Food sensitization and allergy

Defining phenotypes and their risk factors and biomarkers

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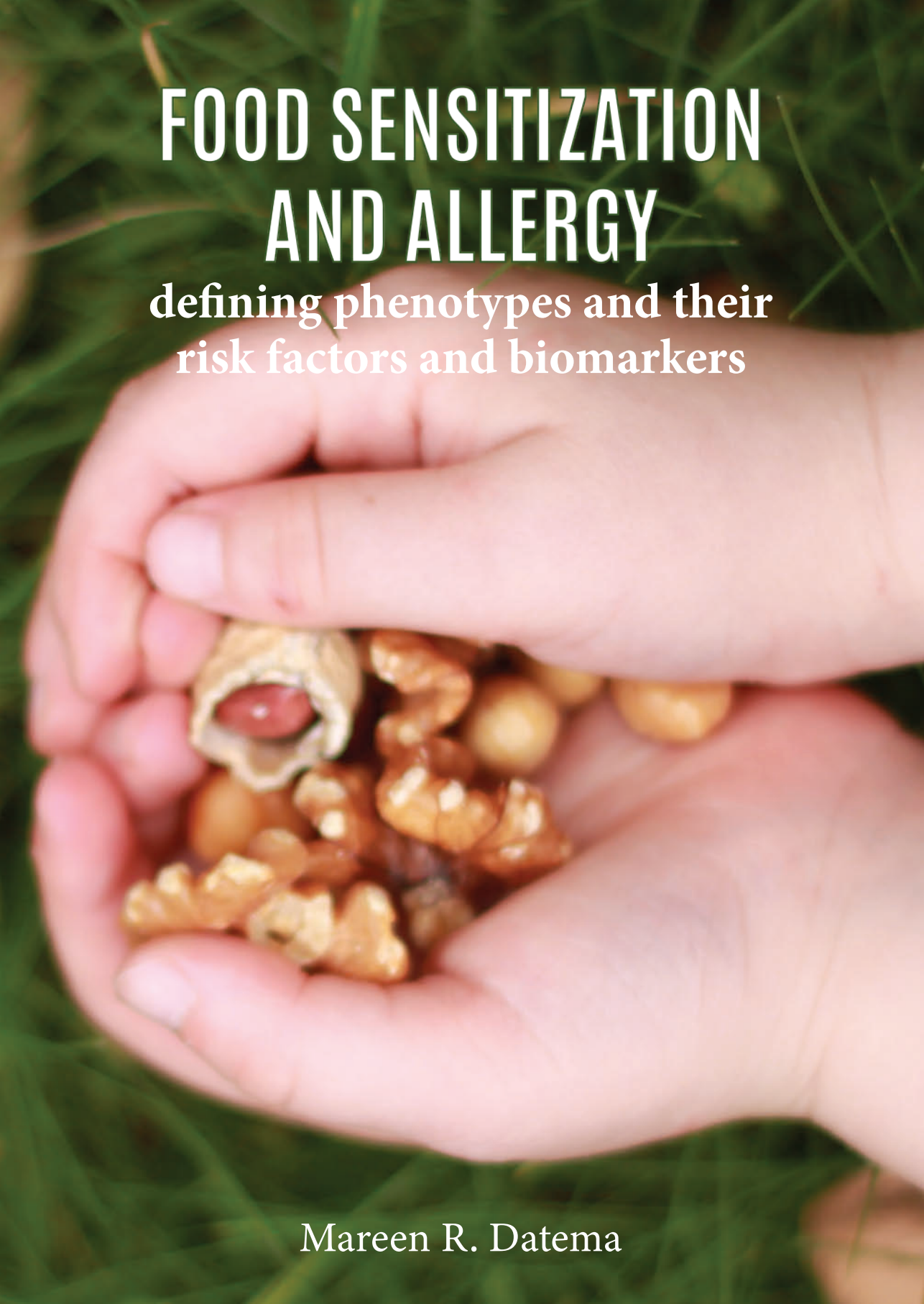
Publication date
2021

Document Version
Final published version



FOOD SENSITIZATION AND ALLERGY

defining phenotypes and their risk factors and biomarkers

Mareen R. Datema

Food sensitization and allergy: defining phenotypes and their risk factors and biomarkers

Mareen Riekelina Datema

The research presented in this thesis were funded by the European Commission under the 6th Framework Programme through EuroPrevall (FP6-FOOD-CT-2005-514000), and the 7th Framework Programme iFAAM (Grant agreement no. 31214)

ISBN: 978-94-93197-80-0
Cover photo: Evert van der Wel
Lay-out: Off Page
Printed by: Off Page, www.offpage.nl

Food sensitization and allergy: defining phenotypes and their risk factors and biomarkers

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. ir. K.I.J. Maex
ten overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op donderdag 11 november 2021, te 13.00 uur

door Mareen Riekelina Datema
geboren te Groningen

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Introduction

Food allergy

Daily, human beings are exposed to innocuous substances in their environment and their food. Our immune system usually does an amazing job in defending us against harmful pathogens but is supposed to ignore these harmless exposures. Sometimes, the immune system nevertheless reacts when exposed to innocuous compounds. Such aberrant hypersensitivity reactions are for example seen in food allergies, where the immune system identifies components in food as dangerous. The two most common immune-mediated hypersensitivity reactions to food are IgE antibody-mediated food allergy, where IgE antibodies are produced against a food, and T-cell driven celiac disease. In this thesis we will focus on IgE-mediated food allergy.

Characteristic for food allergy is its rapid onset upon exposure, usually within minutes. For this reason it is often referred to as immediate-type food allergy.[1,2] Another characteristic is that hypersensitivity reactions are reproducible, i.e. they generally occur again upon repeated exposure. Clinically, food allergy can present itself in many organ systems, including the oral cavity and esophageal tract, the gastro-intestinal tract, the skin, the upper and lower respiratory system, the cardio-vascular system and the neurological system. Most commonly, the symptoms are mild and limited to the oral cavity, the so-called oral allergy syndrome (OAS), which can include pruritus of the lips, tongue, palate, ears and throat and sometimes mild angioedema. When multiple organ systems are involved in the reactions to food we speak of anaphylaxis. When anaphylaxis includes the lower respiratory tract (asthma), the cardiovascular system and/or the neurological system, the reactions can be life-threatening. This is in particular true in case of a so-called anaphylactic shock (loss of consciousness).[2,3]

Managing a food allergy can have a great effect on the quality of life. Forethought about the diet and anxiety about severe reactions upon accidental exposure can affect the emotional and social health of the patients and their families. In some cases, patients outgrow their allergy but unfortunately until today, treatment options are scarce. To avoid symptoms and potentially life-threatening situations, dietary avoidance of the food and access to rescue medication in most cases are the only options. Only for peanut, an oral immunotherapy has recently been granted market authorization.

The study and diagnosis of food allergy is complex. For patient and doctor, it can sometimes be simple and straightforward to identify the causative food underlying a patient's food allergy, but this is certainly not always the case. Multiple foods may be implicated as some foods are known to cluster together such as tree nuts (e.g. hazelnut, walnut, pecan, cashew, and/or pistachio),legumes (e.g. peanut and/or soy) and/or seeds (e.g. sesame). Similarly, patients allergic to one fruit are often but not always also allergic to other fruits and/or vegetables. To support identification of the causative food, IgE antibodies that are produced by the immune system against a food can be measured. But these antibodies can also be present without causing symptoms, making the distinction being one of the major challenges in food allergy diagnosis.

Perhaps even more important in the diagnosis of food allergy is to establish the risk that a patient runs to encounter severe and potentially life-threatening reactions upon exposure. In particular the latter uncertainty has great impact on the anxiety of patients and their quality of life. Complex multifactorial pathogenesis underlies the severity of symptoms and prediction of whether a person will have a mild or severe response has proven to be difficult.

In this thesis, we investigated strategies to provide clinicians and their patients with better tools to predict food allergy and the risk of severe phenotypes of hazelnut, peanut and walnut allergy.

Sensitization, allergy and tolerance

The starting point of the development of IgE-mediated food allergy is called *sensitization*. In this process, antigen-presenting cells called dendritic cells (DC) present protein of a food to T-helper (Th) cells. In contact with DCs, T-cells are skewed towards a Th2-profile. In turn, Th2 cells will instruct B-cells[2,3] to switch to the production of IgE antibodies against the presented protein. Proteins that induce and bind IgE are commonly referred to as *allergens*. After the process of sensitization has occurred, IgE antibodies will bind to high-affinity IgE-receptors on effector cells of the allergic response, mast cells and basophils. Upon re-exposure to the food, the allergen molecules can bind to these effector cell-bound IgE antibodies, thereby potentially cross-linking high-affinity IgE-receptors. Upon cross-linking, effector cells will degranulate, thereby releasing active mediators that trigger allergic reactions (e.g., histamine). This phase of the development of allergy is commonly referred to as *elicitation*. The two phases of sensitization and elicitation are schematically depicted in figure 1.

Not all sensitization leads to clinical symptoms. Some subjects may present with IgE antibodies against a food but not with symptoms upon exposure. These subjects are described clinically as tolerant,[1] similar to non-sensitized subjects. One of the hypotheses to explain why some sensitized subjects are tolerant and others are not, involves another immunoglobulin isotype, i.e. IgG_4. From allergen-specific immunotherapy it is known that allergen-specific IgG_4 antibodies can block IgE-mediated effector mechanisms. IgG_4 antibodies are also induced upon dietary exposure to food proteins. Hence, it has been postulated that this subclass of IgG antibodies may be involved in deciding whether sensitization leads to allergy or not.

Sources and routes of sensitization

Not all proteins in a food have the capacity to induce the production of IgE (sensitize) and the potency to bind to IgE. In the last decades, characterization of food allergens has dramatically increased. Essentially, there are two types of food allergens, those that are capable of inducing IgE sensitization themselves, called primary sensitizers, and those that are not but do bind IgE based on structural similarity to primary sensitizers, referred to as cross-reactive allergens.

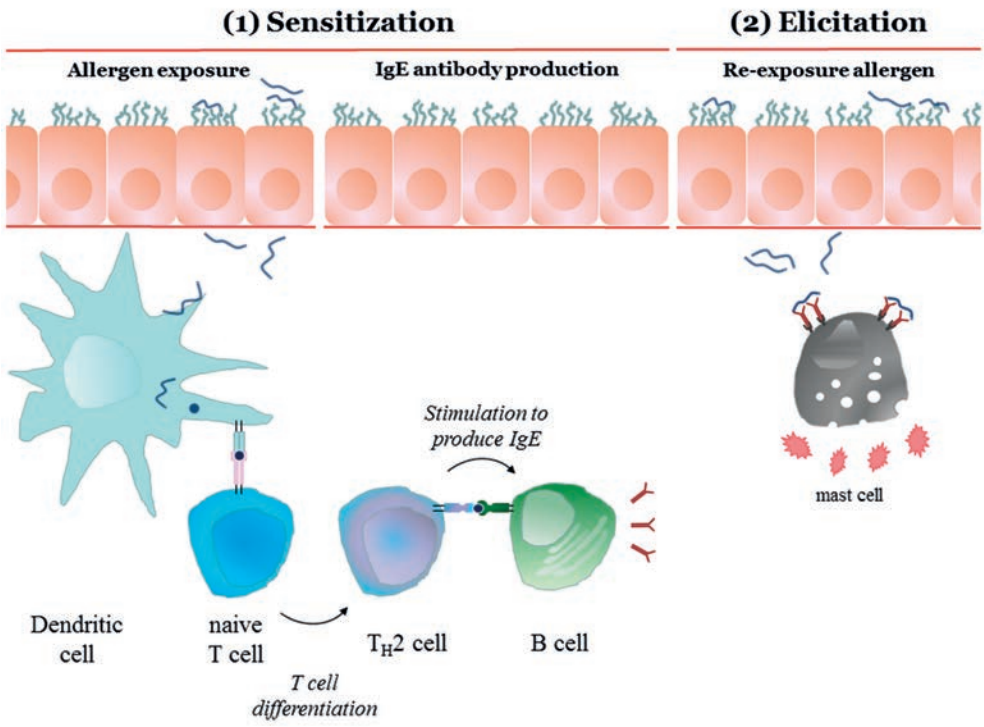

FIGURE I. Schematic view of allergic sensitization. Allergens are transported through the epithelial barrier and taken up by dendritic cells (DC). DCs present peptides of the allergen to naïve T cells that will differentiate to a Th2 phenotype. Th2-cells produce Th2 cytokines such as IL-4 and stimulate B-cells to switch to IgE production. IgE subsequently binds to IgE-receptors on mast cells. Upon re-exposure to allergens, receptor bound IgE will capture allergen. The resulting cross-linking of IgE-receptors will activate the mast cells (degranulation). The activated mast cell will release mediators such as histamine that trigger the allergic symptoms.

There are food-derived and nonfood-derived primary sensitizers that are at the basis of such cross-reactivity. The most common nonfood source of cross-reactivity is pollen. Food allergy as a result of cross-reactivity with pollen allergens is often referred to as pollen food allergy syndrome. Allergic symptoms are usually mild. A major allergen involved in cross-reactivity is Bet v 1, an allergen present in birch tree pollen. Antibodies produced against Bet v 1 can react with allergens from several plant foods (e.g. hazelnut) from the same protein family, the pathogenesis-related protein class 10 (PR-10).[4] Birch pollen allergic patients with IgE antibodies to Bet v 1 often experience (mostly mild) food allergy to plant foods such as apple (Mal d 1), peach (Pru p 1), hazelnut (Cor a 1), walnut (Jug r 5), peanut (Ara h 8) and celery (Api g 1). The second pollen-derived primary sensitizer with homologues in a broad spectrum of foods is profilin, i.e. Bet v 2 in birch pollen, Phl p 12 in grass pollen and Art v 4 in mugwort pollen.[5] A third category of cross-reactive structures in pollen are the so-called cross-reactive

carbohydrate determinants (CCD). These are glycan structures on plant and insect glycoproteins that induce IgE. The consensus is that they are not associated with clinical symptoms.

The best-known food-derived source of cross-reactivity between foods is the lipid transfer protein (LTP). LTPs have been associated with more severe reactions to food, in particular to fruits like peach and apple. Peach LTP (Pru p 3)[6] is considered the most common primary sensitizer amongst the LTPs, leading to cross-reactivity to a broad spectrum of plant foods such as apple (LTP: Mal d 3), hazelnut (Cor a 8), peanut (Ara h 9), tomato (Sola l 3) and walnut (Jug r 3). LTPs were also identified as pollen allergens such as Art v 3 in mugwort pollen.[7] For Chinese patients it has been described that primary sensitization may also start with pollen LTP.[8]

The most important plant food-derived primary sensitizers are storage proteins in tree nuts, legumes and seeds. Three families of allergenic storage proteins have been identified, i.e. the 2S albumins, the 7S globulins (or vicilins) and the 11S globulins (or legumins).[9] IgE antibodies against 2S albumins show limited cross-reactivity between tree nuts, legumes and seeds. The same is true for the 7S globulins (e.g. Cor a 11, Ara h 1 and Jug r 2). The 11S globulins tend to show more cross-reactivity between different foods (e.g. Cor a 9, Ara h 3 and Jug r 4).

Two other important primary sensitizers identified in plant food are the cysteine protease from kiwi, Act d 1, and the omega 5-gliadin from wheat. Finally, the most important animal food-derived primary sensitizers are the parvalbumins from fish (e.g. Gad c 1 from cod and Cyp c 1 from carp), the tropomyosins from crustaceans and mollusks (e.g. Pen a 1 from shrimp), ovomucoid from egg and beta -lactoglobulin and caseins from milk.

An ongoing debate in the field of food allergy is the route of sensitization. Some 20 years ago, the concept was that primary sensitization to food proteins occurs in the gut, and sensitization to the sources of cross-reactivity in pollen via the respiratory tract. The latter is still considered the most likely route of primary sensitization for cross-reactive allergens. For primary sensitization to food proteins, the paradigm has shifted. It is now considered likely that at least part of primary sensitization to food proteins occurs via the skin.[10] Support for the concept of skin sensitizations comes from epidemiological observations. It was discovered that a skin barrier defect caused by single nucleotide polymorphisms (SNP) in the gene for the barrier protein filaggrin is associated with allergies including food allergy.[11,12] In addition, an earlier study had demonstrated that the risk for peanut allergy was associated with the use of ointments containing peanut oil.[13]

Finally, analysis of dust samples collected in the MAAS birth cohort study revealed that peanut allergy in children is associated with the presence of peanut allergen in house dust, but only in the subpopulation with the barrier defect associated filaggrin SNP.[14] Altogether, these observations make the skin a very good candidate to be a (not necessarily exclusive) route for sensitization to food.

Diagnosis of food allergy

A reliable diagnosis of food allergy starts with a thorough medical history.[1] It is well known that self-reported food allergy is unreliable, resulting in a very significant overestimation of its prevalence.[15] The main reasons for this is that (potential) patients are often not aware of the immediate nature of IgE-mediated food reactions, that they confuse non-immune hypersensitivities such as lactose intolerance or T-cell driven immune reactions like celiac disease with IgE-mediated hypersensitivity, and that they mix up adverse reactions of food poisoning with food allergy.[1,2,16,17] It is the task of the clinician to establish what the culprit food most likely is, whether reported reactions are indeed of the immediate type and whether they occur repeatedly upon exposure to the same food. In this way possible food allergy can be separated from reported symptoms that are highly unlikely being IgE-mediated.

To further substantiate a clinical history suggestive of food allergy, clinicians often use skin prick tests (SPT) or serum IgE testing for the implicated or suspected foods to demonstrate the presence of relevant specific IgE (sensitization). In SPT (*in vivo*), a small amount of food extract is pricked into the skin with a special lancet. In case of sensitization to the tested food, the relevant allergens inthe food extract will cross-link IgE-receptors on the mast cells in the skin and trigger mediator release, resulting in an itching red wheal. Serological tests (*in vitro*) quantify IgE antibodies against similar food extracts in a patient's serum. A clear advantage of the SPT over measurement of serum IgE is that the in vivo test gives rapid results that are easily understood by the patient because they actually see and feel a positive response. A major disadvantage of SPT is that the food extracts are usually poorly standardized, vary among batches and that important allergens can be missing.[1,18–20] Serological tests facilitate more accurate quantification of specific IgE and are mostly based on better standardized extracts, resulting in improved reproducibility. When measuring serum IgE, the degree of sensitization can be quantified and IgE levels are in general higher in allergic patients than in patients that tolerate the food.[1] Despite this difference, there often is a large overlap of the IgE-distributions between these two groups. On the other hand it may also occur that IgE levels are not detected in patients that are in fact allergic (false negative).[1] This is, especially in severe cases, of course, not desirable.

A definitive diagnosis however often requires a food challenge, ideally a double-blind placebo-controlled food challenge (DBPCFC).[1] For the challenge, the suspected food is 'hidden' in a food matrix, for example a chocolate bar or a smoothie. The amount of protein of the food that is administered to the patient gradually increases per dose until symptoms occur. In a DPBCFC both the clinician and the patient are blinded; they do not know whether a verum or placebo is administered. Sometimes, for practical reasons, an open oral food challenge (OFC) instead of blinding is an option, especially in young children.

In addition, it is recommended to follow a negative DBPCFC by an OFC.[21] Overall, about 50% of subjects with probable food allergy (convincing history and matching IgE) have a food allergy

confirmed by DBPCFC. Challenges are however time-consuming and expensive, and can be accompanied by severe reactions. To reduce the need for challenges, serum IgE tests with better clinical predictive value would be of great help.

Component-resolved diagnosis

An increasing number of individual food allergens that have been identified and characterized, have become available as purified natural or recombinant allergens. This has revolutionized serum IgE testing, in particular for food allergy. A growing panel of individual food allergen molecules is now used to assess molecular sensitization profiles.

In component-resolved diagnosis (CRD) IgE antibodies against individual allergen components are tested. Traditional serum IgE tests (and SPTs) are based on whole food extracts that contain both allergenic and non-allergenic proteins and cannot distinguish between sensitization against individual allergenic structures, being either primary sensitizers or cross-reactive structures, each having potentially different clinical relevance. These differences in clinical relevance can be explained by (a combination of) properties of the IgE-binding proteins (e.g., stability to gastro-intestinal digestion and processing, and abundance in the food), and/or of the IgE antibodies (e.g. fine-specificity and affinity), as well as in the background of the individual patients (age, sex, genetic predisposition and co-factors such as use of medication, exercise, etc.). Among food allergens, some bind IgE because they are the primary sensitizer, other because they are a cross-reactive homologue of a primary sensitizer from a different allergen source. These IgE-binding proteins can differ significantly in their clinical relevance, being associated with anything between severe life-threatening symptoms and no symptoms at all, which effects their diagnostic accuracy in identifying true allergic patients.

First for peanut allergy, it was demonstrated that a positive serum IgE test for peanut 2S albumin, Ara h 2,[22–25] was a much better predictor for a positive DBPCFC than IgE against peanut extract. Similar observations were later made for hazelnut, where IgE against Cor a 14(2S albumin)[26] and Cor a 9(11S globulin)[26,27] were better predictors. Overall, CRD is increasingly recognized as a diagnostic tool that has improved the clinical predictive potential of serum IgE testing over extract-based testing. CRD may help reducing the need for expensive and time-consuming DBPCFCs.

CRD can also help understanding geographical differences observed for food allergy. When looking at Europe, sensitization to hazelnut is most common among adults from the Northern and Central regions of Europe while in Spain or Greece, peach sensitization is more dominant.[28] These differences are better understood when looking at sensitization at molecular level. In the Northern and Central regions of Europe, sensitization to PR-10 food allergens is frequently observed, while in Mediterranean areas patients usually have no IgE against these Bet v 1-related allergens. It is well accepted that these differences in sensitization to PR-10 allergens are linked to the exposure of birch pollen, which is much higher in the northern and central regions of

Europe than in the Mediterranean.[29] In Mediterranean countries, sensitization to non-specific lipid transfer proteins (LTP) is one of the most dominant sensitizations linked to plant foods.[30–32] This is far less common in Northern and Central Europe. There is consensus that peach is the most likely source of primary sensitization to LTPs, although they also have been identified as allergens in pollen of e.g. mugwort and plane tree.[7,33] Primary sensitization to pollen LTPs is considered to be of minor importance in Europe, but has been proposed to play a role in Northern China, where there is very high exposure to mugwort pollen and sensitization to mugwort LTP (Art v 3) is very prevalent.[8] Overall, these examples illustrates that CRD can help understanding geographical differences in sensitization patterns to foods and what the most likely sources for primary sensitization are that underlie these differences.

Severe allergic reactions to foods

Many foods can initiate allergic reactions of which only some turn out to be severe. Some foods are more likely to induce severe reactions than others, but these foods can vary depending on factors such as age or country. A number of possible risk factors for food allergy and the severity of the reaction that are used for analysis throughout this thesis are discussed in the following section.

