voedingsmiddelen ontstaan. Voorbeelden hiervan zijn kiwi en lupine. Lupine is net als pinda en soja een peulvrucht die sinds de jaren '90 in de voedselindustrie wordt gebruikt. Omdat lupine eiwitrijk, goedkoop en gemakkelijk te telen is, wordt het frequent toegepast als alternatief voor soja. Bovendien is soja soms genetisch gemodificeerd, wat voor de consument minder aantrekkelijk is.
In *hoofdstuk 3* hebben we bestudeerd hoe vaak bij pinda-gesensibiliseerde patiënten tevens sensibilisatie voor lupine (en erwt en soja) voorkomt. Ook is bestudeerd bij hoeveel van de patiënten deze sensibilisatie bovendien ook tot klachten leidt, de zogenaamde klinisch relevante sensibilisatie. Wij toonden aan dat sensibilisatie voor lupine, erwt en soja zeer vaak voorkwam. De sensibilisatie bleek in ongeveer 30% van de patiënten tot klachten te leiden. Dit is een hoog percentage, omdat klinische relevante sensibilisatie voor andere peulvruchten tot nu toe werd gerapporteerd bij slechts ongeveer 5% van de patiënten.
Een opvallende bevinding in deze studie was dat geen enkele van de 39 geïncludeerde patiënten ooit van lupine als ingrediënt in voeding gehoord had. Klachten in mond en keel ontstonden bij sommige patiënten al op zeer geringe hoeveelheden lupinemeel (0.5 mg). Ter vergelijking, een koekje bevat ongeveer 300 mg lupinemeel. Deze kleinste hoeveelheid waarop patiënten reageren wordt de drempelwaarde genoemd. Niemand van de lupineallergische patiënten van *hoofdstuk 3* reageerde op 0.1 mg lupinemeel.
Omdat voor voedselallergie nog geen therapie bestaat, dienen voedselallergische patiënten het betreffende voedingsmiddel waarvoor ze allergisch zijn te vermijden. Als bekend is op welke hoeveelheid een individuele patiënt reageert (drempelwaarde) kan op basis hiervan een gerichter dieetadvies worden gegeven. Voorzichtigheid is echter geboden, omdat niet bekend is hoe stabiel een drempelwaarde blijft in de loop van de tijd. Gegevens over de drempelwaarden zijn ook van groot belang voor

de voedselindustrie en de overheid. Hiermee is het mogelijk om in de toekomst de veiligheid van voedingsmiddelen voor de allergische patiënt te vergroten.
De nieuwe regelgeving omtrent het etiketteren van allergenen helpt patiënten bij het vermijden van een allergeen, omdat de aanwezigheid van de 11 meest voorkomende allergenen, zoals bijvoorbeeld pinda en noten, op voedingmiddelen moet worden weergegeven. De industrie is nog niet verplicht om lupine te etiketteren, maar er zijn al aanbevelingen geweest om ook lupine in de lijst op te nemen. Men moet zich echter realiseren dat etikettering alleen zin heeft als de voedselallergische patiënt zich bewust is van zijn allergie. Dit toont het belang aan van enerzijds goede diagnostiek en anderzijds ook goede voorlichting.
In *hoofdstuk 4* is onderzocht of de lupineallergie bij patiënten met en zonder pinda-allergie wordt veroorzaakt door herkenning van verschillende allergenen in lupine. De IgE-antilichamen van lupineallergische patiënten zonder pinda-allergie bleken aan andere eiwitten te binden dan die van lupineallergische patiënten met pinda-allergie. Het allergeen met een molecuulgewicht van 66 kDa, dat alleen herkend werd door de lupineallergische patiënten zonder pinda-allergie, is nader bestudeerd en lijkt een opslageiwit te zijn. Bij pinda-allergie zijn het ook vooral opslageiwitten, zoals Ara h 1, Ara h 2, Ara h 3/4 en Ara 6, die allergische reacties veroorzaken. Door middel van remmingsproeven (immunoblot inhibities) met het bloed van lupineallergische patiënten die tevens pinda-allergisch waren, kon kruisreactiviteit tussen lupine en pinda niet of nauwelijks worden aangetoond. Er bleek sprake van unieke allergenen. Lupineallergie kan dus voorkomen als enige allergie, maar het kan ook bestaan naast een allergie voor andere peulvruchten (co-allergie).
Huidpriktesten worden nu nog verricht met extracten van voedingsmiddelen. Hierbij kunnen allergenen verloren gaan wat kan resulteren in fout-negatieve uitslagen. Als bekend is wat de relevante allergenen zijn per voedingsmiddel, kunnen de huidpriktesten verricht worden met deze gezuiverde of recombinante allergenen in plaats van met het hele extract waardoor de betrouwbaarheid van deze test wordt vergroot. Voor pinda zijn de belangrijkste allergenen bekend. In *hoofdstuk 5* hebben we onderzocht of sensibilisatie voor deze pinda-allergenen (Ara h 1, Ara h 2, Ara h 3 en Ara h 6) gecorreleerd kan worden aan de ernst van de pinda-allergie. De meeste patiënten herkenden Ara h 2 en Ara h 6. Patiënten met ernstige klachten na inname van pinda toonden in vergelijking met patiënten met milde klachten een sterkere reactie op de huidpriktest met Ara h 2 en Ara h 6 in lage concentraties en op Ara h 1 en Ara h 3 in hoge concentraties. Tevens herkenden patiënten met ernstige klachten meer allergenen dan patiënten met milde klachten. We zagen geen duidelijke verschillen als we patiënten met lage en hoge drempelwaarden met elkaar vergeleken. Uit deze studie concluderen we dat huidpriktesten met gezuiverde pinda-allergenen van belang kunnen zijn bij het voorspellen van de ernst van pinda-allergie, omdat de huidpriktest reactie op alle vier de allergenen die we getest hebben, correleert met de