Geography and allergen molecules

Where peanut is often associated with severe reactions in the UK or The Netherlands, this is rare in Spain or Greece. On the other hand, peach or apple are often associated with severe reactions in Spain but rarely in Northern and Central Europe. CRD has helped explaining these differences. Where severe peanut of tree nut allergic reactions in Northern and Central Europe are associated with sensitization to seed storage proteins like 2S albumins and 7S and 11S globulins, those to fruits in the Mediterranean are associated with sensitization to LTP. On the other hand, sensitization to pollen-cross-reactive allergens such as PR-10 proteins and profilins is rarely associated with severe reactions. The different severity profiles of individual allergen molecules has been proposed to be associated with differences in resistance to protease digestion and to food processing.[34]

In addition, abundance of proteins in the food is likely to play an important role. Where seeds storage proteins are abundant proteins, PR-10 allergens are present at very low concentration. Most likely, for a severe reaction to a food, a sufficiently protease-resistant and sufficiently abundant protein needs to reach to gut immune system to be able to induce a severe reaction. The observation that specific allergen molecules fulfilling these requirements can be linked to specific severity phenotypes has sparked of a lot of interest. Can sensitization to individual allergen molecules be used as biomarker for assessing the risk of severe reactions?

The first individual allergen that was reported to be associated with increased risk of severe reactions was the LTP from apple, Mal d 3.[31] The PR-10 allergen in apple, Mal d 1, was associated with mild symptoms only. Because plant food allergens of the PR-10 protein family are very

susceptible to proteolytic digestion, they usually cause mild symptoms restricted to the oral cavity. In contrast, LTPs such as Mal d 3 are highly resistant to pepsin digestion. Similarly, patients allergic to hazelnuts and sensitized to Cor a 1 almost exclusively report mild symptoms, although more severe symptoms have occasionally been observed in patients that were apparently mono-sensitized to Cor a 1.[26] Not all patients sensitized to LTPs develop severe symptoms, indicating that other factors such as characteristics of the specific IgE antibodies and the clinical and demographic background of the individual patient play a role in the resulting reactions. After the identification of LTP being a risk factor for severity, the first allergen identified to have similar properties was Ara h 2[23,35–37] a 2S albumin seed storage protein. Seed storage proteins have similar protease susceptibility characteristics as the LTPs. Not much later, also sensitization to storage proteins in hazelnut, Cor a 9 (11S globulin) and Cor a 14 (2S albumin),[26,38,39] and in walnut, Jug r 1,[40] have been related to severe allergic reactions although there are also studies that could not find this association.[24,41–43] Finally, Act d 1, a cysteine protease in kiwifruit, has been associated with severe kiwifruit allergy.[44]

Sensitization to food allergens associated with severity (LTPs/seed storage proteins) and to those associated with mild symptoms is frequently seen together in individual patients. It has been suggested that being sensitized to LTP (or storage proteins) in combination with pollen-related allergens (PR-10 and/or profilin), for example sensitization to Pru p 3 (LTP) and Pru p a 1 (PR-10), results in less severe symptoms than when being exclusively sensitized to Pru p 3.[45] If indeed a consistent observation, the mechanism behind will still require some additional research.

Allergen-specific IgG_4 antibodies and severity

To identify those patients at risk for severe reactions, the levels of IgE in serum can be compared between patients with mild and patients with severe reactions. Levels are usually higher in patients with severe symptoms, but there is often a large overlap with the levels of patients with milder symptoms. This overlap makes it difficult to find a cutoff value that leads to a good sensitivity of a test, with few false positives, and a good specificity of a test, with few false negatives. Many patients sensitized to foods also have food-specific IgG_4 antibodies to food. The beneficial effects of allergen-specific immunotherapy have been related to an increase in allergen-specific IgG_4 antibodies[46] and also early introduction of peanut showed an increase of IgG^4 over time together with a decrease in peanut allergy.[47] Although the use of IgG or IgG_4 antibodies as a diagnostic tool is not recommended,[48] it is suggested that the ratio of IgG over IgE levels might more clearly explain the difference between allergic and tolerant and possibly between mild and severe patients.

Age, sex, and genetics

Peanut allergies often start at young age and persist over a lifetime49 but adults tend to experience more severe symptoms than children.[50] It is not straightforward to explain this based on sensitization profiles. On the one hand, allergies to plant foods like hazelnut and apple but also peanut, known to be cross-reactive with birch pollen, often develop later in life and are

mild. On the other hand, young children usually do not react to the PR-10 plant-food proteins but mostly show IgE against the storage proteins, even in birch endemic areas.[26,38,40] From these observations it can be concluded that age is not only a factor in deciding on the severity of reactions based on sensitization profiles. Other age-related factors have to be in play.

Less is known about male and female differences in the risk of food allergy. There are some reports for peanut and tree nut allergy that show differences including that the ratio of male to female patients shifts over time. In children, peanut allergy is more common in boys than in girls, while during adulthood it is the other way around and it is suggested that this reversal is related to hormonal influences.[51]

It is likely that genetic predisposing factors play a role in the development of food allergy but genetic studies for food allergy are scarce to date. Children with family members (parent or sibling) that are peanut allergic have an increased risk to develop food allergy indicating a genetic contribution.[52,53]

Other atopic diseases

Atopic dermatitis (AD) or eczema has often been associated with the development of food allergies.[11,12,54–56] Mutations in a specific gene, filaggrin (FLG), are related to a disrupted skin barrier and presence of eczema[57] and it is believed that allergens in the environment can penetrate the disrupted skin leading to sensitization to food.[10] It is however unknown whether a clinical history of atopic dermatitis is associated with severity of food allergic reactions. Allergic-rhinitis and asthma are also frequently seen in food-allergic patients. Again, it is not really known whether these co-morbidities influence the severity phenotype in food allergic patients.

Predicting severity

In this thesis we have explored whether demographic and clinical phenotype characteristics are associated with severity of reactions to food. We have developed statistical models or algorithms that aim at improving the prediction of the risk of severe reactions to peanut, hazelnut and walnut by combining extract-based and molecular sensitization profiles with clinical and demographic background of patients.

To evaluate how well a test can distinguish whether a patient has the outcome that you want to predict (severe food allergy), Area Under the Receiver Operating Characteristics (ROC) curve can be calculated (see figure 2). The area under the curve (AUC) indicates the overall performance of a test to discriminate between two groups. An AUC of 1 indicates that the test is a perfect discriminator while with an AUC of 0.5 the outcome of the test is random and therefore has no predictive value. To determine whether a test is positive or negative, a threshold value is used. The fraction that is true positive is the *sensitivity* and the fraction that is true negative is the *specificity* of the test. These fractions depend on the distribution of the values of

the test that is evaluated and on how much these values overlap between the 2 groups that are compared. This is illustrated in Figure 2.

Figure 2A shows a fictive IgE distribution in patients with a mild food allergy (blue line) and patients a severe food allergy (red line). The dashed line indicates a threshold of 6 kU_A/L IgE; this means that all patients with IgE levels below 6 have a negative test (no severe allergy) and those with levels of 6 and higher are diagnosed as positive (severe allergy). In this example, 92/100 patients witha severe food allergy (red line) have levels above 6 kU_A/L, the green area on the right side of the line. In the group with mild allergic symptoms (red line), 70/100 have levels below 6 kU_A/L. This gives the following 2x2 table:

TABLE I 2*2 TABLE

	Severe allergy	Mild allergy
≥ 6 kU_A /L	92	30
< 6 kU_A /L	8	70
	100	100

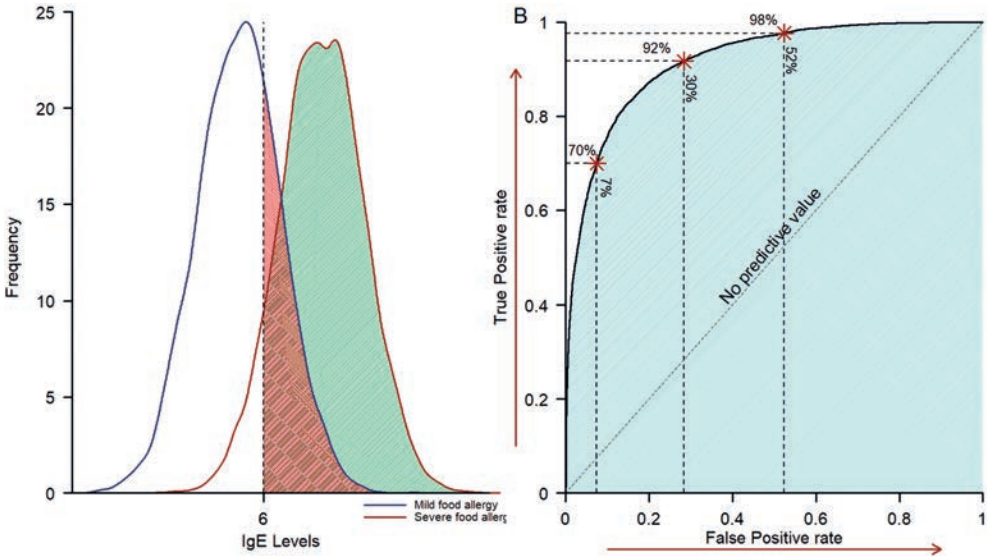

FIGURE II. Example illustration of a distribution of IgE levels in 2 groups from (**A**) which the Receiver Operating Characteristics (ROC) curve (**B**) is built from all possible cutoff values. The dashed line in the distribution on the left panel (**A**) shows a cut-off value at 6 kU_A/L. All patients with IgE levels above 6 are classified as severity allergic. This corresponds to a true positive rate of 92% (green area) and false positive rate of 30% (red area) which are both the coordinates of the middle star in the ROC curve. The Area Under the Curve (AUC) is marked in blue.

The line of the ROC curve in figure 2B is drawn from all possible thresholds of the test. The coordinates of the line are drawn from the true positive (TP) rates (y-axis) and the false positive (FP) rates (x-axis). At the example threshold of 6 kU_A /L, the TP is 92, meaning that 92% of the allergic patients are correctly classified as positive. Of the tolerant patients, 70% are correctly classified as negative (not allergic) but 30% of the non-allergic patients have levels above 6 kU_A /L and have a false positive test (red area). The TP rate of 92% and FP rate of 30% are the coordinates of one points of the ROC line of figure 2B. When moving the threshold line in figure 2A to the left, the TP rate will go up but the FP rate will also increase. Figure 2B shows that at a TP rate of 98%, the FP is 52%. The other way around, when setting a higher threshold, the FP rate go down but it will also affect the TP rate because less severely allergic patients will be identified.

ROC analysis can also be used for prediction models. The goal of a prediction model is to include multiple demographic and clinical factors (for example age, sex, atopic dermatitis) and IgE test results to predict the outcome more accurately as compared to a single test. The combination of all the factors and IgE measurements (hereinafter referred to as variables) needs to be translated to one single outcome, which in this thesis will be the probability of severe allergic reactions.

To create such a model, a selection of variables that are most strongly related to the outcome has to be made. If too many variables are included in a model, it cannot make a reliable prediction and results in an overfitted model with large variance. Additionally, in clinical practice it would be unpractical to use too many different variables to make a prediction of a patient's allergic status. We used two methods to build prediction models: multivariate logistic regression using a backwards selection and LASSO (Least Absolute Shrinkage and Selection Operator) logistic regression. Both methods start by including (all available) clinical factors and IgE tests (hereinafter referred to as variables) in the model and calculate a value (*coefficient*) for each variable that most accurately classifies those with severe food allergy with a probability as close as possible to *1*, and the other patients as close as possible to *0*.

The LASSO is a regularization method and it uses shrinkage on coefficients to reduce variance and prevents overfitting. Values are shrunken towards a central value (population mean) by using a penalty term also called tuning parameter (λ). It means that the size of the coefficients is limited and values will be closer together, therefore its variance will be less. This penalty term that is used, is equal to the sum of the absolute values (distances of the coefficient to *0*) of the coefficients. Some coefficients are shrunken to be equal to zero and that means that the associated variables are eliminated from the model. If λ=0, no variables are excluded. If λ increases, shrinkage increases and the bigger the amount of shrinkage is, the more variables are eliminated. The optimum penalty term to select the best model is somewhere in between *0* (all variables included) and *1* (all variables excluded) can be found by cross-validation which assesses how the models generalize best to new data.

The result of both backward selection and LASSO regression analysis is a model including a subset of variables of which the outcome is the probability of having a severe food allergy.

EuroPrevall project

The thesis is largely based on data analyses that capitalize on existing databases, coming from an earlier EU project, EuroPrevall. EuroPrevall was a multidisciplinary project on food allergy across Europe that took place from May 2005 until December 2010. The main aim of the project was to fill in knowledge gaps and improve the quality of information to deliver tools to effectively manage food allergies.[58]

Three types of surveys were carried out:

- longitudinal birth cohort (9 countries)
- cross-sectional community survey, including cases and controls (8 countries)
- cross-sectional outpatient clinics survey (12 countries)

The project studied 24 foods, but its most detailed investigations were directed towards nine foods, i.e. peach, apple, celery, peanut, hazelnut, fish, shrimp, egg and milk (Table I).

The analyses in this thesis were carried out on the outpatient clinics survey.[59] The recruitment of patients took place in outpatient clinics from 12 European cities to represent different geographical regions of Europe:

Athens (Greece)
Łódź (Poland)
Madrid (Spain)
Manchester (United Kingdom)
Milan (Italy)
Prague (Czech Republic)
Sofia (Bulgaria)
Strasbourg (France)
Reykjavik (Iceland),
Utrecht (The Netherlands)
Vilnius (Lithuania)
Zürich (Switzerland)

iFAAM project

The analyses of EuroPrevall further contributed to a follow-up EU project, iFAAM. iFAAM stands for Integrated Approaches to Food Allergen and Allergy Risk Management. One of the aims of this project was to integrate and share data from previous and ongoing studies. EuroPrevall was integrated in iFAAM with other observational and interventional surveys. Within iFAAM, a grading system for classifying the severity of allergic reaction using the EuroPrevall data was developed (Table II).

TABLE I. THE 24 FOODS, 12 INHALANT ALLERGENS AND LATEX STUDIED IN THE EUROPREVALL STUDY

EuroPrevall foods	Inhalant allergen sources & latex	Studied allergen components Chapters 2-6		Protein family
Peach*	Birch	Hazelnut	Cor a 1	PR-10†
Apple*	Chenopodium		Cor a 2	Profilin
Kiwi	Cypress		Cor a 8	Lipid transfer protein
Banana	Mugwort		Cor a 9	11S globulin§
Melon	Olive		Cor a 11	7S globulin§
Celery*	Parietaria		Cor a 12	Oleosin
Carrot	Plane tree		Cor a 14	2S albumin§
Tomato	Ragweed			
Corn	Timothy grass	Walnut	Jug r 1	2S albumin§
Lentil			Jug r 2	7S globulin§
Soybean	House Dust mite		Jug r 4	11S globulin§
Peanut*	Cat		Jug r 5	PR-10†
Walnut	Dog*		Jug r 6	7S globulin§
Hazelnut*			Jug r 7	Profilin
Sesame seed				
Sunflower		Peanut	Ara h 1	7S globulin§
Poppy seed			Ara h 2	2S albumin§
Mustard			Ara h 3	11S globulin§
Wheat			Ara h 6	2S albumin§
Buckwheat			Ara h 8	PR-10†
Shrimp				
Fish*				
Egg*				
Milk				

†PR-10: Pathogenesis-related protein.

§Storage protein

*Most detailed investigated foods, including a Double Blind Placebo Controlled Food challenge

TABLE II. SEVERITY CLASSIFICATION OF SYMPTOMS

Oral allergy syndrome	Rhinitis Conjunctivitis	Pruritus Flushing/ Erythema Angioedema Urticaria	Dysphagia Diarrhea Cramps Gastric pain and/or burning Abdominal pain Emesis/ Vomiting Nausea	Asthma (dyspnea, wheezing, cough, chest tightness) Bronchospasms Tightness of the Throat	Disorientation Confusion Dizziness Loss of consciousness Incontinence Seizures	Cardiac arrhythmia Myocardial ischaema Hypotension
Oral	Rhino-conjunctival	Skin	Gastrointestinal	Laryngeal/Bronchial	Neurological	Cardio-vascular
Grade I	**Grade II (1 organ system involved)** **Grade III (>1 organ system involved)**			**Grade IV**	**Grade V**	

2 Hazelnut allergy across Europe dissected molecularly: a EuroPrevall outpatient clinic survey

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Journal of Allergy and Clinical Immunology 2015; 136:382-391
DOI: 10.1016/j.jaci.2014.12.1949

Abstract

Background: Hazelnut allergy is birch pollen-driven in Northern/Western Europe and LTP-driven in Spain and Italy. Little is known about other regions and other allergens.

Objective: Establishing a molecular map of hazelnut allergy across Europe.

Methods: In twelve European cities, subjects reporting reactions to hazelnut (n=731) were evaluated and sensitization to 24 foods, 12 respiratory allergen sources and latex was tested by SPT and ImmunoCAP. A subset (124/731) underwent a double-blind placebo controlled food challenge (DBPCFC) to hazelnut.Sera of 423/731 subjects were analyzed for IgE against 7 hazelnut allergens and CCD by ImmunoCAP.

Results: Hazelnut allergy was confirmed in 70% of those undergoing DBPCFCs. Birch-pollen driven hazelnut sensitization (Cor a 1) dominated in most cities, except in Reykjavik, Sofia, Athens and Madrid, where reporting of hazelnut allergy was less frequent anyhow. In Athens, IgE against Cor a 8 dominated and strongly correlated with IgE against walnut, peach and apple and against Chenopodium, plane tree and mugwort pollen. Sensitization to seed storage proteins was observed in < 10%, mainly in children and correlated with IgE to nuts, seeds and legumes. IgE to Cor a 12, observed in all cities (10-25%), correlated with IgE to nuts, seeds and pollen.

Conclusion: In adulthood, importance of hazelnut sensitization to storage proteins, oleosin (Cor a 12) and Cor a 8 is diluted by the increased role of birch pollen cross-reactivity with Cor a 1. Cor a 8 sensitization in the Mediterranean is probably driven by diet in combination with pollen exposure. Hazelnut oleosin sensitization is prevalent across Europe; however the clinical relevance remains to be established.

Key messages

- Similar to what has been described for North-Western Europe, birch pollen exposure drives hazelnut allergy in Central and North-Eastern Europe.
- Sensitization to hazelnut storage proteins is observed across Europe with its relative importance decreasing with age due to the increasing role of pollen cross-reactivity.
- As reported for Spain and Italy, hazelnut allergy in Greece is an LTP-driven phenomenon, closely associated not only with peach but also with walnut, and to lesser extent pollen sensitizations.

Introduction

From 2005 to 2010, the multicenter and multidisciplinary EuroPrevall project was conducted, aiming to investigate the prevalence, cost and basis of food allergy across Europe.[58] Several large-scale multi-center epidemiological surveys were performed, including birth cohort surveys,[60] cross-sectional community surveys in school-aged children and adults,[61] and an outpatient clinic survey in twelve cities across Europe.[59] The project studied 24 foods, but its most detailed investigations were directed towards nine foods,i.e. egg, milk, fish, shrimp, peanut, hazelnut, celery, peach and apple.[58,59] In the current paper we describe the main characteristics of hazelnut allergy across Europe.

Hazelnut allergy is one of the more common food allergies in Europe, but most studies so far have been limited to European countries like Sweden, Germany, The Netherlands, Switzerland (North-western and Alpine) and Spain and Italy (Western Mediterranean).[32,38,62–65] These studies have established that hazelnut allergy in the former countries is dominated by cross-reactivity between birch pollen Bet v 1 and hazelnut Cor a 1[62,63] and in the latter by cross-reactivity between peach Pru p 3 and hazelnut Cor a 8.[66] Little is known about hazelnut allergy in Central and Eastern European countries, and North-Western (Iceland) and South Eastern extremes of Europe (Greece). The EuroPrevall consortium set out to fill these gaps.

Additionally, very little has been reported about the importance across Europe of hazelnut allergens other than Cor a 1 and Cor a 8. Besides profilin (Cor a 2), first described around two decades ago,[62] several non-pollen related allergens have now been identified and characterized. These include the seed storage proteins Cor a 9 (11S globulin),[67] Cor a 11 (7S vicilin-like)[68] and Cor a 14 (2S albumin).[69] More recently also oil-body associated oleosins have been identified as hazelnut allergens, i.e. Cor a 12 and Cora 13.[70,71] Here, we investigate the full spectrum of hazelnut allergens as is known to date (Cor a 1, Cor a 2, Cor a 8, Cor a 9, Cor a 11, Cor a 12 and Cor a 14). Where the origin of sensitization to hazelnut Cor a 1 is generally accepted to be Fagales tree pollen, in particular birch pollen, it is less well-established for the other hazelnut allergens. Sensitization to profilin is thought to be closely linked to grass pollen sensitization, but a role for other pollens cannot be excluded.5 The concept of peach lipid transfer protein (LTP) Pru p 3 inducing sensitization to fruit, vegetable, nut and seed LTPs is quite firmly established, but involvement of other foods or pollens as primary sensitizer cannot be ruled out.[72] In Northern China, mugwort pollen was recently shown to to induce LTP-reactive IgE, resulting in cross-reactivity to peach.[8] Some studies demonstrated that IgE responses to the storage proteins are more common in children than in adults[26,38] and pollen-related cross-sensitization first occurs at later age. The age composition in the EuroPrevall population with around 17% children allowed us to verify this.

In twelve EuroPrevall outpatient clinic surveys, all enrolled subjects were tested by skin prick testing SPT and ImmunoCAP on 24 foods, 12 respiratory allergen sources and latex. We aimed

to identify differences in hazelnut sensitization patterns between European cities and possible associations between IgE against hazelnut components, and IgE against pollen, latex and/or other foods, providing insight into probable primary sensitizers. To the best of our knowledge this is the first detailed clinical and serological study of hazelnut allergy with such a broad geographic, socio-economic, cultural and lifestyle spectrum across Europe.

Methods

Study design

This survey is part of the EuroPrevall project.[58] Subjects were prospectively recruited from 2006 towards the end of 2009 in outpatient clinics from 12 European cities: Madrid (Spain), Sofia, (Bulgaria), Reykjavik (Iceland), Athens (Greece), Prague (Czech Republic), Łòdź (Poland), Utrecht (The Netherlands), Strasbourg (France), Manchester (United Kingdom), Milan (Italy), Zürich (Switzerland) and Vilnius (Lithuania). Participating subjects reported immediate adverse reactions ≤ 2 hours after ingestion of any food. The population was further complemented with subjects enrolled in the EuroPrevall community surveys in adults and children.[59,61] In the end, 2273 subjects were enrolled in the survey (Figure 1; also see the Methods section in the Supplemental Files). In the present study, we included 731 subjects reporting reactions to hazelnut. Local ethical committees approved all studies and written informed consent was obtained from all subjects or their legal representatives.