ernst van de reactie op pinda.
In *hoofdstuk 6* hebben we de waarde onderzocht van kernspinresonantie (NMR) in combinatie met multivariate data analyse (MVDA) als een nieuwe diagnostische methode voor pinda-allergie. NMR is een techniek die het mogelijk maakt om stoffen van biologische processen in lichaamsvloeistoffen te identificeren en te meten. Eerder werd met deze techniek aangetoond dat er verschillen zijn in urinesamenstelling tussen patiënten met osteoarthritis en gezonde controles. In ons onderzoek kon worden aangetoond dat bloed en speeksel van pinda-allergische patiënten, zowel voor als na pinda consumptie, een ander biomarkerprofiel lieten zien dan niet pinda-allergische controles. Deze niet-invasieve methode zou in de toekomst kunnen bijdragen aan het diagnosticeren van pinda-allergie.
Dit proefschrift illustreert dat het gebruik van gezuiverde allergenen en technieken zoals NMR de potentie heeft om de huidige diagnostische mogelijkheden te verbeteren. Het geeft ook weer dat lupineallergie een vrij veel voorkomende allergie is in Nederland. Karakteristieken van lupineallergie, zoals drempelwaarde en klachtenpatroon, lijken sterk op die van pinda-allergie terwijl patiënten zich niet bewust zijn van de aanwezigheid van lupine in hun voeding.
Lupineallergie kan voorkomen als geïsoleerde allergie, maar ook in de context van een multipele peulvruchtenallergie. De verantwoordelijke allergenen werden geïdentificeerd en bleken verschillende, maar in beide gevallen unieke allergenen te zijn. Lupineallergie komt dus voor als een co-allergie en is niet zozeer gebaseerd op kruisreactiviteit.

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Curriculum Vitae

De auteur van dit proefschrift werd geboren op 16 juni 1976 te Venray. Na het behalen van het gymnasium diploma aan het Boschveldcollege te Venray, begon zij in 1994 aan de studie geneeskunde aan de Universiteit Utrecht. Haar wetenschappelijke stage deed zij bij de afdeling Dermatologie/Allergologie van het Universitair Medisch Centrum Utrecht onder begeleiding van Drs. I.M.L. Majoie en Prof. dr. W.A. van Vloten. Zij onderzocht of er genotoxische schade ontstond bij fotodynamische therapie met 5-aminolevulinezuur. In 1999 behaalde zij haar doctoraalexamen. Voordat zij in 2000 met haar co-assistentschappen kon beginnen, verrichtte zij in het St. Elisabeth Hospitaal te Curaçao onder begeleiding van Dr. A.J. Duits, onderzoek naar de rol van enkele cytokinen bij de ziekte van Rendu-Osler-Weber. In mei 2002 behaalde zij haar artsexamen, waarna zij gedurende 2 maanden als AGNIO interne geneeskunde heeft gewerkt in het Diakonessenhuis te Zeist.
In september 2002 is zij gestart met het onderzoek dat tot dit proefschrift heeft geleid. Drie jaar later begon zij aan de opleiding tot dermatoloog in het Universitair Medisch Centrum Utrecht met Prof. dr. C.A.F.M. Bruijnzeel-Koomen als opleider.