Clinical evaluation and DBPCFC

Allergy specialists in the outpatient clinics applied standardized case-report forms to collect a detailed medical history.[59] All subjects underwent SPT and serum IgE testing to detect sensitization to 24 foods, latex and 12 inhalants allergen sources (see Supplemental Table S1). We asked all subjects to undergo a double-blind placebo controlled food challenge (DBPCFC) to hazelnut and 124 consented (see also the Methods section in the Supplemental Material). Those with a history of severe anaphylaxis[73] to hazelnut were excluded from the challenge (n=22). Both a positive challenge or history of severe anaphylaxis to hazelnut was considered as confirmed hazelnut allergy.

Skin-prick testing

Skin prick test (SPT) reagents were kindly provided by ALK-Abelló (Madrid, Spain). Details of the procedure are described in the Methods section in the Supplemental Files. SPT results were expressed as allergen/histamine wheal ratios with a ratio ≥ 0.5 designated as positive.

Specific IgE measurements

Specific IgE (sIgE) antibodies to foods and respiratory allergens sources and latex were tested by ImmunoCAP following the manufacturer's instructions (Thermo Fisher Scientific, Uppsala, Sweden). For component-resolved diagnosis (CRD), we tested the following hazelnut

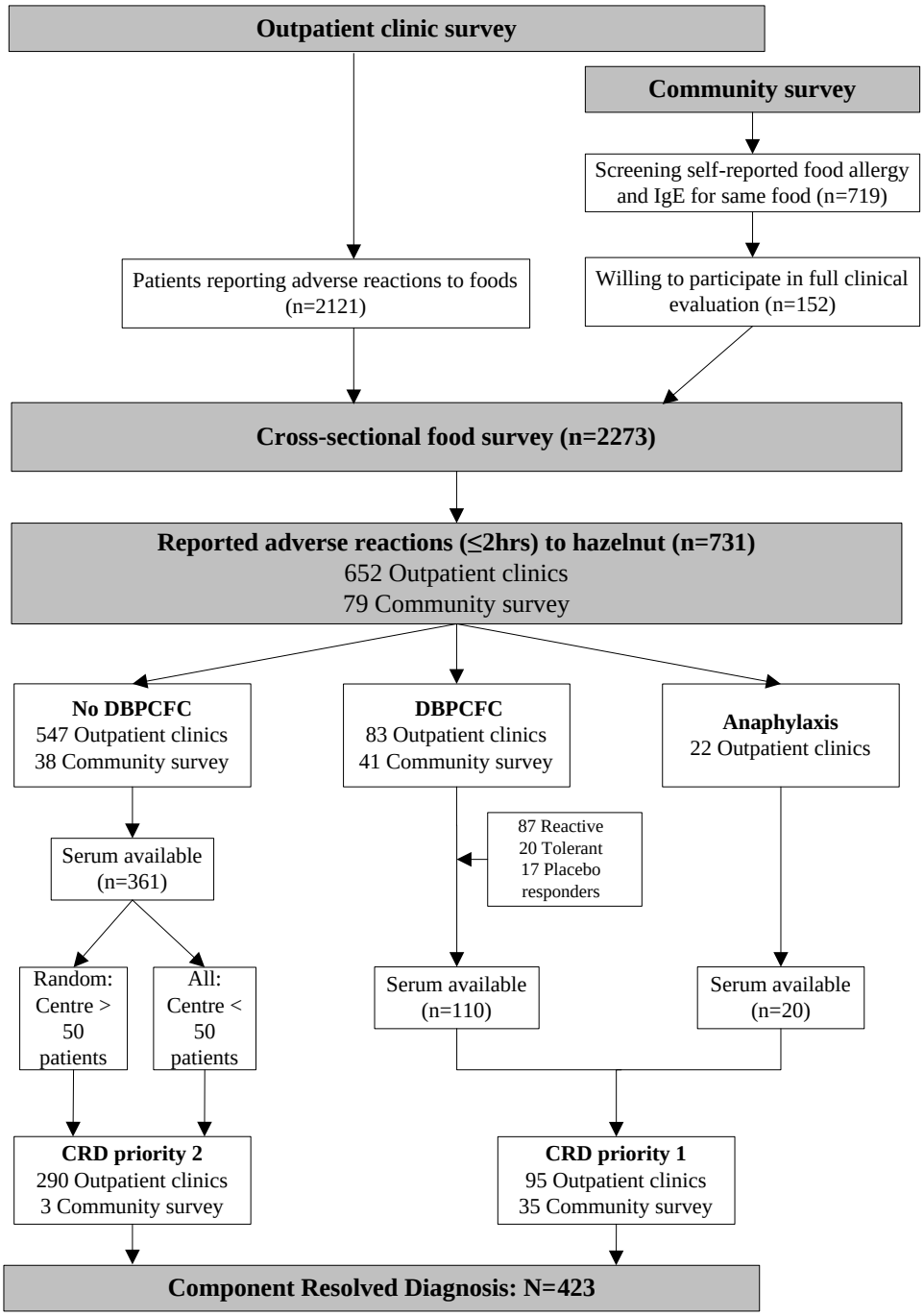

FIGURE I. Flowchart showing the selection of subjects in the out-patient and community survey. The number of included subjects with reported adverse reactions (≥ 2hrs) to hazelnut. The full clinical evaluation included SPT and serum IgE testing for 24 foods, 12 inhalant sources and latex. A subset also underwent a DBPCFC to the food to which they reported symptoms.

components: rCor a 1 (Bet v 1 homologue), rCor a 2 (profilin), rCor a 8 (LTP), nCor a 9 (11S globulin), nCor a 11 (7S globulin), nCor a 12 (oleosin) and rCor a 14 (2S albumin). In addition, IgE against bromelain was used as measure for sIgE against cross-reactive carbohydrate determinants (CCD). Details of serology measurements are presented in the Methods section in the Supplemental Files. IgE levels ≥ 0.35kU$_A$/L were considered as positive.

Serum selection for component-resolved diagnosis

Due to restricted availability of experimental custom-made ImmunoCAP tests, not all 731 sera were tested with CRD. Sera with sufficient volumes from subjects that had undergone a DBPCFC (110/124) and anaphylactic subjects (20/22) were tested with priority. Remaining ImmunoCAP tests were used for analysis of samples (n=293) selected so as to achieve a more balanced representation of the 12 cities. In those with low numbers (<50) of subjects with sufficient serum volume (Sofia, Madrid, Reykjavik, Athens, Prague, Utrecht, Strasbourg, Manchester, Milan) all sera were tested. A random sample from subjects of the remaining cities (Łódź, Zürich and Vilnius) was drawn.

Allergens

Hazelnut allergens were produced and purified as described elsewhere.[69,71,74,75]

Statistical analysis

Differences between cities in characteristics and proportions of positive and negative test results were tested using the Pearson χ^2 test and ANOVA (age). We calculated medians and interquartile ranges and used Kruskal-Wallis test to compare differences in IgE levels to hazelnut between cities. Correlations between IgE levels to hazelnut and pollen of 9 different species, 23 different foods and latex (Table S1) were analyzed using the Spearman correlation coefficient (*Rho*). To accommodate possible differences between cities affecting the overall results, we also assessed the correlations using random effects models. As no significant differences between the two methods were observed, the Spearman's *Rho* correlations are reported. *P*-values ≤ 0.05 were considered significant. For analyzes including multiple comparisons, *p*-values adjusted according to the Bonferroni method were calculated. We used R software version 3.1.0 for all statistical analyzes.

Results

Population characteristics and hazelnut sensitization

Hazelnut was the most reported food allergy in the EuroPrevall outpatient clinic survey (32%). Differences in frequencies between European cities were however considerable, ranging from 68.4% in Vilnius to 5.7% in Madrid (Supplemental Figure S1). The population included more females (63.1%) than males (36.9%) (Table I). The majority was adult (83.6%) and among the 120 children (<18 years), 22 were below 7 (3-6 years).

TABLE I. CHARACTERISTICS OF THE EUROPREVALL HAZELNUT STUDY POPULATION STRATIFIED BY CITY.

	European city						
Characteristic	**All (n=731)**	**Madrid (n=15)**	**Sofia (n=12)**	**Reykjavik (n=13)**	**Athens (n=23)**	**Prague (n=29)**	**Łódź (n=70)**
Type of clinic/department		Allergy	Allergy	Pulmonology & Allergy	Allergy	Pediatrics	Immunology, Rheumatology, & Allergy
Age (y), mean ± SD	32.3 ± 14.8	24.7 ± 14.0	21.5 ± 15.0	32.7 ± 17.1	27.8 ± 12.7	17 ± 10.8	32.2 ± 18.1
<18 y, n/N (%)	120/731 (16.4)	5/15 (33.3)	6/12 (50.0)	3/13 (23.1)	5/23 (21.7)	16/29 (55.2)	18/70 (25.7)
Female gender	461/731 (63.1)	9/15 (60.0)	8/12 (66.7)	8/13 (61.5)	12/23 (52.2)	19/29 (65.5)	52/70 (74.3)
Hazelnut sensitization							
SPT n/N (%)	556/718 (77.4)	11/15 (73.3)	3/12 (25.0)	10/13 (76.9)	20/23 (87)	20/29 (69.0)	37/70 (52.9)
sIgE n/N (%)	585/669 (83.7)	10/15 (66.7)	3/11 (27.3)	10/12 (83.3)	14/21 (66.7)	23/28 (82.1)	41/69 (59.4)
DBPCFCs hazelnut							
Completed n/N (%)	124/731 (16.9)	8/15 (53.3)	1/12 (8.3)	6/13 (46.2)	3/23 (13.0)	5/29 (17.2)	17/70 (25.0)
Reactive n/N (%)	87/124 (70.2)	5/8 (62.5)	0/1 (0.0)	4/6 (66.7)	1/3 (33.3)	3/5 (60.0)	8/17 (47.1)
Anaphylaxis n/N (%)	22/713 (3.0)	–	1/12 (8.3)	–	3/23(13.0)	2/29(6.9)	2/70(2.9)

TABLE I. CHARACTERISTICS OF THE EUROPREVALL HAZELNUT STUDY POPULATION STRATIFIED BY CITY. (CONTINUED)

	European city						
Characteristic	Utrecht (n=80)	Strasbourg (n=70)	Manchester (n=51)	Milan (n=50)	Zürich (n=177)	Vilnius (n=141)	*P*-value*
Type of clinic/department	Dermatology	Pulmonology	Allergy & Clinical Immunology	Allergy	Allergy/ Dermatology	Allergy	
Age (y), mean ± SD	32 ± 10.9	34.2 ± 13.0	31.2 ± 15.8	40.5 ± 14.2	36 ± 14.3	29.9 ± 13.7	<0.001
<18 years n/N (%)	5/80 (6.2)	7/70 (10.0)	11/51 (21.6)	1/50 (2.0)	14/177 (7.9)	30.141 (21.3)	<0.001
Female gender	54/80 (67.5)	54/70 (77.1)	33/51 (64.7)	34/50 (68.0)	105/177 (59.3)	73/141 (51.8)	<0.001
Hazelnut sensitization							
SPT n/N (%)	62/73 (84.9)	60/69 (87.0)	38/51 (74.5)	48/50 (96.0)	129/173 (74.6)	118/140 (84.3)	<0.001
sIgE n/N (%)	75/80 (93.8)	63/68 (92.6)	40/48 (83.3)	47/50 (94.0)	149/176 (84.7)	110/121 (90.9)	<0.001
DBPCFCs hazelnut							
Completed n/N (%)	20/80 (25.0)	9/70 (12.9)	0/51 (0.0)	13/50 (26.0)	41/177 (23.2)	1/141 (0.7)	<0.001
Reactive n/N (%)	15/20 (75.0)	8/9 (88.9)	–	8/13 (61.5)	34/41 (82.9)	1/1 (100)	
Anaphylaxis n/N (%)	2/78 (2.6)	1/69 (1.4)	7/51(13.7)	2/50 (4.0)	2/177 (1.1)	–	<0.001

sIgE, Specific IgE ≥ 0.35kU$_A$/L

*P-values were calculated by using ANOVA and Pearson χ^2 test and show an overall difference in characteristics between the cities.

Patients most commonly reported symptoms of the oral mucosa (84.4%), of which around half without other symptoms. Upper airway (rhinitis, rhino-conjunctivitis), skin and digestive symptoms were reported by 20.7-35.4%. More severe symptoms were less often reported, with 13.5% reporting asthma and 3% cardio-vascular or neurological symptoms.

Symptoms to hazelnut were supported by sensitization for the vast majority (88.2%), as detected in 566/718(77.4%) by SPT and 585/699(83.7%) by ImmunoCAP (496 subjects were positive to both tests). In 11.8% of all tested subjects we did not find evidence for hazelnut sensitization. Assessing sensitization by CRD in 423 patients (excluding CCD), 86.5% was positive. CRD detected IgE to individual hazelnut components in 15/68 (22%) with a negative hazelnut ImmunoCAP and 49/91 (54%) of SPT-negatives (more details in Supplementary Tables S2 and S3). Because only 3/11 patients from Sofia had detectable IgE against hazelnut, they were excluded from further statistical analysis of serological data.

To confirm hazelnut allergy, 124/731 subjects underwent a DBPCFC (Table S4). Hazelnut DBPCFC was positive for 87/124 patients (70.2%). Including the 22 anaphylactic subjects, we confirmed hazelnut allergy in 109 patients of which 95% had evidence for hazelnut sensitization by either SPT (87.0%), ImmunoCAP (89.9%) or CRD (93.8%). Sensitivity of CRD was significantly higher than of both other tests but specificity was significantly lower (for details see Supplemental Tables S5 and S6).

Patterns of recognition of individual hazelnut allergens in European cities

Hazelnut sIgE showed a clear variation across European cities (Figure 2). The pattern closely followed that of sensitization to birch pollen and IgE levels significantly correlated (*Rho* = 0.88, p <0.001). IgE levels to hazelnut were significantly lower in Athens and Madrid and, although not significantly, also in Reykjavik compared to the other cities.

Figure 3 shows the frequency and level of sensitization to individual hazelnut allergens and CCD. Sensitization to Cor a 1 was most prevalent (74.3%), followed at distance by both other pollen-related allergens Cor a 2 (19.6%) and CCD (10.2%). IgE levels against Cor a 1 were 5 to 10 times higher than those against other hazelnut allergens. Cor a 1 was dominant (≥60%) in all cities except Athens and Madrid (<10%) (Figure 4). In contrast, sensitization to Cor a 8 dominated in Athens 15/18 (83%) and to a lesser extent Madrid 4/11 (36%), while this was rare in other cities (<15%). Almost all patients sensitized to Cor a 14 were sensitized to Cor a 9 (20/22) and IgE levels closely correlated (*Rho* 0.74; p< 0.001). Cor a 9 and/or Cor a 14 sensitization was more common in Prague, Reykjavik, Utrecht, Manchester and Madrid (18.2-27.3%) than in other cities (<7%). Sensitization to Cor a 11 only reached a frequency >10% in Prague. Finally, sensitization to Cor a 12 was observed all over Europe in around 10-25% of the patients, except in Łódź and Strasbourg (<8%).

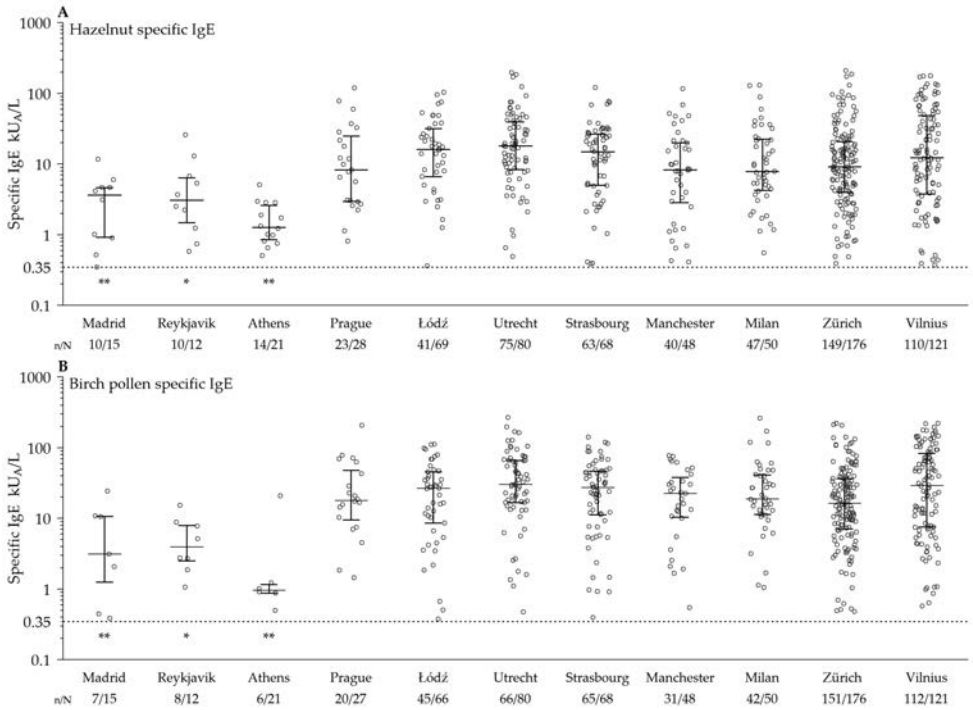

FIGURE II. IgE levels of all subjects with specific IgE to hazelnut (A) and birch (B) (≥ 0.35 kU$_A$/L). The black lines indicate the median IgE values and the interquartile range. For each city, the number of positive responders (n), total tested (N) and the proportion positives of the total (%) are shown.
*Significantly different from Łódź, Utrecht, Strasbourg, Manchester, Milan, Zürich and Vilnius.
**Significantly different from Prague, Łódź, Utrecht, Strasbourg, Milan, Zürich and Vilnius.

Age differences in hazelnut sensitization

Details of age related sensitization are shown in Table S7. Sensitization to Cor a 1 was less common in children (<18 years) than in adults (61.5% vs 76.2%; p<0.02). Children (<18 years) were significantly more often sensitized to Cor a 9 and/or Cor a 14 than adults (42.0% vs 5.8%; p<0.001), with the exception of Utrecht, where 9/10 sensitized to Cor a 9/Cor a 14 were older than 18 years. In addition, children were more often sensitized to Cor a 12 than adults (34% vs 11.4%, p <0.001).

Correlations between IgE to pollens and hazelnut allergens

Sensitization to all pollen extracts was observed in all centers (Table S8). Birch pollen sensitization was the most frequent amongst the nine pollens species tested (80.3%) followed by grass pollen sensitization, ranging from just under 50% to over 80%. To evaluate which of the 9 tested pollen species may be implicated in cross-reactivity to hazelnut allergens, IgE correlations were investigated. Figure 5A shows the strength of each correlation between a hazelnut allergen and a pollen extract (for exact correlation coefficients see Table S9).

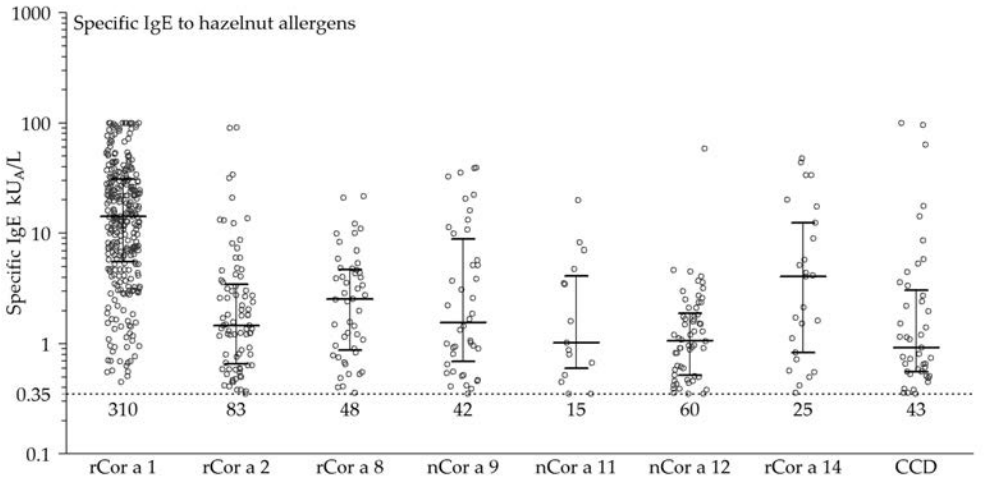

FIGURE III. Specific IgE to hazelnut allergens in a subset of the population with hazelnut allergy (n=423). Median sIgE values and interquartile range are indicated with black lines. The dotted lines indicate the cut-off IgE at 0.35 (kU$_A$/L). The number with positive IgE (≥ 0.35 kU$_A$/L) is indicated for each hazelnut allergen.

IgE against Cor a 1 correlated only with IgE against birch pollen (*Rho*=0.92, p<0.001), but such correlation was lacking in Athens and Madrid. Almost all birch-hazelnut co-sensitized patients (301/318) were sensitized to Cor a 1 (median IgE 14.60 kU/l, IQR 6.08-31.20). On the other hand, 5/30(16.1%) subjects sensitized to hazelnut but not birch pollen had IgE against Cor a 1 (median 4.07 kU/l, IQR 0.85-6.48).

IgE to Cor a 8 correlated weakly (*Rho* < 0.55) with that to *Chenopodium*, plane tree, mugwort and *Parietaria* pollen. Sensitization to these pollen was frequent in Athens, Madrid and Milan (see Table S8 and Figure S2 in the Supplemental Files) but only in Athens these correlations were stronger compared to the total population (*Rho* 0.78, 0.71, 0.71 and 0.66, respectively, p=0.001).

Patients sensitized to profilin (Cor a 2) and to CCD were sensitized to virtually all pollen species (92-100%). Surprisingly, correlations between IgE against Cor a 12 and pollens followed a very similar pattern. IgE to Cor a 9, Cor a 11 and Cor a 14 showed only very weak correlations to those against pollen.

Sensitization to other foods in a molecular perspective

IgE to other foods was observed in almost all subjects sensitized to hazelnut (92.9%), but the pattern of food sensitizations varied with the spectrum of hazelnut allergens recognized (Figure 5B and Supplemental Table S10). Peach and apple IgE correlated with Cor a 1 and Cor a 2, although not in Athens, Madrid, Milan. In those cities, IgE against these peach and apple

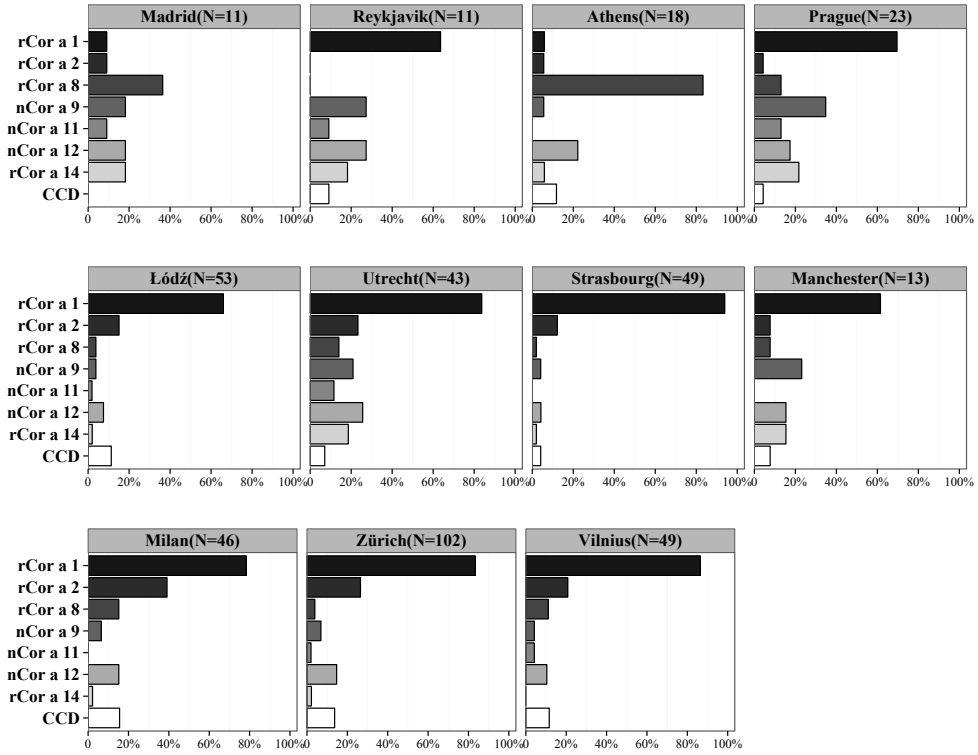

FIGURE IV. Sensitization to hazelnut allergens stratified by city. Sensitization to single hazelnut allergens was measured in a subpopulation (Sofia excluded) of the hazelnut patients. The bars show the percentage of positive test results patients for each allergen.

correlated with that against Cor a 8 in Athens (*Rho* 0.95 and 0.94, $p<0.001$) and Milan (*Rho* 0.68 and 0.66, $p<0.001$). In Madrid, only IgE to peach correlated with Cor a 8 (*Rho* 0.63, $p<0.04$).

Overall however, IgE to Cor a 8 most closely correlated with IgE to walnut, corn and lentil. Walnut sensitization was very common in Athens (92.8%) and Madrid (100.0%) compared to other cities (14.3 - 38%). IgE to walnut and Cor a 8 correlated tightly in Athens (*Rho* 0.94, $p<0.001$), but no significant correlation was found in Madrid (see Figure S3).

IgE responses to Cor a 9 and 14 showed weak correlations with those to tree nuts (*Rho* ≤ 0.57), seeds and legumes. IgE correlations between walnut and Cor a 9 were stronger in Utrecht and Prague (*Rho* = 0.70 and 0.78) than in the total population (*Rho* = 0.57). No such correlations were observed in Athens and Madrid.

IgE to Cor a 12 correlated moderately with IgE to oil-rich foods like tree nuts, seeds and legumes, but surprisingly also with melon and banana. No city-specific differences were observed for

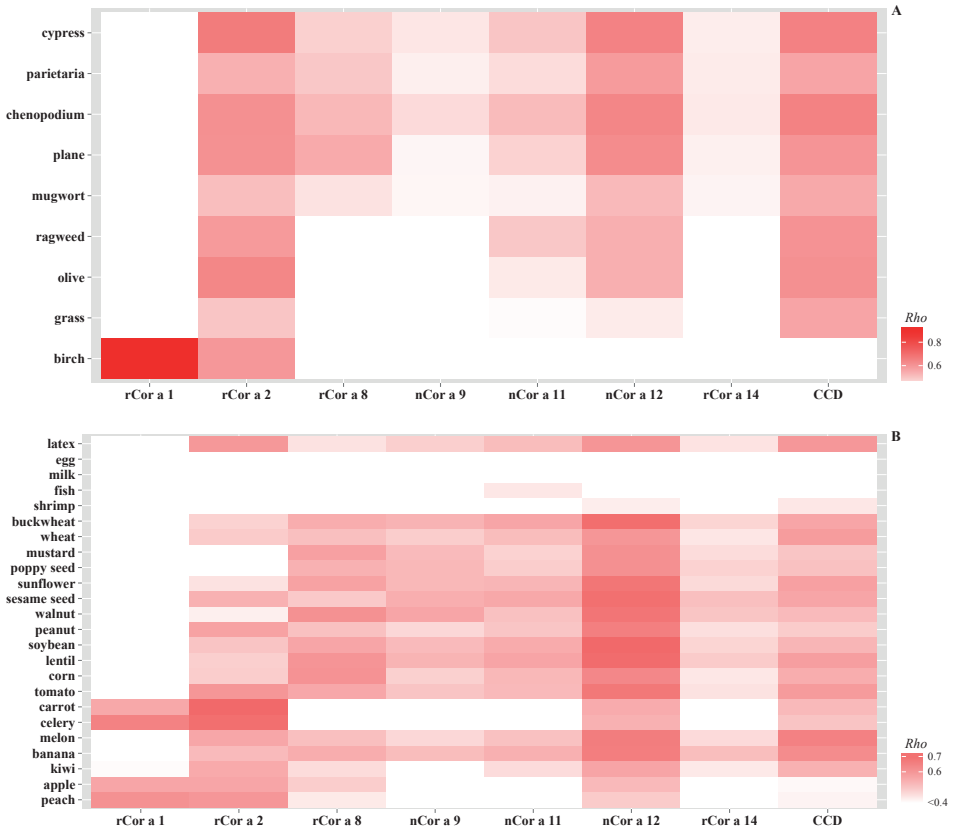

FIGURE V. Heat plots. (A) correlation between IgE to individual hazelnut allergens and 9 pollens and (B) the correlation between single hazelnut allergens and 23 foods and latex. Each color indicates the strength in correlation of IgE levels to hazelnut allergens with pollen, foods and latex . White: Spearman's *Rho* < 0.4; red-colors: Spearman's *Rho* 0.4-0.92

these correlations Figure S4 in the Supplemental Files shows correlations between IgE to walnut and soybean and Cor a 9 and Cor a 12). Finally, latex IgE correlated with all hazelnut allergens, except Cor a 1 and was strongest for Cor a 12, Cor a 2 and CCD.

Discussion

In the present study, the largest case series on hazelnut allergy ever performed were analyzed across Europe. Although the study was not a general population-based survey, the inclusion of consecutive patients coming into the clinic over a longer period of time gives an indication on the magnitude of the problem of hazelnut allergy across Europe. This study indicates that hazelnut allergy is far less common in cities like Athens, Madrid, Reykjavik and Sofia than in other European cities, similar to what has been reported in population-based surveys.[28,76] One

probable explanation is the low exposure to birch pollen in these four cities[29] as IgE levels against hazelnut and birch are much lower thanin other cities (median IgE < 4 vs 8-30 kU_A/L).

Birch pollen exposure has a dominant role in the occurrence of hazelnut allergy.[28,76] The high frequency and magnitude of IgE responses against Cor a 1 clearly supports that, in most of our cities, birch pollen sensitization (i.e. Bet v 1 - Cor a 1 cross-reactivity) is the driving force. In a small group, Cor a 1 sensitization was observed in the absence of birch sensitization. This has also been reported in hazelnut-sensitized children from the Netherlands.[77] Although we cannot exclude direct food-driven sensitization to hazelnut Cor a 1, it is perhaps more likely that sensitization to pollen of other *Fagales* species, such as hazel, oak, alder or beech might induce IgE antibodies cross-reactive with Cor a 1.

Where the role of birch pollen as dominant source of sensitization for Cor a 1 is not disputed, the situation is more complex for Cor a 8. A longstanding assumption is that sensitization to LTP is a Mediterranean phenomenon.[32] The dominant Cor a 8 profile in Athens and Madrid confirms this. The currently prevailing opinion is that peach Pru p 3 induces sensitization to other food LTPs. In Athens and Madrid, IgE to Cor a 8 correlates strongly with that to peach (*Rho* = 0.68 and 0.95, respectively). However, the high frequency of walnut sensitization (93%) and the strong correlation between Cor a 8 and walnut IgE (*Rho* = 0.94) in Athens, suggests that a walnut rich diet could also be relevant for hazelnut sensitization. On the other hand, several studies have demonstrated that sensitization to mugwort and plane tree pollen LTP also plays a role in the "LTP-syndrome".[7,8,33,78] We found correlations between IgE to Cor a 8 and IgE to weed and tree pollen, although weaker than those to peach, apple and walnut. Whether the associated foods or pollens act as primary cause of sensitization to Cor a 8 cannot easily be inferred from available data. Mediterranean patients are perhaps geographically not likely to develop LTP-driven food allergies, but less prone to develop birch-pollen associated food allergies. Future studies, in particular IgE inhibition assays, are needed to unravel probable primary source of sensitization to LTPs.

The geographical distribution of sensitization to seed storage proteins (Cor a 9, Cor a 11 and Cor a 14) is less clear. Although relatively more subjects from Prague, Reykjavik, Utrecht, Manchester and Madrid were sensitized to these allergens, the total numbers are low (n=2-9). Other studies have shown sensitization for Cor a 9 and Cor a 14 to occur preferentially in younger children[26,38,79] and we also observed significant differences between children and adults (42% vs 5.8%). These proportional differences can be explained by more Bet v-1 related sensitization later in life (adults showed significantly higher proportions of Cor a 1 sensitization) causing a diluting effect of hazelnut storage protein sensitization in adults. Storage protein IgE correlates relatively weakly to other foods. This may indicate that sensitization to these proteins is driven by hazelnut consumption, and sensitization to other foods are independent co-sensitizations.

Of interest are the sensitization patterns observed for hazelnut oleosin (Cor a 12). To date, very little is known about allergic reactions to oleosin in foods and have so far only been reported for peanut,[80] sesame seed[81] and buckwheat.[82] Hazelnut oleosin was first described by Akkerdaas and collegues[70] and has recently been associated with severe symptoms.[71] Our data show that hazelnut oleosin sensitization is not uncommon in Europe. IgE against Cor a 12 correlated with sensitization to many foods, in particular oil-rich tree nuts, seeds and legumes. Interestingly, the pattern of associations to pollen sensitization was very similar to that observed for Cor a 2 and CCD. Oleosins have been identified in pollen as well[83] so it cannot be excluded that pollen play a role in sensitization to oleosins.

IgE against both Cor a 2 and CCD were associated with all pollen species as true pan allergenic structures. Surprisingly, the closest correlation was with olive and cypress pollen and not with grass pollen. Foods that have previously been linked to profiling sensitization were closely associated in the present study as well: carrot, celery, peach, tomato and melon.[65,84–88]

Some limitations of the present study have to be considered. Although we performed the largest series of DBPCFCs for hazelnut, still 83% of those reporting hazelnut allergy were not challenged. Moreover, the number of challenges carried out was unbalanced between cities. However, this is the most comprehensive standardized study so far with respect to sensitization and allergy to hazelnut. What can we say about the place of the three test for sensitization in an outpatient clinic setting? Sensitivity of CRD with 7 allergens together is higher than conventional hazelnut ImmunoCAP or SPT, but probably too costly for routine application. A more realistic approach, with a minimal loss of sensitivity (93.8% vs 91.2%), would be to test IgE against Cor a 1, Cor a 8 and Cor a 14. For simply assessing if reported hazelnut allergy is supported by sensitization, SPT or hazelnut ImmunoCAP are most likely appropriate and more feasible. None of the three tests has a useful specificity (10%-30%), maintaining the DBPCFC still remains an important diagnostic procedure in cases where to establish clinical relevance. Having said that, CRD has revealed associations between the outcome of a DBPCFC including severity and specific allergens.[26] This certainly is an added value of CRD, allowing better assessment of the risk of severe reactions. Currently, we are analyzing the patients' sensitization pattern reported here for associations of reported symptoms in real life and during DBPCFC with specific IgE against individual allergens.

In conclusion, our study has mapped hazelnut allergy across Europe. Major differences in the number of cases were observed across Europe, which are largely explained by differences in exposure to birch pollen. This dominant cross-reactive phenomenon explains the lower sensitization rates to storage proteins, oleosins and LTPs. A dominance of Cor a 8 (LTP) was confirmed for the Mediterranean basin, in particular for Athens but the source of primary sensitization is still not completely certain. Finally, oleosin-sensitization is observed across the whole of Europe but whether pollen plays a role in sensitization to oleosins needs to be established.

Acknowledgements

We thank ALK-Abelló for providing the SPT reagents for this study. We also thank Lothar Vogel for his Involvement in the serum screening and selection and thank all the patients who volunteered to participate in the EuroPrevall studies.

Supplemental files

Supplemental Methods

2

Study Design

The EuroPrevall outpatient clinic survey included 2273 subjects reporting adverse reactions to a food. Background and case-report forms were completed for 2261 subjects. This population consisted of 1615 adults (median age 32.9; IQR: 18.23) and 646 children below 18 years old (median age 8.3; IQR 10.70). Of those children, 148 were younger than 4 years.

Of the 2261, 740 food forms for hazelnut were completed. For this study, we included subjects when the reported time between the food intake and onset of symptoms was within 2 hours and 731 met these criteria.

Skin-prick testing

Commercially available extracts (ALK-Abelló, Madrid, Spain) were used to measure skin reactivity against 24 foods and 12 inhalants (Table S1). All centers were provided with the same batch of SPT reagents for the study. At each clinical center, a single investigator carried out all SPTs following the recommendations of the European Academy of Allergology and Clinical Immunology.[89] The same type of lancets were used in all centers (ALK-Abelló). Histamine hydrochloride (10 mg/mL) and saline were used aspositive and negative controls, respectively.

Specific IgE measurements

Sera from patients were sent to the Paul Ehrlich institute (Germany). Using uniform batches of reagents, all sera were tested for the presence of IgE against 24 food extracts and 12 inhalant extracts (Table S1). All sera from the general population survey were first tested with five mixtures, containing 5 (in one case 6) different foods as described earlier (Table S1).[61] If a mix was positive (≥ 0.35 kU_A/L), the individual foods present in that mix were tested separately. To save serum, the same procedure was performed for outpatient clinic subjects with limited serum volume (< 2ml).

For the component-resolved diagnosis, commercially available ImmunoCAPS were used to measure IgE levels to rCor a 1, rCor a 8 and MUXF3(CCD). Custom-made ImmunoCAPs were used to measure IgE against rCor a 2, nCor a 9, nCor a 11, nCor a 12 and rCor a 14. All CRD measurements were carried out at the Academic Medical Center (the Netherlands) on an ImmunoCAP 250 instrument.

DBPCFC

The active and placebo provocations with hazelnut were done on 2 different randomly assigned days. The first dose started at 3μg of hazelnut protein and the following 8 doses gradually

increased until the top dose of 3 g protein (corresponding to 15 hazelnut kernels). The next dose was given after 20 minutes or if any previous reaction has disappeared. The challenge was stopped when an objective reaction occurred or convincing subjective reactions lasted for more than 45 minutes.

Supplemental Results

Diagnostic performance of the SPT, ImmunoCAP and CRD

The differences in performance between the hazelnut SPT, ImmunoCAP and CRD (positive to any allergen) are shown in table S3. The majority of the allergic patients were positive for all test, with a sensitivity of 87.0% (95% CI: 79.2-92.7) by SPT, 89.9% (82.5-94.8) by ImmunoCAP and 93.8% by CRD using a cut-off of 0.35 kUA/L. Among the tolerant cases, the percentage of those with positive IgE was high, resulting in a low specificity for SPT (30%), ImmunoCAP (25%) and CRD (16.7%). When using 0.1 kUA/L as the cut-off IgE value, almost all tolerant patients (15/18) were classified as 'positive' resulting in a specificity of 10% (1.2-37.7%). Seven challenged patients that had IgE levels to hazelnut between 0.1 and 0.35 and 4/7 were reactive during the challenge. When combining the SPT and ImmunoCAP (cut-off 0.50 and 0.35, respectively), the NPV improved compared to the individual tests. The specificity for CRD (16.7%) was significantly lower compared to the SPT tests (textit p =0.001) and ImmunoCAP(p=0.04).

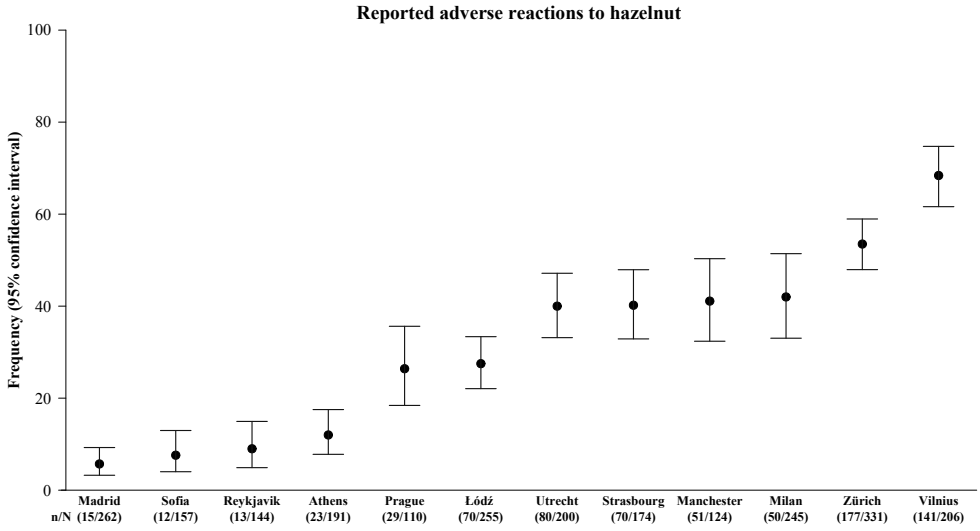

FIGURE S1. Frequency of subjects reporting hazelnut allergy. The proportion of patients reported hazelnut allergy for each clinical center is shown, with bars indicating the 95% confidence interval. The numbers below the cities show the number of subjects with convincing hazelnut allergy(n) and the total number of subjects included in the EuroPrevall survey(N).

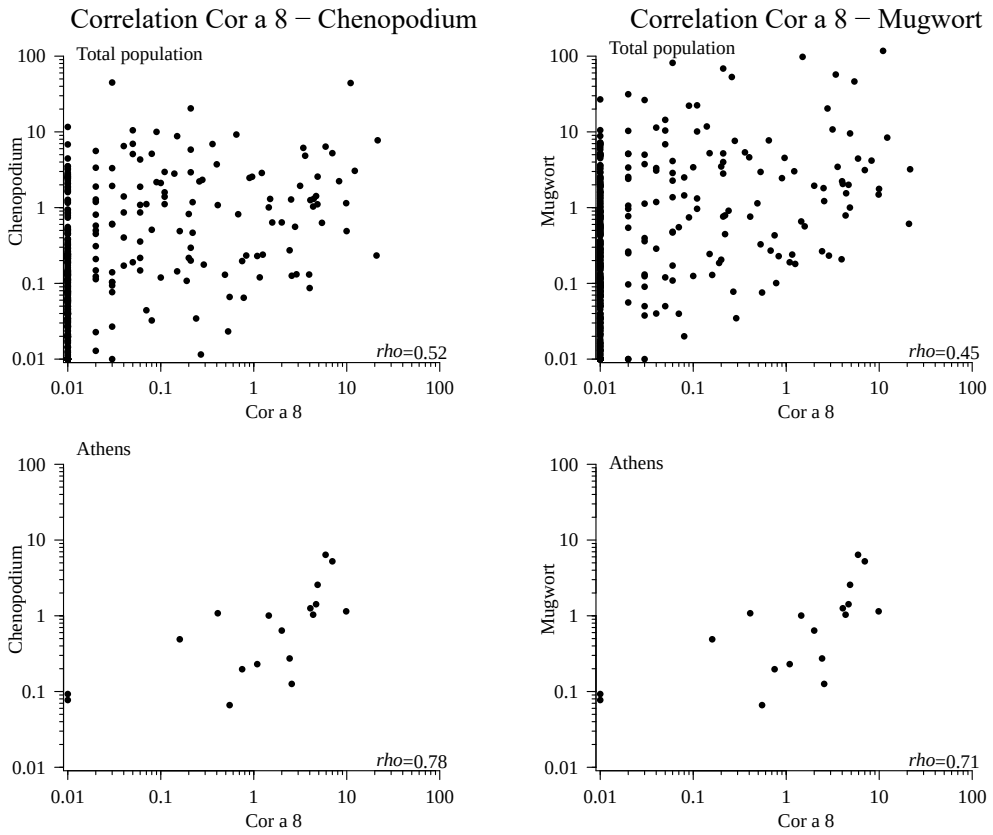

FIGURE S2. Scatterplots showing the correlation between IgE against Cor a 8 and Chenopodium pollen and Mugwort pollen. The upper graphs present all subjects in the population, the lower graphs are specific for subjects from Athens. The x and y axis show the IgE levels on a logaritmic scale.

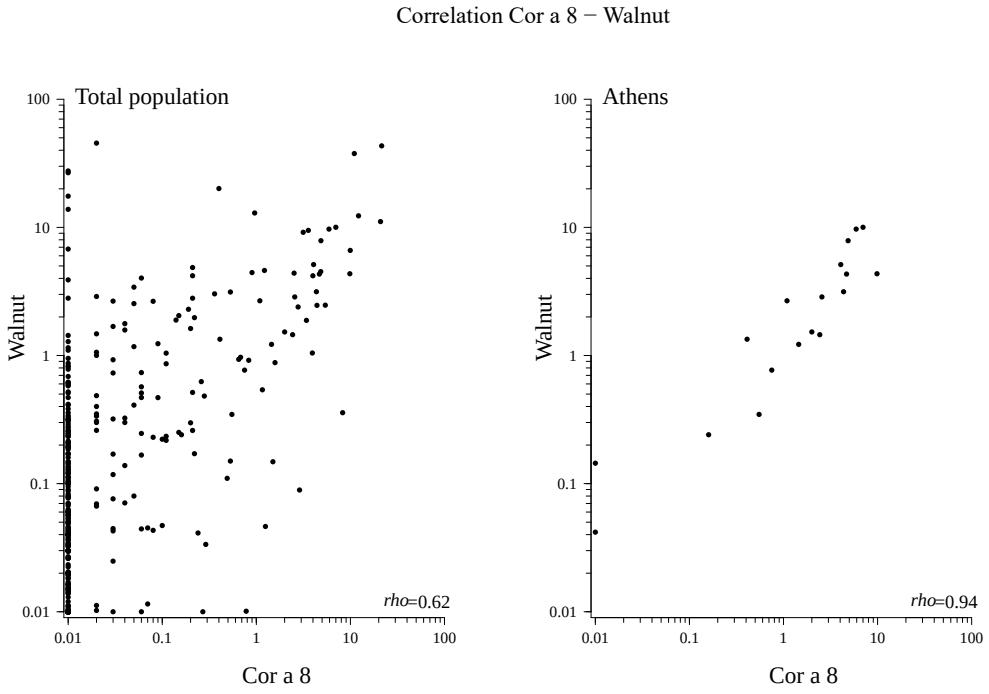

FIGURE S3. Scatterplots showing the correlation between IgE against Cor a 8 and walnut in the total population and in Athens. The x and y axis show the IgE levels on a logaritmic scale.

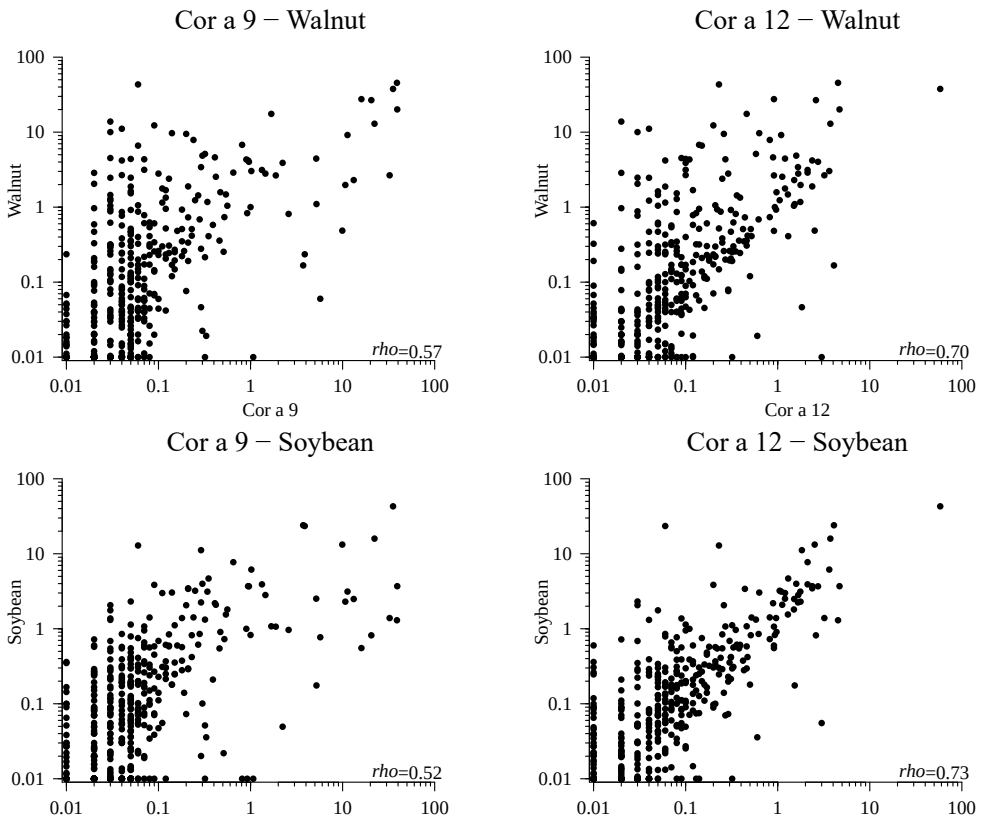

FIGURE S4. Scatterplots showing the correlation between IgE against hazelnut allergens Cora 9 and Cor a 12 with IgE against walnut and soybean. The x and y axis show the IgE levels on a logaritmic scale.

TABLE S1. THE 24 FOODS, 12 INHALANT ALLERGENS AND LATEX MEASURED BY SKIN PRICK TESTING AND IMMUNOCAP .

All foods	Composition of the food mixes		Inhalent allergen sources & latex
Peach	Mix 1	Hazelnut	Birch[§]
Apple		Tomato	Chenopodium[*§]
Kiwi		Carrot	Cypress[*§]
Banana		Celery	Mugwort[§]
Melon		Walnut	Olive[*§]
Celery	Mix 2	Shrimp	Parietaria[§]
Carrot		Mustard	Plane tree[*§]
Tomato		Poppy seed	Ragweed[*§]
Corn		Lentil	Timothy grass[§]
Lentil		Sunflower	
Soybean	Mix 3	Apple	House Dust mite
Peanut		Kiwi	Cat
Walnut		Melon	Dog[*]
Hazelnut		Banana	
Sesame seed		Peach	Latex[*§]
Sunflower	Mix 4	Egg white	
Poppy seed		Milk	
Mustard		Fish (cod)	
Wheat		Peanut	
Buckwheat		Soybean	
Shrimp		Wheat	
Fish	Mix 5	Wheat	
Milk		Maize, Corn	
Egg		Rice	
		Sesame seed	
		Buckwheat	

Panel of foods, inhalants and latex that were tested for sensitization by Skin Prick Testing and serum IgE. The mixes were only used for serological analysis in patients that were first selected in the general population survey or those with a limited serum volume (< 2ml).

[*]Subjects from the general population survey were not tested for sensitization against these allergens.

[§]Analyzed for correlation between specific IgE and IgE against hazelnut allergens.

TABLE S2. SENSITIZATION TO HAZELNUT MEASURED BY CRD, SKIN PRICK TESTING AND EXTRACT.

CRD vs Skin prick testing		
	Skin Prick Test*	
	Positive	**Negative**
CRD Positive	316	49
CRD Negative	14	42
Total	330	91
CRD vs Extract		
	Extract**	
	Positive	**Negative**
CRD Positive	349	15
CRD Negative	4	53
Total	353	68
CRD vs ImmunoCAP		
	SPT and/or IgE levels Extract	
	Positive	**Negative**
CRD Positive	360	6
CRD Negative	16	41
Total	376	47

SPT: Skin Prick Test; CRD: Component Resolved Diagnosis

All positive tests results were based on sIgE ≥0.35kUA/L and SPT allergen/histamine ratio ≥ 0.5

*Two subjects with missing data for ImmunoCAP

**Three subjects with missing data for ImmunoCAP

TABLE S3. SENSITIZATION TO HAZELNUT ALLERGENS IN PATIENTS WITHOUT DETECTABLE SIGE TO HAZELNUT EXTRACT AND NEGATIVE SPT.

	Negative IgE levels Extract			Negative SPT			Negative SPT and IgE levels Extract		
		IgE levels			IgE levels			IgE levels	
	N	Median	IQR	N	Median	IQR	N	Median	IQR
Total	68			91			47		
CRD positive	15			49			5		
rCor a 1	3	0.67	0.63-4.00	38	4.69	1.34-13.68	1	0.63	-
rCor a 2	7	2.22	1.63-2.93	13	1.79	0.80-2.61	5	2.22	1.79-2.68
rCor a 8	5	0.83	0.68-1.10	1	3.41	-	0	-	-
nCor a 9	1	1.07	-	9	0.96	0.56-3.88	0	-	-
rCor a 11	0	-	-	2	3.98	2.43-5.53	0	-	-
rCor a 12	0	-	-	12	1.40	0.52-2.54	0	-	-
rCor a 14	0	-	-	1	1.12	-	0	-	-
CCD	0	-	-	6	1.36	0.80-2.92	0	-	-

SPT: Skin Prick Test; CRD: Component Resolved Diagnosis.

The number of subjects with IgE to a single hazelnut allergen but with negative tests results to hazelnut SPT or Extract (IgE < 0.35kU$_A$/L). The first rows shows the total number of negative hazelnut SPTs and Extract based tests of the 423 CRD tested subjects. The second row shows the number of the negative tested subjects that were positive in the CRD. Then for each allergens the number of positives to that allergen where shown. Some subjects responded to more than 1 hazelnut allergen.

TABLE S4. DOUBLE BLIND PLACEBO-CONTROLLED CHALLENGES TO HAZELNUT AND ANAPHYLACTIC PATIENTS.

	European city						
DBPCFCs hazelnut	**All n=731**	**Madrid n=15**	**Sofia n=12**	**Reykjavik n=13**	**Athens n=23**	**Prague n=29**	**Łódź n=70**
Completed n/N (%)	124/731 (16.9)	8/15 (53.3)	1/12 (8.3)	6/13 (46.2)	3/23 (13.0)	5/29 (17.2)	17/70 (25.0)
Reactive n/N (%)	87/124 (70.2)	5/8 (62.5)	0/1 (0.0)	4/6 (66.7)	1/3 (33.3)	3/5 (60.0)	8/17 (47.1)
Anaphylaxis n/N (%)	22/731 (3.0)	-	1/12 (8.3)	-	3/23(13.0)	2/29(6.9)	2/70(2.9)

	European city					
DBPCFCs hazelnut	**Utrecht n=80**	**Strasbourg n=70**	**Manchester n=51**	**Milan n=50**	**Zürich n=177**	**Vilnius n=141**	**P-value**
Completed n/N (%)	20/80 (25.0)	9/70 (12.9)	0/51 (0)	13/50 (26.0)	41/177 (23.2)	1/1441(0.7)	<0.001
Reactive n/N (%)	15/20(75.0)	8/9 (88.9)	-	8/13 (61.5)	34/41 (82.9)	1/1 (100.0)	
Anaphylaxis n/N (%)	2/78(2.6)	1/69(1.4)	7/51(13.7)	2/50(4.0)	2/177(1.1)	-	<0.001

*P-values were calculated by Pearson χ^2 test and show an overall difference between the cities.

TABLE S5. SENSITIVITY AND SPECIFICITY OF THREE DIFFERENT HAZELNUT TESTS TO MEASURE SENSITIZATION.

		Allergic	Tolerant	Sensitivity (CI95%)		Specificity (CI95%)	
SPT(0.5)	Positive	94	14	87.0	(79.2-92.7)	30.0	(11.9-54.3)
	Negative	14	6				
ImmunoCAP (0.35)	Positive	97	15	89.8	(82.5-94.8)	25.0	(8.7-49.1)
	Negative	11	5				
ImmunoCAP (0.10)	Positive	102	18	94.4	(88.3-97.9)	10.0	(1.2-31.7)
	Negative	6	2				
SPT(0.5) + ImmunoCAP (0.35)	Positive	106	16	97.2	(92.2-99.4)	20.0	(5.7-43.7)
	Negative	3	4				
CRD* (0.35)	Positive	91	6	93.8	(87.0-97.7)	16.7	(3.6-41.4)
	Negative	15	3				
CRD* (0.1)	Positive	94	17	96.9	(91.2-99.4)	5.6	(0.1-27.3)
	Negative	3	1				
Cora 1/Cor a 8/ Cor a 14§	Positive	89	12	91.2	(84.3-96.4)	33.3	(13.3-59.0)
	Negative	8	16				

SPT: Skin Prick Test; CRD: Component Resolved Diagnosis

*Sensitization to any of the seven hazelnut allergens measured by component-resolved diagnosis (CRD)

Sensitivity and specificity were calculated for SPT using allergen/histamine wheal ratio ≥ 0.5 and for serology testing IgE levels ≥ 0.35 and ≥ 0.1 kUA/L as a cut-off value.

§sensitization to Cor a 1, Cor a 8 or Cor a 14 using (≥ 0.35 kUA/L)

TABLE S6. SENSITIZATION AGAINST HAZELNUT ALLERGENS.

	Reactors		Anaphylaxis		Tolerant	
	N	%	N	%	N	%
rCor a 1 (positive)	69/77	89.6	10/20*	50.0	11/18	61.1
rCor a 2 (positive)	14/77	18.2	3/20*	15.0	4/18	22.2
rCor a 8 (positive)	7/77	9.1	5/20*	25.0	1/18	5.6
nCor a 9 (positive)	5/77	6.5	5/20*	25.0	3/18	16.7
rCor a 11 (positive)	5/77	6.5	2/20*	10.0	1/18	5.6
nCor a 12 (positive)	9/77	11.7	6/20*	30.0	2/18	11.1
rCor a 14 (positive)	5/72	6.9	5/17*	29.4	0/17	0.0
CCD (positive)	4/77	5.2	5/20*	25.0	2/18	11.1

*7/10 subjects also had IgE against one of the non-pollen related allergens Cor a 8, Cor a 9, Cor a 11, Cor a 12 or Cor a 14, implying that 3 were selectively sensitized to Cor a 1 amongst the 7 allergens we tested.

TABLE S7. AGE RELATED SENSITIZATION TO SINGLE HAZELNUT ALLERGENS.

	Adults		<18 years		< 7years	
	N	%	N	%	N	%
Extract (positive)	502/593	84.7	83/106	78.3	3/18	44.4
rCor a 1 (positive)	278/365	76.2	32/52	61.5	3/7	42.9
rCor a 2 (positive)	76/369	20.6	7/53	13.2	0/7	0.0
rCor a 8 (positive)	41/367	11.2	7/52	13.5	1/7	14.3
nCor a 9 (positive)	21/369	5.7	21/53	39.6	4/7	57.1
nCor a 11 (positive)	10/369	2.7	5/53	9.4	1/7	14.3
nCor a 12 (positive)	42/368	11.4	18/53	34.0	2/7	28.6
rCor a 14 (positive)	11/360	3.1	14/50	28.0	2/7	28.6
CCD (positive)	36/362	9.9	7/52	13.5	0/7	0.0

Sensitization to hazelnut Extract, individual allergens and CCD are shown for all adults, children younger than 18 years (including <7 years) and a group of subjects younger than 7 years.

TABLE S8. FREQUENCY OF POLLEN SENSITIZATION STRATIFIED BY CITY.

		European city										
	Total	**Athens**	**Madrid**	**Reykjavik**	**Milan**	**Manchester**	**Prague**	**Zürich**	**Vilnius**	**Strasbourgh**	**Łódź**	**Utrecht**	***P*-value***
Birch	80.3	28.6	46.7	66.7	84.0	64.6	74.1	85.8	92.6	95.6	68.2	82.5	<0.001
Grass	65.0	61.9	80.0	50.0	68.0	81.2	66.7	71.0	62.8	58.8	47.0	68.8	<0.001
Olive	58.0	61.9	66.7	33.3	70.0	64.6	55.6	71.2	52.9	55.9	29.7	62.8	<0.001
Ragweed	47.0	57.1	66.7	41.7	76.0	31.2	51.9	50.4	55.4	23.5	35.9	37.2	<0.001
Mugwort	46.3	66.7	60.0	25.0	66.0	31.2	59.3	44.9	61.2	26.5	42.4	35.0	0.001
Plane tree	40.0	66.7	83.3	25.0	68.0	41.7	37.0	41.7	38.8	22.1	26.6	34.9	<0.001
Chenopodium	35.1	57.1	58.3	25.0	50.0	29.2	40.7	41.0	35.5	17.6	21.9	34.9	<0.001
Parietaria	34.4	66.7	60.0	16.7	56.0	29.2	29.6	37.5	33.1	25.0	24.2	27.5	<0.001
Cypress	29.9	33.3	58.3	16.7	50.0	25.0	22.2	34.5	26.4	14.7	18.8	34.9	<0.001

**P*-values were calculated by using Pearson χ^2 test and show the overall proportional differences in sensitization (IgE ≥0.35kUA/L) between cities.

TABLE S9. CORRELATIONS BETWEEN SIGE TO HAZELNUT ALLERGENS AND INHALANTS.

	Hazelnut allergen							
	rCor a 1	**rCor a 2**	**rCor a 8**	**nCor a 9**	**nCor a 11**	**nCor a 12**	**rCor a 14**	**CCD**
Birch	0.92	0.60	0.04[$]	0.15	0.23	0.34	0.25	0.36
Grass	0.20	0.50	0.31	0.33	0.40	0.43	0.35	0.57
Olive	0.34	0.64	0.37	0.35	0.43	0.54	0.35	0.61
Ragweed	0.23	0.59	0.38	0.40	0.49	0.54	0.37	0.61
Mugwort	0.20	0.51	0.45	0.41	0.42	0.52	0.42	0.56
Plane tree	0.21	0.61	0.55	0.42	0.47	0.63	0.42	0.61
Chenopodium	0.17	0.61	0.52	0.46	0.52	0.64	0.43	0.65
Parietaria	0.16	0.54	0.49	0.42	0.45	0.59	0.43	0.57
Cypress	0.31	0.67	0.48	0.44	0.50	0.65	0.42	0.65

The values represent the Spearman's *Rho* correlation between IgE levels.

All *p*-values <0.001. [$]no significant correlation

TABLE S10. CORRELATIONS BETWEEN SIGE TO HAZELNUT ALLERGENS AND 23 FOODS AND LATEX.

	Hazelnut allergen							
	rCor a 1	**rCor a 2**	**rCor a 8**	**nCor a 9**	**nCor a 11**	**nCor a 12**	**rCor a 14**	**CCD**
Peach	0.62	0.61	0.43	0.24	0.29	0.49	0.32	0.42
Apple	0.57	0.57	0.48	0.27	0.30	0.53	0.32	0.41
Kiwi	0.41	0.56	0.46	0.40	0.46	0.57	0.44	0.54
Banana	0.21	0.52	0.55	0.52	0.54	0.67	0.52	0.63
Melon	0.21	0.57	0.51	0.47	0.51	0.67	0.46	0.66
Celery	0.66	0.71	0.25	0.26	0.37	0.54	0.33	0.50
Carrot	0.56	0.73	0.23	0.30	0.40	0.55	0.35	0.53
Tomato	0.22	0.61	0.56	0.50	0.53	0.68	0.45	0.59
Corn	0.08§	0.48	0.61	0.48	0.53	0.65	0.44	0.55
Lentil	0.17	0.48	0.61	0.54	0.57	0.72	0.49	0.59
Soybean	0.19	0.50	0.57	0.52	0.56	0.73	0.47	0.53
Peanut	0.28	0.58	0.51	0.47	0.50	0.67	0.45	0.49
Walnut	0.10*	0.42	0.62	0.57	0.51	0.70	0.50	0.52
Sesame seed	0.18**	0.54	0.49	0.55	0.56	0.70	0.51	0.57
Sunflower	0.11*	0.45	0.58	0.53	0.53	0.69	0.46	0.58
Poppy seed	0.04§	0.36	0.54	0.53	0.49	0.62	0.47	0.51
Mustard	0.07§	0.31	0.58	0.52	0.47	0.62	0.46	0.50
Wheat	0.12*	0.49	0.51	0.48	0.52	0.61	0.44	0.59
Buckwheat	0.12*	0.48	0.55	0.54	0.57	0.71	0.47	0.57
Shrimp	0.21	0.31	0.29	0.39	0.39	0.43	0.36	0.44
Fish	0.19	0.30	0.26	0.28	0.44	0.40	0.33	0.36
Milk	0.14*	0.26	0.31	0.33	0.35	0.29	0.34	0.30
Egg	0.14*	0.24	0.34	0.39	0.37	0.34	0.37	0.29
Latex	0.18	0.60	0.45	0.48	0.52	0.61	0.44	0.60

The values represent the Spearman's Rho correlation between IgE levels.

All *p*-values <0.001; **p<0.01; *p<0.05; §no significant correlation.

3 Component-resolved diagnosis and beyond: multivariable regression models to predict severity of hazelnut allergy

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Allergy 2018; 73(3):549-559
DOI: 10.1111/all.13328

Abstract

Background: Component-resolved diagnosis (CRD) has revealed significant associations between IgE against individual allergens and severity of hazelnut allergy. Less attention has been given to combining them with clinical factors in predicting severity.

Aim: To analyze associations between severity and sensitization patterns, patient characteristics and clinical history, and to develop models to improve predictive accuracy.

Methods: Patients reporting hazelnut allergy (n=423) from 12 European cities were tested for IgE against individual hazelnut allergens. Symptoms (reported and during Double-blind placebo-controlled food challenge [DBPCFC]) were categorized in mild, moderate and severe. Multiple regression models to predict severity were generated from clinical factors and sensitization patterns (CRD- and extract-based). Odds ratios (ORs) and areas under receiver operating characteristic (ROC) curves (AUCs) were used to evaluate their predictive value.

Results: Cor a 9 and 14 were positively (OR 10.5 and 10.1 respectively), and Cor a 1 negatively (OR 0.14) associated with severe symptoms during DBPCFC, with AUCs of 0.70-073. Combining Cor a 1 and 9 improved this to 0.76. A model using a combination of atopic dermatitis (risk), pollen allergy (protection), IgE against Cor a 14 (risk) and walnut (risk), increased the AUC to 0.91. At 92% sensitivity, the specificity was 76.3% and the positive and negative predictive values 62.2% and 95.7%, respectively. For reported symptoms, associations and generated models proved to be almost identical but weaker.

Conclusion: A model combining CRD with clinical background and extract-based serology is superior to CRD alone in assessing the risk of severe reactions to hazelnut, particular in ruling out severe reactions.

Key messages

- Severe allergic symptoms to hazelnut are reproducibly associated with high IgE against Cor a 9 and 14 and low against Cor a 1, but predictive accuracy of stand-alone CRD is poor.

- A model that combines sensitization to Cor a 14 with atopic dermatitis (risk factor), pollen allergy (protective) and IgE against walnut (risk factor) significantly improves severity risk assessment.

Introduction

In the EuroPrevall project, symptoms and sensitization to hazelnut were most frequently reported and observed.58,76,90 Symptoms can vary from mild oral symptoms to more severe symptoms such as bronchospasm and, in some cases, life-threatening anaphylaxis. An area that has drawn much attention in predicting the risk of severe reactions is the IgE recognition profile of individual allergen molecules in foods. These profiles, usually referred to as component-resolved diagnosis (CRD), may facilitate better distinction of patients with severe reactions from those with milder reactions, or reactive from tolerant among sensitized patients.

There is growing evidence that the seed storage proteins Cor a 9 (11S globulin) and Cor a 14 (2S albumin) are associated with more severe reactions26,38,39 similar to lipid transfer protein (LTP) Cor a 8 in the Mediterranean area.32,64,91 In Northern and Central Europe, sensitization to the Bet v 1-homologue Cor a 1 is typically seen in adolescents and adults with sensitization to birch pollen and oral symptoms.32,62,63 Less is known about the clinical relevance of profilin (Cor a 2),63,88 Cor a 11(vicilin-like protein),68,90 and the oleosins Cor a 12 and Cor a 13.70 Although multiple studies have shown an association between severity and sensitization to specific hazelnut allergens,26,27,38,39,71 these reports are mostly in children and limited to patient populations with specific geographical background. To establish broader applicability of CRD, it is important to confirm these associations in larger populations with different geographical backgrounds. Additionally, it is relevant to evaluate the accuracy in classifying between mild-tomoderate allergy and severe allergy because markers associated with clinical outcomes, such as severity, are not necessarily good diagnostic tools as well.92

In clinical practice, a thorough clinical history is the starting point. Here, we aim to investigate whether a combination of a detailed anamnesis and molecular and/or extract-based sensitization patterns can lead to better prediction of the risk of severe reactions. The EuroPrevall outpatient clinic survey, in which detailed clinical histories and sensitization profiles were recorded in twelve European cities, allowed us to investigate this and validate factors related to severe hazelnut allergy in a large and demographically diverse study population.59,90 We evaluated clinical and serological data (24 foods,12 inhalants, and latex) on 731 outpatients that reported allergic 1 symptoms to hazelnut and that were enrolled across Europe, using a standardized protocol. Molecular diagnostics using rCor a 1, rCor a 2, rCor a 8, nCor a 9, nCor a 11, rCor a 12, rCor a 14, and CCD was performed for 423 subjects. For 124 of these, a double-blind placebo-controlled food challenge (DBPCFC) was performed, allowing us to establish whether associations with severity during challenge were similar to those found for reported food allergy.

Methods

Patient selection

In the outpatient clinic survey of the EuroPrevall project58, subjects reporting allergic reactions to food were prospectively recruited between 2006 and 2009 in 12 outpatient clinics: Athens (Greece), Łódź (Poland), Madrid (Spain), Manchester (United Kingdom), Milan (Italy), Prague (Czech Republic), Reykjavik (Iceland), Sofia, (Bulgaria), Strasbourg (France), Utrecht (The Netherlands), Vilnius (Lithuania) and Zürich (Switzerland). Details are presented elsewhere.59,61,90

Here, we evaluate 731 subjects reporting immediate adverse reactions (≤ 2hrs) to hazelnut. Of these patients 652 were recruited in the outpatient clinics, the remaining 79 patients selected for detailed clinical evaluation during the EuroPrevall general population surveys.90 There was no difference in reported symptom severity between both groups, and therefore the groups were analyzed together. For two patients, data concerning symptoms were missing, and they were excluded from statistical analyses. Of the remaining 729 patients, 120 (16.5%) were children (< 18 years).

Sera of 423 patients (12.5% children) were available for CRD, as detailed elsewhere.90 Patients evaluated by CRD were older and had lower frequencies of an atopic family background and atopic dermatitis (AD) than those that were not. They also showed some differences in the severity classification (see Supplemental Table S1).

Clinical evaluation and double blind placebo-controlled food challenges (DBPCFC)

The protocol used for clinical evaluation has been described in detail elsewhere.59 In short, allergy specialists used standardized case-record forms for a detailed description of hazelnut-induced symptoms (type, age of onset, and duration), family members with atopy (father, mother, sibling) and of co-existing non-food-induced atopic co-morbidities (current or ever) including asthma, allergic rhinitis and AD. Patients were classified as pollen-, house dust mite- and/or latex-allergic when they reported respiratory symptoms to that source supported by matching sensitization.

All patients were asked to undergo a DBPCFC for hazelnut and 124 patients (18 <18 years) consented to do so. Hazelnut challenges were performed by trained physicians using the same protocol and challenge meals in all clinics.21 Details are provided in the Methods section in the Supplemental Material. The backgrounds of patients undergoing DBPCFC were comparable to those not undergoing the procedure, except that they had a lower frequency of AD and slightly higher matching sensitization to hazelnut (Table S2). Patients with positive DBPCFC (n=87) and patients with a convincing history of severe anaphylaxis59 to hazelnut (n=22, excluded from DBPCFC) were considered true hazelnut allergic patients. From 1/87 patients, clinical data was

not recorded and this patient was excluded in the statistical analyses. We classified symptoms (reported and during the challenge) as follows: isolated symptoms of the oral cavity as mild, symptoms of skin, upper airway and gastro-intestinal as moderate, and laryngeal, bronchial, cardiovascular and neurological symptoms as severe. The patients classified as "anaphylaxis" were included in the severe group.

Specific IgE antibody measurements

Hazelnut sensitization was evaluated by skin prick testing (SPT) and specific IgE (sIgE) in serum. Additionally, sIgE to 23 other foods, 12 inhalant allergens and latex was also measured. The descriptive outcomes of these analyses were reported previously.90 In addition, CRD was performed for the following purified natural (n) or recombinant (r) hazelnut components produced and purified as described elsewhere69,70,74,75: rCor a 1 (Bet v 1 homologue), rCor a 2 (profilin), rCor a 8 (LTP), nCor a 9 (11S globulin), rCor a 11 (7S vicilin), nCor a 12 (oleosin) and rCor a 14 (2S albumin). All sIgE measurements were performed by ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden). IgE levels ≥ 0.35 kU_A/L were considered positive.

Statistical analysis

Differences in patient characteristics (age, sex, family member with atopy, atopic dermatitis and allergy to pollen, house dust mite and latex) of the 731 patients between mild, moderate and severe symptoms were evaluated by chi-square test and ANOVA.

Differences in IgE levels were analyzed using non-parametric tests (Mann-Whitney *U* and Kruskal-Wallis test). Multinomial logistic regression models were used to determine associations between severity and clinical characteristics, and sensitization to hazelnut (extract and allergens). Having mild symptoms was the reference category.

The ability of sIgE levels against hazelnut allergens to discriminate between mild-to-moderate and severe symptoms was evaluated by receiver operating characteristic (ROC) analysis and sensitivity, specificity, positive and negative predictive values (PPV and NPV) were determined. Because the main focus was prediction of severe reactions, patients with mild and moderate reactions to hazelnut were grouped together. Additionally, we generated four multivariate logistic regression models. In each model, only variables that were univariate associated with severity ($p < 0.1$) were added, followed by backward selection. The first model (model 1) tested the predictive accuracy of combinations of sensitization to allergen molecules as a comparison to the use of single allergens. In the next step, we used clinical variables coming from medical history (model 2) sensitization data (model 3 and 4). Model 2 was generated using clinical variables only. Model 3 included selected clinical variables from model 2 to which hazelnut sensitization markers (SPT and sIgE to hazelnut extract and to the allergens) were added. Model 4 was built from the remaining variables in model 3 and sIgE against 23 food, 12 inhalant and latex extracts.

We performed these analyses for both reported symptom severity and for symptom severity recorded during the DBPCFC. The predictive accuracy of the models was quantified with the AUC and AUCs were compared using DeLong tests. Specificity, PPV and NPV at the threshold at which 95% sensitivity was obtained, or the maximum sensitivity that could be achieved. Prediction formulas are provided in the Supplemental Material. All statistical analyses were done in R software version 3.2.4.

Results

Patient characteristics associated with severe symptoms

Table 1 summarizes all patient characteristics. Mild symptoms (Isolated Oral allergy syndrome [OAS]) were reported by 350 patients, moderate by 263 and severe by 116. Severe reactions were unevenly distributed over distributed over the 12 centers (Figure S1).

AD was more frequently seen in patients with moderate (34%) and severe (37%) reactions to hazelnut, as compared to those with mild symptoms (20%), with an odds ratio (OR) of 2.1 and 2.3, respectively (Figure 1A). Latex allergy was more frequently seen in patients with severe (11%) compared to patients with mild and moderate symptoms (5% and 4%, respectively) with an OR of 2.7 (95%CI 1.2-6.0). In contrast, pollen allergy was significantly (around two-fold) less often reported by patients with moderate and severe symptoms.

Of the 86 DBPCFC-positive patients, 36 experienced mild oral symptoms, and 40 skin, gastrointestinal or upper airway symptoms (moderate). The severe group included 10 patients with lower respiratory and neurological symptoms during challenge, and 22 patients with a convincing history of anaphylactic reaction to hazelnut. Except for latex allergy, similar associations between the severity of reactions observed during a DBPCFC with patient characteristics as for reported reactions, although with wider confidence intervals (Figure 1B).

Sensitization to hazelnut allergens univariately associated with severe symptoms

Of patients reporting severe symptoms, 21% were negative on all seven hazelnut allergens investigated, compared to 9% of patients reporting mild symptoms ($p = 0.061$). Sensitization to the seed storage proteins Cor a 9 and Cor a 14 was positively associated with severe symptoms compared to mild symptoms (Figure 1A) with odds ratios (OR) of 2.9 (95% CI 1.3-6.3) when sensitized to Cor a 9 and 4.7 (95% CI 1.8-12.4) when sensitized to Cor a 14. Sensitization to Cor a 1 and to Cor a 2 were negatively associated with severe symptoms (OR 0.4, 95% CI 0.20-0.64 and 0.5, 95% CI 0.2-1.0, respectively). Although the majority (176/309) of Cor a 1-sensitized patients had mild symptoms, 41 reported severe symptoms of which 26 had no detectable IgE to the other tested allergens.

In sera of patients sensitized to Cor a 9 (≥ 0.35 kU_A/L), specific IgE levels were significantly higher in those with severe symptoms (median 11.3 kU_A/L) than in patients with moderate and

TABLE 1. CHARACTERISTICS IN PATIENTS REPORTING MILD, MODERATE AND SEVERE SYMPTOMS TO HAZELNUT

	Severity classification†						
Characteristic	**Mild N= 350**		**Moderate N=263**		**Severe N=263**		***p*-value**
Age (y), mean ± SD	33.1	± 13.4	30.9	± 16.5	32.8	± 14.8	0.426
< 18 (y), n/N (%)	42/350	(12.0)	63/263	(24.0)	15/116	(12.9)	**<0.001**
Female gender, n/N (%)	228/350	(65.1)	158/263	(60.1)	73/116	(62.9)	0.438
Atopy family, n/N (%)	247/350	(70.6)	183/263	(69.6)	84/116	(72.4)	0.856
Atopic diseases, n/N (%)							
Atopic dermatitis (ever)	68/339	(20.1)	86/253	(34.0)	42/114	(36.8)	**<0.001**
Pollen allergy‡	301/350	(86.0)	206/263	(78.3)	82/116	(70.7)	***0.001***
House dust mite allergy‡	170/350	(48.6)	105/263	(39.9)	57/116	(49.1)	0.073
Latex allergy‡	14/314	(4.5)	10/236	(4.2)	12/108	(11.1)	**0.019**
Cat / dog sensitization	146/346	(42.0)	100/252	(39.7)	53/114	(46.5)	0.471
Hazelnut sensitization, n/N (%)							
SPT	291/345	(84.3)	191/256	(74.6)	89/115	(77.4)	***0.011***
ImmunoCAP	297/337	(88.1)	195/246	(79.3)	92/114	(80.7)	0.010
Single hazelnut molecules,* n/N, %							
Any	202/223	(90.6)	107/127	(84.3)	56/71	(78.9)	0.045
rCor a 1	176/219	(80.4)	92/127	(72.4)	41/69	(59.4)	0.002
rCor a 2	55/222	(24.8)	18/127	(14.2)	10/71	(14.1)	0.024
rCor a 8	24/220	(10.9)	15/127	(11.8)	9/70	(12.9)	0.899
nCor a 9	16/222	(7.2)	13/127	(10.2)	13/71	(18.3)	0.025
nCor a 11	7/222	(3.2)	2/127	(1.6)	6/71	(8.5)	0.047
nCor a 12	26/221	(11.8)	19/127	(15.0)	15/71	(21.1)	0.142
rCor a 14	8/217	(3.7)	7/125	(5.6)	10/66	(15.2)	0.006
CCD	23/216	(10.6)	13/127	(10.2)	7/69	(10.1)	0.989

SPT: Skin Prick Test.

† Missing data 2 patients; ‡ Reported symptoms + matching sensitization by SPT or ImmunoCAP

* In 423 patients, IgE against hazelnut allergen molecules were tested by Component-resolved diagnosis (CRD). Not all patients had complete data for all allergens measured.

Bold *p*-values: remained significant after Bonferroni correction.

Italics: positive associations with severity.

mild symptoms (median 1.68 and 0.73 kU_A/L, respectively; see Figure 2). A similar difference was observed for Cor a 14 although not significant. No significant differences in IgE levels against hazelnut extract and the other hazelnut allergens were observed between patients of different severity. Compared to reported symptoms, Cor a 9 and Cor a 14 showed even greater ORs for severe symptoms observed during challenge (10.5, 95% CI 1.2-91.4 and 10.1, 95% CI 1.1-91.5, respectively, Figure 1B). Also, for Cor a 1 sensitization, a stronger negative association was found (OR 0.14, 95% CI 0.03-0.55), this was not observed for Cor a 2 and hazelnut extract.

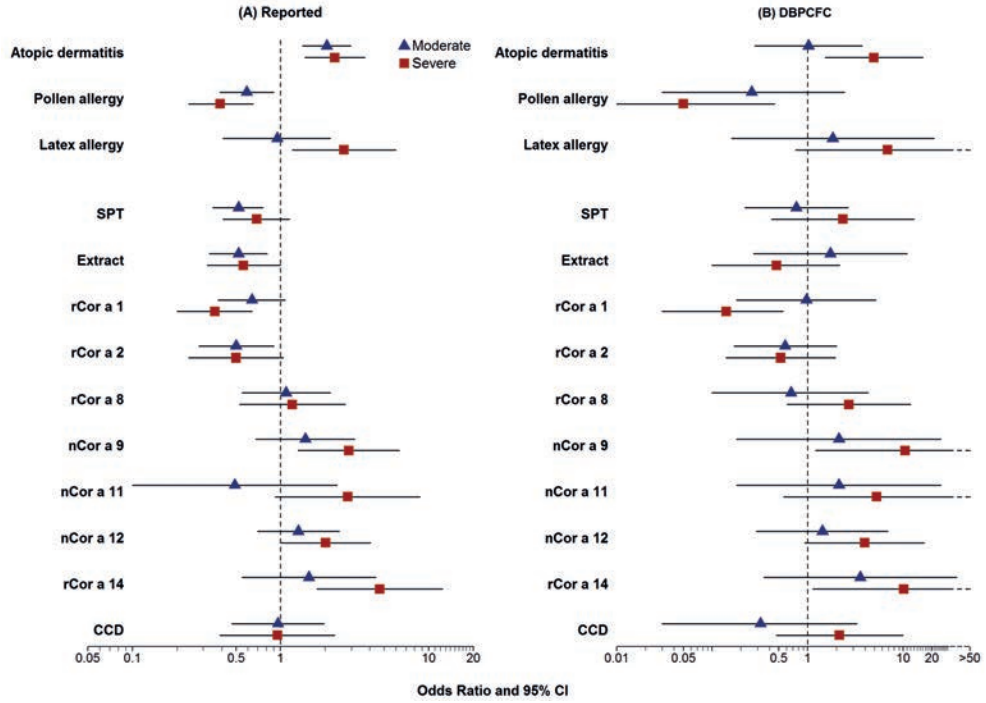

FIGURE I. Clinical factors and hazelnut sensitization (IgE ≥ 0.35 kU_A/L) associated with severe symptoms in the univariate analysis. The x-axis represents odds ratios (ORs). The symbols indicate the OR on moderate (triangle) and severe (square) symptoms compared to mild symptoms when sensitized to an allergen. The lines present the 95% confidence interval (CI). CI lines that cross the hatched line indicate no significant association, and upper limits were truncated at 20.

Impact of inhalant sensitization on reported severity

We investigated whether milder symptoms in pollen-sensitized cross-reactive patients were also observed when there was co-sensitization to non-pollen associated hazelnut allergens. We compared severity reported by patients sensitized to hazelnut storage proteins (Cor a 9, 11 and 14), oleosin and/or LTP that were either co-sensitized to (birch) pollen-associated Cor a 1, Cor a 2 and/or CCD or not. In addition the same analysis was performed for presence or absence of co-sensitization to birch pollen, to any pollen or to HDM, cat and/or dog (indoor allergen sensitization). Severity was being less frequently reported in patients co-sensitized to pollen-associated allergens, to birch pollen or to any pollen, but this was not the case when co-sensitization was to indoor allergens (Figure 3). IgE levels to non-pollen associated hazelnut allergens were not significantly different between patients co-sensitized to pollen-associated allergens and those without that cross-reactive response.

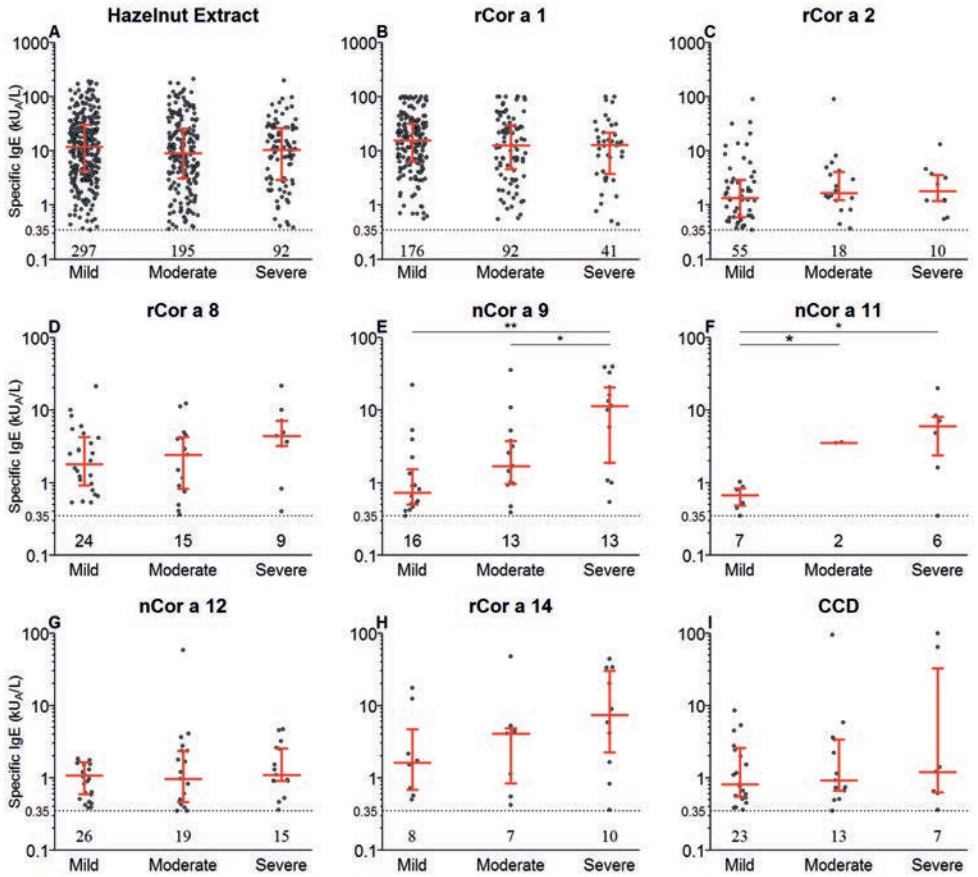

FIGURE II. IgE levels to hazelnut in subjects with mild moderate and severe symptoms. The dots present the level of IgE to hazelnut measured by hazelnut extract, Cor a 1, Cor a 2, Cor a 8, Cor a 9, Cor a 11, Cor a 12 and CCD. The y-axis presents the IgE in kU$_A$/L on a common log scale. The horizontal red lines represent the median and interquartile IgE levels for each symptom group within those that are positively sensitized (≥ 0.35 kU$_A$/L). The black top lines show the significant difference in IgE levels between groups*($p \leq 0.05$) **($p \leq 0.001$). The numbers indicate the total number of sensitized subjects within the groups.

Models to improve the discriminatory ability to assess the risk of severe reactions

AUCs of the ROC curves of single hazelnut allergens in discriminating between mild-to-moderate and severe symptoms were poor, i.e. between 0.57 and 0.62 (Figure 4 and see also Supplemental Table S3). For symptoms recorded during DBPCFC, the AUCs were higher (0.70-0.73). AUCs were also higher when a sub-analysis was performed for reported symptoms of children (n=53) in case of Cor a 9 (AUC 0.70, 95%CI 0.48-0.92) and Cor a 14 (AUC 0.70, 95%CI 0.53-0.88), but not for Cor a 1 (AUC 0.58, 95%CI 0.39-0.72).

We evaluated whether combining IgE responses to different allergens in a model would improve the predictive accuracy (model 1). Although some improvement was achieved, the best

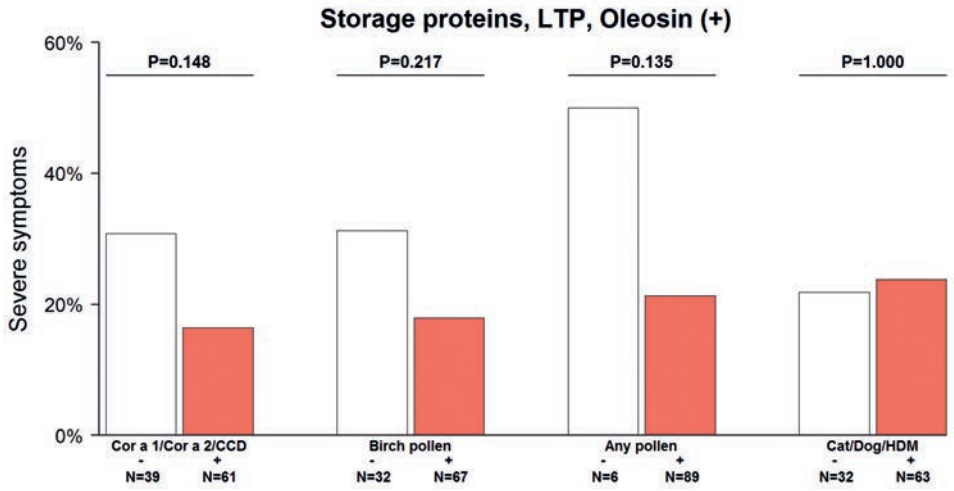

FIGURE III. Severe symptoms to hazelnuts and inhalant allergen co-sensitization in patients sensitized to hazelnut storage proteins, LTP and/or oleosin. The bars show the proportion of patients reporting severe symptoms. All patients are sensitized to Cor a 8, Co r a 9, Cor a 11, Cor a 12 and/or Cor a 14. Orange bars indicate co-sensitization either against Cor a 1, Cor a 2 and/or CCD, or against birch pollen, or against any pollen or against indoor allergens (house dust mite, cat and/or dog). The white bars indicate absence of co-sensitization to these allergen sources.

combination of Cor a 1 (protective) and Cor a 9 (risk) only marginally increased the AUC from 0.62 to 0.66, ($p < 0.05$). In model 2 AD, latex allergy (both risk factors) and pollen allergy (protective factor) were included, resulting in an AUC of 0.62 (95%CI: 0.57-0.68). For severity in DBPCFC, the AUC of model 2 was 0.75 (95%CI: 0.66-0.85), without latex allergy being included. In model 3, adding IgE against Cor a 14 resulted in a significantly higher AUC for reported (0.70, 95%CI 0.63-0.77) and challenge-recorded symptoms (AUC 0.86, 95%CI 0.77-0.95) as compared to model 1 and 2. Finally, in model 4 sIgE to walnut, cat and milk (for details, Supplemental Table S4) slightly increased the AUC for reported symptoms (from 0.70 to 0.72). For symptoms during challenge, just sIgE to walnut increased the AUC to 0.91 (95%CI: 0.84-0.97).

The predictive probability on severe allergic reactions generated from the models are Supplemental Table S5 and illustrated in Figure S2 for models 3 and 4. Using model 4, a probability of 8% or higher on severe symptoms, based on DBPCFCs, corresponded to 96% sensitivity, 76% specificity and a PPV and NPV of 62% and 96%, respectively (see also Supplemental Table S6). All patients in which severe symptoms were excluded (probability < 8%), were pollen allergic and had no AD, latex allergy or detectable IgE against Cor a 14.

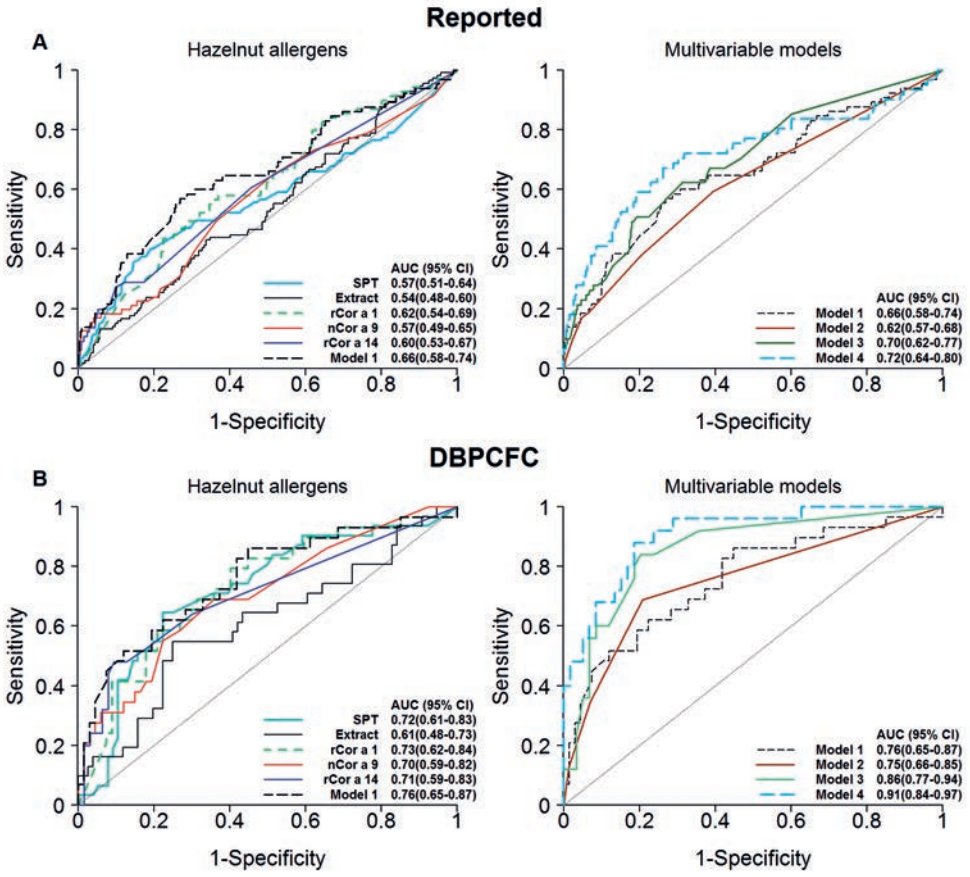

FIGURE IV. Receiver operating characteristic (ROC) curves. (A) Results from patients reporting symptoms to hazelnut and tested with CRD (N=423). (B) A subset (n=124) of patients that underwent a DBPCFC for hazelnut. Area under the curves (AUC) are shown for hazelnut extract, Cor a 1, Cor a 9, Cor a 14 and the combination of Cor a 1 and 14 (model 1). The other models were built from atopic dermatitis (AD), pollen allergy and latex allergy (model 2); AD, pollen allergy, latex allergy and Cor a 14 (model 3); AD, pollen allergy, latex allergy, Cor a 14, and IgE against walnut, cat and milk (model 4).

Discussion

Measurement of specific IgE against individual allergen molecules is now widely recognized as a valuable tool in the diagnosis of food allergy.26,27 In the present study, we had the unique opportunity to evaluate associations between IgE responses to seven individual hazelnut allergens and symptom severity in patients in twelve European centers with very diverse climatic and cultural backgrounds. Moreover, around 30% of patients were also undergoing a DBPCFC, allowing comparison of associations with severity for both reported and challenge-recorded symptoms.

Our study confirmed that IgE responses against Cor a 1 are associated with mild symptoms and against Cor a 9 and Cor a 14 (and to a lesser extent Cor a 11 and Cor a 12) with severe symptoms to hazelnut, both reported and challenge-induced. Although responders to Cor a 1 mostly suffer from mild symptoms, 60% of the patients with severe symptoms also recognize Cor a 1, of which a significant number exclusively. Also, it is important to note that overall 12% of the patients were negative for all seven hazelnut allergens tested, and that this percentage was significantly higher in those with severe symptoms (21%) than mild (9%), suggesting that one or more allergens with relevance for severity may still be missing.

Although associations with severity for Cor a 9 and 14 were quite strong (OR reported: 2.9 and 4.7; OR DBPCFC: 10.5 and 10.1, respectively), the predictive accuracy of isolated serological tests for these allergens was relatively poor, with AUCs at best around 0.60 for reported symptoms, increasing to 0.70 for children, and around 0.70 for DBPCFC symptoms (almost only adults). Similar to our results, Masthoff *et al.*26 found AUC in adults for Cor a 9 and Cor a 14 of 0.66 and 0.67, respectively. For children, the AUCs were higher (0.87 and 0.80, Cor a 9/Cor a 14) than we observed, but their analysis was based on DBPCFC which in our analyses for the whole population also resulted in higher AUCs compared to reported symptoms

Patients sensitized to birch pollen or pollen-related allergens (like Cor a 1) and to hazelnut storage proteins less frequently reported severe symptoms than those without co-sensitization to pollen, although this difference did not reach significance. Interestingly, there was no difference in IgE levels against the hazelnut allergens between both groups. A protective phenomenon by pollen co-sensitization was for the first time described amongst Pru p 3 sensitized peach allergic patients that displayed less severe phenotypes if co-sensitized to birch pollen.45 It is still unclear why, amongst patients becoming sensitized to hazelnut storage proteins, severe reactions would be less frequent if they also have IgE against pollen-related allergens. A number of co-sensitizations to other foods were univariate associated ($p < 0.1$) with severity of reactions to hazelnut: soybean, walnut, sesame seed, poppy seed and buckwheat (Table S4). It has been reported that patients with broader spectra of sensitization to tree nuts, seeds and legumes have more severe phenotypes.94 In the models developed in the present study, only walnut co-sensitization significantly contributed to the prediction of severity. Whether recognition of cross-reactive epitopes on walnut and hazelnut storage proteins or a propensity to develop co-sensitization to multiple tree nuts explains the association with severity remains to be established. Currently, walnut ImmunoCAP is quite insensitive for picking up typical Bet v 1-related cross-reactivity. 95 Positive walnut IgE tests are therefore most likely dominated by IgE against walnut storage protein. In the past hazelnut ImmunoCAP had similar problems and is therefore now spiked with rCor a 1. If a similar approach would be taken for walnut, the association with severity of hazelnut allergy may disappear.

We aimed to improve the predictive accuracy by combining clinical history, CRD and extract-based serology. Interestingly, AD and latex allergy were positively related with severe symptoms whereas pollen allergy proved to have a negative association. It is well-established that AD is a risk factor for the development of food allergy in young children12 and that mutations in the filaggrin gene predispose for the development of both AD and food allergy.11 It is interesting to speculate that this route of sensitization, associated with AD and skin permeability, might be a risk factor for severe food allergy.

In the multivariable regression models combining clinical background, CRD and other (extract-based) sensitizations, a combination of AD, pollen allergy and sIgE against Cor a 14 and walnut resulted in the best predictive accuracy of severity with an AUC of 0.91. The model performs particularly well in excluding the risk of severe reactions with a NPV of 96% at a sensitivity of 92%. Specificity (76.3%) and PPV (62.2%) are less favourable. The prevalence of severity is however relatively low (16%), which impacts sensitivity and PPV. Therefore, a relatively large number of patients are falsely indicated as being severely allergic. All patients that are classified as 'severe' will thus need additional testing. Nevertheless, the model(s) developed perform better than CRD alone and will now be validated in different study populations of hazelnut allergic patients that have been studied in the EU-funded project iFAAM (http://research.bmh.manchester.ac.uk/iFAAM).

It is important to realize that we evaluated patients from tertiary clinics heavily dominated by the typical Northern and Central European adult patients with birch pollen-associated hazelnut allergy. Removing Spanish and Greek patients from the analyses did not significantly change the predictive accuracy of the models (data not shown). The number of patients from Madrid and Athens was too small (38 of which 6 reported severe symptoms) to reliably evaluate the performance the models generated in the present study.

The strength of the present study is the evaluation and confirmation of previously reported CRD findings in more selected populations, but now in a much larger number of patients with a diverse pan-European background. On top of that, not only serological tests but also patients' characteristics were evaluated, showing that this approach significantly improves the identification of patients with increased risk on experiencing severe reactions.

A limitation of our study is that only a subset of the patients volunteered to undergo a DBPCFC which could introduce bias resulting in less generalizable results. Additionally, stopping criteria makes DBPCFC less accurate for establishing real-life severity, our associations with severity found in reported and challenge-recorded symptoms were similar. These findings however, need to be further validated.

In summary, we have confirmed the important role of Cor a 9 and Cor a 14 in severe hazelnut allergy, and have developed models incorporating clinical background, molecule and extract-based sensitizations to more accurately predict the risk of severe reactions. A patient sensitized to Cor a 14 and walnut, with a history of AD but no pollen allergy, should be further evaluated for the risk on severe reactions, whereas this risk is very low in pollen and hazelnut allergic patients without sensitization to Cor a 14 and walnut.

Acknowledgements

We would like to thank all the patients for their participation in the study. We would like to thank ALK Abelló (Madrid, Spain) for their generous gift of SPT reagents. We acknowledge the support by the 6th and7th Framework Programs of the EU, for EuroPrevall (FP6-FOOD-CT-2005-514000) and iFAAM (Grant agreement no. 312147), respectively.

Supplemental files

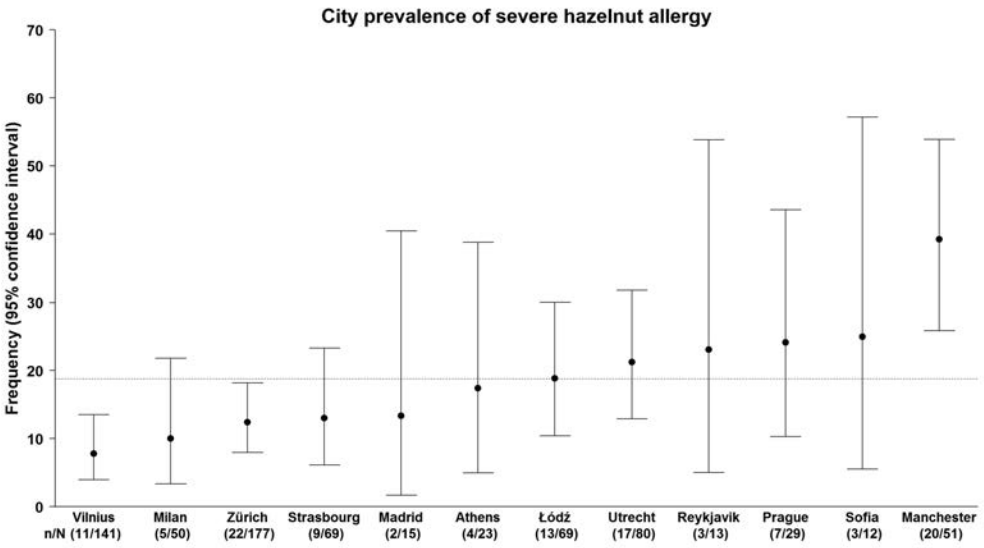

FIGURE S1. Severity of hazelnut allergy in European cities. Frequency of severe reported symptoms and 95%CI. Hatched line shows the mean frequency of all the cities.

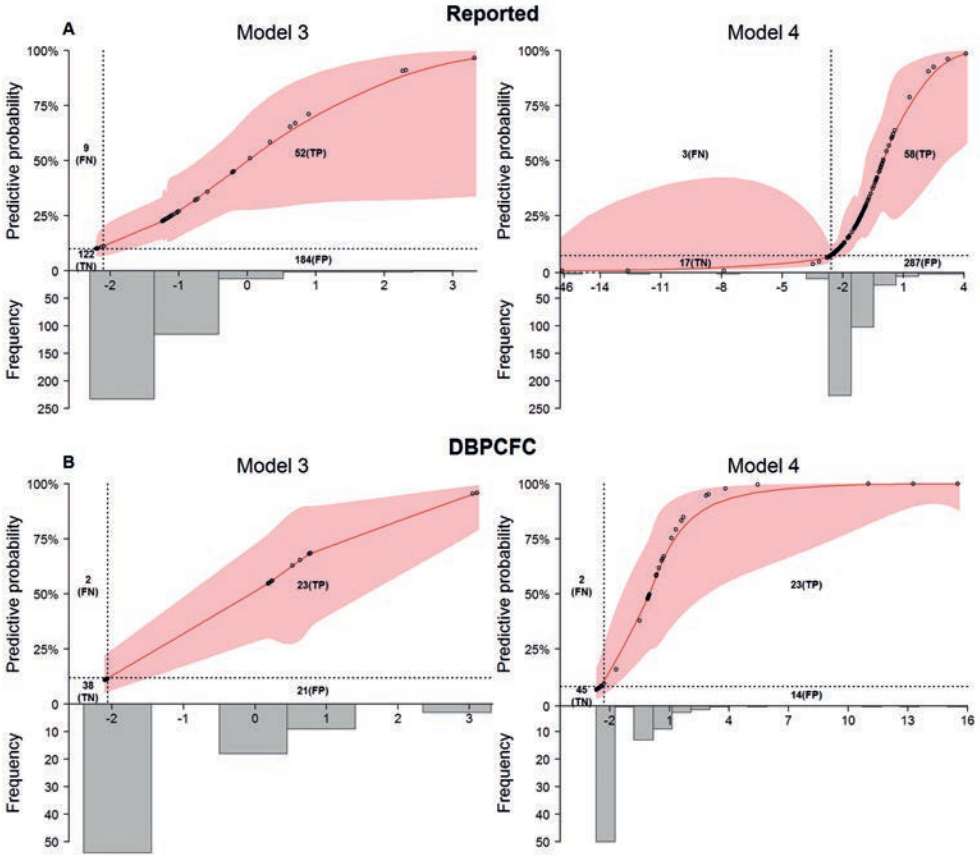

FIGURE S2. Probability of having severe allergic reactions to hazelnut based on (A) reported symptoms and (B) symptoms during a challenge. The red line represents the probability and 95% confidence interval on severe allergic symptoms to hazelnut. For reported symptoms, model 3 severity was estimated including atopic dermatitis (yes/no), pollen allergy (yes/no), latex allergy (yes/no), sIgE levels to Cor a 14. Model 4 also included sIgE levels to walnut, cat and milk. For challenged symptoms, model 3 included atopic dermatitis (yes/no), pollen allergy (yes/no), sIgE levels to Cor a 14 and model 4 additionally included sIgE levels to walnut. The combination of the weight of the variables is indicated by the x-axis and the distribution of this score is depicted in the histogram. The hatched lines indicate the predictive probability that gives the highest possible sensitivity to classify severe patients. The numbers show the classification of the patients using that probability as at cut-point; TP (true positive), FP (false positive), TN (true negative), FN (false negative).

TABLE S1. DIFFERENCES IN CHARACTERISTICS BETWEEN SUBSET WITH AND WITHOUT CRD

	CRD (n=423)*		No CRD (n=308)		
Characteristic	**Mean**	**SD**	**Mean**	**SD**	***p* -value**
Age (mean, sd)	33.7	14.2	30.3	15.5	**0.003**
< 18 years (n/N, %)	53/423	12.5	67/308	21.8	0.096
Female gender (n/N, %)	278/423	65.7	183/308	59.4	0.096
Atopy family (n/N, %)	284/423	67.1	231/308	75.0	0.027
Atopic diseases (n/N, %)					
Atopic dermatitis	87/410	21.2	109/298	36.6	<0.01
Pollen allergy‡	391/423	92.4	280/308	90.9	0.545
House dust mite allergy‡	193/423	45.6	139/308	45.1	0.954
Latex allergy‡	19/394	4.8	17/266	6.4	0.487
Symptom classification (n/N, %)*					
Mild	223/421	53.0	127/308	41.2	<0.001
Moderate	127/421	30.2	136/308	44.2	
Severe	71/421	16.9	45/308	14.6	
Hazelnut sensitization (n/N, %)					
SPT	342/422	81.0	230/296	77.7	0.622
ImmunoCAP	353/421	83.8	232/278	83.5	0.973

SPT: Skin prick test.

*Missing data 2 patients

‡ Reported symptoms + matching sensitization by SPT or ImmunoCAP

p-values < 0.05 are indicated in **bold**

TABLE S2. DIFFERENCES IN CHARACTERISTICS OF SUBJECTS WHO RECEIVED A DBPCFC

	DBPCFC (n=124)		No DBPCFC (n=585)*		
Characteristic	**Mean**	**SD**	**Mean**	**SD**	***p* -value**
Age (mean, sd)	33.1	14.1	32.2	15.0	0.527
< 18 years (n/N, %)	18/124	14.5	98/585	16.8	0.633
Female gender (n/N, %)	82/124	66.1	366/585	62.6	0.519
Atopy family (n/N, %)	86/124	69.4	413/585	70.6	0.867
Atopic diseases (n/N, %)					
Atopic dermatitis	23/119	19.3	165/567	29.1	**0.039**
Pollen allergy‡	119/124	96.0	532/585	90.9	0.071
House dust mite allergy‡	55/124	44.4	268/585	45.8	0.844
Latex allergy‡	4/115	3.5	28/523	5.4	0.488
Symptom classification					
Mild	61/123	49.6	289/584	49.5	0.333
Moderate	41/123	33.3	222/584	38.0	
Severe	21/123	17.1	73/584	12.5	
Hazelnut sensitization (n/N, %)					
SPT	107/123	87.0	445/573	77.7	0.056
ImmunoCAP	111/123	90.2	439/534	82.2	**0.041**

SPT: Skin prick test.

*22 subjects with a convincing history of anaphylaxis were excluded for a DBPCFC.

p-values < 0.05 are indicated in **bold**

TABLE S3. PREDICTIVE ACCURACY INDIVIDUAL HAZELNUT ALLERGENS AND MULTIVARIABLE MODELS

	Reported					DBPCFC				
	AUC	**CI95%**				**AUC**	**CI95%**			
SPT	0.57	0.51-0.64	§	†	‡	0.72	0.61-0.83			‡
Extract	0.54	0.48-0.60	§	†	‡	0.61	0.48-0.73		†	‡
rCor a 1	0.62	0.54-0.69	§	†	‡	0.73	0.62-0.84			‡
rCor a 2	0.54	0.46-0.61	§	†	‡	0.54	0.41-0.67	§	†	‡
rCor a 8	0.51	0.44-0.58	§	†	‡	0.62	0.50-0.74		†	‡
nCor a 9	0.57	0.49-0.65	§	†	‡	0.70	0.59-0.82			‡
nCor a 11	0.50	0.42-0.57	§	†	‡	0.48	0.34-0.62	§	†	‡
nCor a 12	0.52	0.43-0.60	§	†	‡	0.55	0.41-0.68	§	†	‡
rCor a 14	0.60	0.53-0.67	§	†	‡	0.71	0.59-0.83		†	‡
CCD	0.55	0.47-0.62	§	†	‡	0.53	0.41-0.65	§	†	‡
Model 1	0.66	0.58-0.74			‡	0.76	0.65-0.87			‡
Model 2	0.62	0.57-0.68				0.75	0.66-0.85		†	‡
Model 3	0.70	0.63-0.77				0.86	0.77-0.94			
Model 4	0.72	0.64-0.80				0.91	0.84-0.97			

§ significantly different from model 2

† significantly different from model 3

‡ significantly different from model 4

Model 1: Cor a 1 and Cor a 9.

Model 2: *Reported*: Atopic dermatitis, pollen allergy, latex allergy; *DBPCFC:* Atopic dermatitis, pollen allergy .

Model 3: *Reported*: Atopic dermatitis, pollen allergy, latex allergy, Cor a 14; *DBPCFC*: Atopic dermatitis, pollen allergy, Cor a 14.

Model 4: *Reported*: Atopic dermatitis, pollen allergy, latex allergy, Cor a 14, walnut, milk, cat; *DBPCFC*: Atopic dermatitis, pollen allergy, Cor a 14, walnut.

TABLE S4. SENSITIZATION TO FOOD AND POLLEN AND OTHER INHALANT SOURCES

	Severity Classification				
	Mild/moderate		Severe		
	Median	IQR	Median	IQR	*p* -value
Peach	1.41	0.31-4.58	1.40	0.11-4.26	0.101
Apple	0.73	0.16-2.65	0.69	0.05-2.37	0.192
Kiwi	0.24	0.04-0.80	0.21	0.04-1.14	0.960
Banana	0.11	0.04-0.33	0.13	0.04-0.41	0.688
Melon	0.09	0.05-0.25	0.09	0.05-0.31	0.967
Carrot	0.36	0.07-1.35	0.29	0.05-1.36	0.385
Celery	0.45	0.09-1.74	0.44	0.03-1.68	0.220
Corn	0.13	0.06-0.44	0.17	0.06-0.79	0.181
Tomato	0.16	0.04-0.75	0.23	0.04-1.23	0.319
Lentil	0.08	0.02-0.31	0.10	0.02-0.78	0.174
Soybean †	**0.09**	**0.02-0.33**	**0.15**	**0.03-0.67**	**0.039**
Peanut	0.25	0.06-1.33	0.50	0.06-1.47	0.394
Walnut ‡	***0.08***	***0.02-0.36***	***0.10***	***0.02-0.88***	***0.071***
Sesame seed †	***0.23***	***0.11-0.74***	***0.29***	***0.12-1.66***	***0.028***
Sunflower	0.10	0.05-0.36	0.12	0.05-0.72	0.147
Poppy seed †	***0.07***	***0.03-0.24***	***0.11***	***0.03-0.66***	***0.015***
Mustard	0.04	0.02-0.11	0.05	0.02-0.19	0.212
Wheat	0.17	0.07-0.56	0.19	0.07-0.58	0.450
Buckwheat †	***0.10***	***0.04-0.31***	***0.15***	***0.05-0.60***	***0.021***
Fish	0.00	0.00-0.03	0.00	0.00-0.03	0.790
Shrimp	0.04	0.01-0.16	0.05	0.02-0.17	0.401
Egg	0.06	0.04-0.10	0.06	0.05-0.14	0.135
Milk‡	**0.06**	**0.04-0.10**	**0.06**	**0.05-0.16**	**0.045**
Birch†	**14.38**	**1.96-41.6**	**5.62**	**0.47-29.75**	**0.006**
Grass	2.4	0.1-13.08	2.81	0.14-13.56	0.624
Mugwort	0.27	0.06-1.49	0.23	0.07-1.33	0.987
Parietaria	0.14	0.04-0.61	0.14	0.06-0.83	0.506
Plane tree	0.21	0.06-1.36	0.19	0.05-1.27	0.564
Ragweed	0.29	0.07-1.58	0.29	0.07-1.12	0.422
Chenopodium	0.14	0.04-0.71	0.15	0.04-0.95	0.572
Cypress	0.12	0.04-0.43	0.08	0.03-0.39	0.328
Olive	0.59	0.11-2.74	0.53	0.07-1.61	0.103
Cat‡	***0.11***	***0.03-1.18***	***0.29***	***0.03-2.24***	***0.068***
Dog	0.33	0.1-1.38	0.35	0.12-1.86	0.497
House dust mite †	***0.14***	***0.04-1.73***	***0.40***	***0.05-5.69***	***0.041***
Latex	0.11	0.07-0.48	0.11	0.07-0.39	0.864

The *p* -value indicates the difference between mild-to-moderate and severe patients.

Variables included in regression modelling are indicated in **bold**.

† Variables included in the multivariable logistic regression model ($p < 0.1$, negative associations with severity indicated in *Italics*).

‡ Variables selected in the final model after a backwards selection method (negative associations with severity indicated in *Italics*).

None of the markers remained significant after Bonferroni correction ($p < 0.0013$).

TABLE S5. MULTIVARIABLE LOGISTIC REGRESSION MODELS

Reported symptoms

			95% CI around exp(β)	
Model I: CRD only	β	exp(β)	**Lower**	**Upper**
Cor a 1 IgE Levels	-0.016	0.98	0.97	0.99
Cor a 9 IgE Levels	0.117	1.12	1.05	1.20
Intercept	*-1.528*			

Probability severe hazelnut allergy

$1/(1+(e^{\wedge}-(-1.528+ (\text{Cor a 1} * -0.016) + (\text{Cor a 9} * 0.117))))$

			95% CI around exp(β)	
Model II: Clinical background	β	exp(β)	**Lower**	**Upper**
Atopic dermatitis (AD)	0.591	1.81	1.14	2.86
Pollen allergy (PA)	-0.846	0.43	0.26	0.70
Latex allergy (LA)	0.953	2.60	1.22	5.50
Intercept	*-1.201*			

Probability severe hazelnut allergy

$1/(1+(e^{\wedge}(-(-1.201 + (\text{AD} * 0.591) + (\text{PA} * -0.846) + (\text{LA} * 0.953))))$

			95% CI around exp(β)	
Model III: Model II + sensitization to hazelnut	β	exp(β)	**Lower**	**Upper**
Atopic dermatitis (AD)	1.026	2.79	1.45	5.35
Pollen allergy (PA)	-0.964	0.38	0.20	0.72
Latex allergy (LA)	0.013	1.01	0.29	3.49
Cor a 14 IgE Levels	0.103	1.11	1.01	1.22
Intercept	*-1.242*			

Probability severe hazelnut allergy

$1/(1+(e^{\wedge}(-(-1.242 + (\text{AD} * 1.026) + (\text{PA} * -0.964) + (\text{LA} * 0.013) + (\text{Cor a 14} * 0.103))))$

			95% CI around exp(β)	
Model IV: Model III + sensitization to other sources	β	exp(β)	**Lower**	**Upper**
Atopic dermatitis (AD)	1.161	3.19	1.61	6.33
Pollen allergy (PA)	-1.088	0.34	0.17	0.66
Latex allergy (LA)	0.350	1.42	0.38	5.27
Cor a 14 IgE Levels	0.075	1.08	0.97	1.19
Walnut IgE Levels	0.068	1.07	1.00	1.14
Milk IgE Levels	-1.323	0.27	0.07	0.98
Cat IgE Levels	0.025	1.03	1.01	1.04
Intercept	*-1.231*			

Probability severe hazelnut allergy

$1/1+(e^{\wedge}(-(-1.231 + (\text{AD} * 1.161) + (\text{PA} * -1.088) + (\text{LA} * 0.350) + (\text{Cor a 14} * 0.075) + (\text{Walnut} * 0.068) + (\text{Cat} * 0.025))))$

TABLE S5. MULTIVARIABLE LOGISTIC REGRESSION MODEL (CONTINUED)

DBPCFC symptoms				
			95% CI around exp(β)	
Model 1: CRD Only	**β**	**exp(β)**	**Lower**	**upper**
Cor a 1	-0.031	0.97	0.94	1.00
Cor a 9	0.079	1.08	1.00	1.18
Intercept	*-0.631*			
Probability severe hazelnut allergy $1/(1+(e^{\wedge}(-(0.631 + (\text{Cor a 1} * -0.031) + (\text{Cor a 9} * 0.079))))$				
			95% CI around exp(β)	
Model II: Clinical background	**β**	**exp(β)**	**Lower**	**upper**
Atopic dermatitis (AD)	1.699	5.47	1.98	15.13
Pollen allergy (PA)	-2.076	0.125	0.04	0.44
Intercept	*0.378*			
Probability severe hazelnut allergy $1/(1+(e^{\wedge}(-(0.378 + (\text{AD} * 1.699) + (\text{PA} * -2.076))))$				
			95% CI around exp(β)	
Model III: Model II + hazelnut sensitization	**β**	**exp(β)**	**Lower**	**upper**
Atopic dermatitis (AD)	2.290	9.87	2.66	39.69
Pollen allergy (PA)	-2.862	0.06	0.01	0.28
Cor a 14 IgE levels	0.010	1.01	0.94	1.08
Intercept	*0.757*			
Probability severe hazelnut allergy $1/(1+(e^{\wedge}(-(0.757 + (\text{AD} * 2.290) + (\text{PA} * -2.862) + (\text{Cor a 14} * 0.010))))$				
			95% CI around exp(β)	
Model IV: Model III + Sensitization to other sources	**β**	**exp(β)**	**Lower**	**upper**
Atopic dermatitis (AD)	2.574	13.11	3.04	56.54
Pollen allergy (PA)	-3.005	0.05	0.01	0.28
Cor a 14 IgE levels	-0.074	0.93	0.80	1.08
Walnut IgE levels	0.400	1.49	1.10	2.01
Intercept	*0.338*			
Probability severe hazelnut allergy $1/(1+(e^{\wedge}(-(-0.338 + (\text{AD} * 2.574) + (\text{PA} * -3.005) + (\text{Cor a 14} * -0.074) + (\text{Walnut} * 0.400))))$				

Results from the multivariable regression model in predicting severe allergic reaction to hazelnuts.
Calculations are based on a model combining absence (0) or presence (1) of atopic dermatitis, pollen allergy, latex allergy, and Cor a 14, Walnut, Milk and Cat IgE levels in in kU_A/L. For the quantitative variables of serology, the models show the effect of a change in 1 kU_A/L

TABLE S6. ACCURACY OF HAZELNUT ALLERGEN IGE TESTS AND MODELS FOR SEVERITY OF HAZELNUT ALLERGY

		Reported symptoms											
Allergens	**Positivity threshold**		**Mild/ Moderate**	**Severe**	**Sensitivity (95% CI)**		**Specificity (95% CI)**		**PPV (95% CI)**		**NPV (95% CI)**	
Extract	0.35	≥	492	92	80.7	72.2 - 87.5	15.6	12.8 - 18.9	15.8	12.9 - 19.0	80.5	72.0 - 87.4
		<	91	22								
CRD (any)	0.35	≥	309	56	78.9	67.6 - 87.7	11.7	8.54 - 15.5	15.3	11.8 - 19.5	73.2	59.7 - 84.2
		<	41	15								
rCor a 1	0.35	≥	268	41	59.4	46.9 - 71.1	22.5	18.2 - 27.3	13.3	9.7 - 17.6	73.6	64.1 - 81.7
		<	78	28								
rCor a 2	0.35	≥	73	10	14.1	7.0 - 24.4	79.1	74.4 - 83.2	12.1	5.9 - 21.0	81.9	77.4 - 85.9
		<	276	61								
rCor a 8	0.35	≥	39	9	12.9	6.0 - 23.0	88.8	85.0 - 91.9	18.8	8.9 - 32.6	83.5	79.3 - 87.1
		<	308	61								
nCor a 9	0.35	≥	29	13	18.3	10.1 - 29.3	91.7	88.3 - 94.4	30.9	17.6 - 47.1	84.7	80.6 - 88.1
		<	320	58								
nCor a 11	0.35	≥	9	6	8.4	3.2 - 17.4	97.4	95.2 - 98.8	40.0	16.3 - 67.7	84.0	80.0 - 87.4
		<	340	65								
nCor a 12	0.35	≥	45	15	21.1	12.3 - 32.4	87.1	83.1 - 90.4	25.0	14.7 - 37.9	84.4	80.2 - 88.0
		<	303	56								
rCor a 14	0.35	≥	15	10	15.2	7.5 - 26.1	95.6	92.9 - 97.5	40.0	21.1 - 61.3	85.4	81.4 - 88.8
		<	327	56								
CCD	0.35	≥	36	7	10.1	4.2 - 19.8	89.5	85.8 - 92.5	16.3	6.8 - 30.7	83.2	79.0 - 86.9
		<	307	62								

TABLE S6. ACCURACY OF HAZELNUT ALLERGEN IGE TESTS AND MODELS FOR SEVERITY OF HAZELNUT ALLERGY (CONTINUED)

Models	Positivity threshold		Mild/ Moderate	Severe	Sensitivity (95% CI)		Specificity (95% CI)		PPV(95% CI)		NPV (95% CI)	
Model 1	6%	≥	316	62	95.4	87.1 - 99.0	5.4	3.2 - 8.4	16.4	12.8 - 20.5	85.7	63.7 - 97.0
		<	18	3								
Model 2	19%	≥	210	63	59.4	49.5 - 68.9	60.5	56.2 - 64.7	23.1	18.2 - 28.5	88.2	84.5 - 91.3
		<	322	43								
Model 3	10%	≥	184	52	85.3	73.8 - 93.0	39.9	34.3 - 45.6	22.0	16.9 - 27.9	93.1	87-36 - 96.8
		<	122	9								
Model 4	7%	≥	287	58	95.1	86.3 - 99.0	5.6	3.3 - 8.8	16.8	13.0 - 21.2	85.0	62.1 - 96.8
		<	17	3								
					DBPCFC symptoms							
Allergens	Positivity threshold		Mild/ Moderate	Severe	Sensitivity (95% CI)		Specificity (95% CI)		PPV(95% CI)		NPV (95% CI)	
Extract	0.35	≥	71	26	83.9	66.3 - 94.55	6.6	2.2 - 14.7	26.8	18.3 - 36.8	50.0	18.7 - 81.3
		<	5	5								
CRD (any)	0.35	≥	65	25	86.2	68.3 - 96.1	3.0	0.4 - 10.4	27.8	18.9 - 38.2	33.3	4.3 - 77.7
		<	2	4								
rCor a 1	0.35	≥	61	17	58.6	38.9 - 76.5	9.0	3.4 - 18.5	21.8	13.2 - 32.6	33.3	13.3 - 59.0
		<	6	12								
rCor a 2	0.35	≥	13	4	13.8	3.9 - 31.7	80.6	69.1 - 89.2	23.5	6.8 - 49.9	68.3	56.9 - 78.4
		<	54	25								
rCor a 8	0.35	≥	5	6	20.7	8.0 - 39.72	92.5	83.4 - 97.5	54.5	23.4 - 83.2	72.9	62.2 - 8 2.0
		<	62	23								
nCor a 9	0.35	≥	3	7	24.1	10.3 - 43.5	95.5	87.5 - 99.1	70.0	34.8 - 93.3	74.4	63.9 - 83.2
		<	64	22								
nCor a 11	0.35	≥	3	4	13.8	3.9 - 31.7	95.5	87.5 - 99.1	57.1	18.4 - 90.1	71.9	61.4 - 80.9
		<	64	25								

TABLE S6. ACCURACY OF HAZELNUT ALLERGEN IGE TESTS AND MODELS FOR SEVERITY OF HAZELNUT ALLERGY (CONTINUED)

Allergens	Positivity threshold		Mild/ Moderate	Severe	Sensitivity (95% CI)		Specificity (95% CI)		PPV(95% CI)		NPV (95% CI)	
nCor a 12	0.35	≥	7	8	27.6	12.7 - 47.2	89.5	79.6 - 95.7	53.3	26.6 -78.7	74.1	63.1 - 83.2
		<	60	21								
rCor a 14	0.35	≥	4	6	24.0	9.4 - 45.1	93.7	84.5 - 98.2	60.0	26.2 - 87.8	75.6	64.6 - 84.7
		<	59	19								
CCD	0.35	≥	4	5	17.2	5.8 - 35.8	94.0	85.4 - 98.3	55.6	21.2 - 86.3	72.4	61.8 - 81.5
		<	307	62								
Models	Positivity threshold		Mild/ Moderate	Severe	Sensitivity (95% CI)		Specificity (95% CI)		PPV(95% CI)		NPV (95% CI)	
Model 1	22%	≥	46	27	93.1	77.2 - 99.2	31.3	20.6 - 43.8	37.0	26.0 - 49.1	91.3	72.0 - 98.9
		<	21	2								
Model 2	50%	≥	15	22	68.8	50.0 - 83.9	79.2	68.0 - 87.8	59.5	42.1 - 75.2	85.1	74.3 - 92.6
		<	57	10								
Model 3	11%	≥	21	23	92.0	72.0 - 98.9	64.4	50.9 - 76.5	52.3	36.7 - 67.5	95.0	83.1 - 99.4
		<	38	2								
Model 4	8%	≥	14	23	92.0	76.5 - 99.1	76.3	63.4 - 86.4	62.2	44.8 - 77.5	95.7	85.5 - 99.5
		<	45	2								

Measures of accuracy were calculated for each of the individual diagnostic IgE tests and for models including a combination of clinical history, sensitization to hazelnut and sensitization to other food and non-food sources.

Model 1: Cor a 1 and Cor a 9

Model 2: *Reported*: Atopic dermatitis, pollen allergy, latex allergy; *DBPCFC:* Atopic dermatitis, pollen allergy;

Model 3: *Reported:* Atopic dermatitis, pollen allergy, latex allergy, Cor a 14; *DBPCFC:* Atopic dermatitis, pollen allergy, Cor a 14 IgE;

Model 4: *Reported*: Atopic dermatitis, pollen allergy, latex allergy, Cor a 14, walnut, milk, cat; *DBPCFC*: Atopic dermatitis, pollen allergy, Cor a 14 IgE, walnut IgE;

4 Walnut allergy across Europe: distribution of allergen sensitization patterns and prediction of severity

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Journal of Allergy and Clinical Immunology: In Practice 2021; 225-235e10
DOI: 10.1016/j.jaip.2020.08.051

Abstract

Background: Walnut allergy is common across the globe, but data on the involvement of individual walnut components is scarce.

Objectives: To identify geographical differences in walnut component sensitization across Europe, explore co-sensitization and cross-reactivity, and assess associations of clinical and serological determinants with severity of walnut allergy.

Methods: As part of the EuroPrevall outpatient surveys in 12 European cities, standardized clinical evaluation was conducted in 531 individuals reporting symptoms to walnut, with sensitization to all known walnut components assessed in 202 subjects. Multivariable Lasso regression was applied to investigate predictors for walnut allergy severity.

Results: Birch-pollen related walnut sensitization (Jug r 5) dominated in Northern and Central Europe; LTP sensitization (Jug r 3) in Southern Europe. Profilin sensitization (Jug r 7) was prominent throughout Europe. Sensitization to storage proteins (Jug r 1, 2, 4 and 6) was detected in up to 10% of subjects. The walnut components that showed strong correlations with pollen and other foods differed between centres. The combination of determinants best predicting walnut allergy severity were: symptoms upon skin contact with walnut, atopic dermatitis (ever), family history of atopic disease, mugwort pollen allergy, sensitization to cat/dog, positive SPT to walnut, and IgE to Jug r 1, 5, 7 or carbohydrate determinants (AUC = 0.81 [95%-CI 0.73-0.89]).

Conclusions: Walnut allergic subjects across Europe show clear geographical differences in walnut component sensitization and co-sensitization patterns. A predictive model combining results from component-based serology testing with results from extract-based testing and information on clinical background, allows for good discrimination between mild-to-moderate and severe walnut allergy.

Key messages

- Molecular diagnostics in walnut allergy reveal varied patterns of sensitization across Europe.

- Molecular diagnostics and can help accurately distinguish mild-to-moderate from severe walnut allergy when considered in combination with extract-based testing and clinical background

Introduction

Walnut is one of the tree nuts most often reported to elicit food allergic reactions in European countries and globally.[76,96,97] Ongoing developments in food allergy diagnostic testing, make it possible to assess IgE sensitization to a broadening spectrum of specific food allergens, commonly referred to as component-resolved diagnostics (CRD). At the time of this study, seven components of the "English" walnut, *Juglans regia*, had been characterised: Jug r 1 (2S albumin), Jug r 2 (vicilin-like 7S globulin), Jug r 3 (lipid transfer protein [LTP]), Jug r 4 (legumin-like 11S globulin), Jug r 5 (PR-10 protein), Jug r 6 (vicilin-like 7S globulin), and Jug r 7 (profilin).

Studies on geographical differences in sensitization patterns to walnut components across Europe, are scarce.[98] One study investigated sensitization to walnut components in 91 walnut-allergic patients from three European regions, and described a particularly high occurrence of Jug r 3 sensitization in Spain, and Jug r 5 sensitization in Germany and Switzerland.[40] However, geographical comparisons were limited by the fact that only children were included in Germany, and only adults in Switzerland. Larger studies, with standardized cross-border inclusion criteria, and a broader geographical distribution including Northern and Eastern Europe, are needed to substantiate previous findings and expand data on international comparisons.

CRD can be of help in distinguishing primary from cross-reactive walnut sensitization,[99,100] but also in predicting severity of food allergic reactions.[26,101] For walnut, literature suggests that IgE to the seed storage proteins Jug r 1, Jug r 2, Jug r 4, and Jug r 6, is associated with more severe reactions,[40,102] but data is limited. A recent study evaluated CRD data in combination with other serological measurements and clinical factors for predicting severity of hazelnut allergy, and found that a model combining IgE to Cor a 14, IgE to walnut extract, atopic dermatitis, and pollen allergy, performed well.[101] Such a predictive model has not yet been elaborated for walnut allergy.

In this study, we explored walnut allergy through data collected during the standardized EuroPrevall outpatient project, from 12 geographically, culturally and socio-economically diverse regions across Europe. Our aim was three-fold: 1. to identify differences in sensitization patterns to walnut components across Europe; 2. to assess relationships between IgE to walnut components, and IgE to pollen and foods other than walnut, providing insight into possible primary sensitizers; and 3. to optimally predict severity of walnut allergy using data from clinical history and IgE responses to walnut and walnut components.

Methods

Study design, setting and subjects

Participants of the EuroPrevall outpatient clinic study reporting adverse reactions within two hours of ingestion of walnut, were evaluated in this study. A detailed methodology of the standardized EuroPrevall outpatient food allergy work-up, was published previously.[59]

Data were collected between 2006 and 2009 in 12 European allergy clinics, in Athens (Greece), Łódź (Poland), Madrid (Spain), Manchester (United Kingdom), Milan (Italy), Prague (Czech Republic), Reykjavik (Iceland), Sofia, (Bulgaria), Strasbourg (France), Utrecht (The Netherlands), Vilnius (Lithuania) and Zürich (Switzerland).

Ethical approval and written informed consent were obtained in each centre and from each participating subject.

Data collection

A detailed questionnaire was completed for each subject by a trial physician, and focused on demographic data, reaction characteristics, and personal and family history of atopy.

IgE sensitization was assessed through skin prick testing (SPT) and serum analyses, according to the same standardized approach in all centers (details in the Supplementary methods on data collection), using extracts from food (including walnut) and inhalant allergens that are commonly implicated in food allergy across Europe. Additional prick-to-prick testing (PTP) with fresh walnut was performed in case of negative SPT with walnut extract as indicated by local practice. Additional testing of sera for IgE to walnut components Jug r 1, Jug r 2, a low-molecular-weight fragment of Jug r 2 (Jug r 2 LMW), Jug r 3, Jug r 4, Jug r 5, Jug r 6, and Jug r 7, was performed in January 2008 with all sera collected at that time. Jug r 2 LWM is described in supplement 1. SPT results were expressed as allergen/histamine wheal ratios, and a ratio ≥0.5 was considered positive. IgE levels ≥0.35 kU_A/L were considered positive.

Definitions

Probable walnut allergy was defined as a combination of reported symptoms to walnut and matching sensitization, as demonstrated by a positive walnut SPT, PTP, and/or presence of serum IgE against walnut extract and/or one or more individual walnut components as tested by ImmunoCAP.

Reactions to walnut were classified as *severe* if subjects reported dysphagia, dysphonia, lower airway, cardiovascular, or neurological symptoms, or anaphylaxis (specifically severe laryngeal oedema, severe bronchospasm, or hypotensive shock). All other symptoms were considered *mild-to-moderate*: isolated oral allergy symptoms, symptoms of the skin, eyes, upper airway, or gastro-intestinal system (details in supplement 1).[103-104]

Allergy to inhalant allergen sources and to latex was defined as symptoms and matching IgE sensitization in SPT and/or ImmunoCAP to the respective allergen source.

Statistical analyses

Walnut sensitization patterns across Europe

Demographics, reaction severity, and proportions of positive test results, were explored for each participating centre. Medians and interquartile ranges were calculated to evaluate IgE levels for walnut extract and walnut components. Differences between centres in levels of IgE to walnut extract were tested using the Kruskal-Wallis test with Bonferroni correction.

Relationship between IgE to walnut components and other allergens

Spearman *Rho* coefficients were calculated to evaluate relationships between levels of IgE to walnut components, and levels of IgE to food, latex, and pollen extracts. Bonferroni correction was used to correct for multiple comparisons.

Predictors for severity of walnut allergy

Only subjects conforming to the definition of 'probable walnut allergy' were included for prediction of severity of walnut allergy. Univariable logistic regression was performed to explore crude associations between demographics, clinical history variables, walnut sensitization patterns, and severity of walnut allergy.

To identify the most discriminative combination of predictors for severity of walnut allergy, Least Absolute Shrinkage and Selection Operator (Lasso) regression was applied. Lasso regression is a form of penalized regression, which selects only the most contributive predictors, and applies shrinkage of regression coefficients through cross-validation, to limit overfitting.[105] In order to enable the use of all data and increase power for this predictive analysis, multiple imputation of sporadically missing data on predictor variables was performed (10 imputations by Chained Equations using the R package *mice*).[106] Missing data is described in Supplemental Table S1.

A three-step approach to model building was taken. In model I, all demographic and clinical variables were entered, and Lasso regression selected the most discriminative combination of predictors. In model II, variables on IgE sensitization to walnut extract as assessed by SPT and ImmunoCAP were entered, along with the variables selected in model I. In model III, ImmunoCAP results for walnut components, and IgE to Ana c 2 (bromelain) as a measure for cross-reactive carbohydrate determinants (CCD), were added to the variables remaining after selection in model II. Predictor variables selected in at least seven of the ten imputed datasets were included in each model, and their coefficients and 95% confidence intervals (CI) were pooled, using Rubin's rules.

To assess how well each model could discriminate between mild-to-moderate and severe walnut allergy, the area under the curves (AUC) of the receiving operating characteristics (ROC) and

corresponding 95% CIs were calculated and pooled over the ten imputed datasets. DeLong's test was used to compare AUC values.[107]

Analyses were conducted with SPSS version 25 and R version 3.4.1.

Results

Population characteristics

As the fourth most commonly reported causative food in the EuroPrevall outpatient clinic study, walnut was reported to elicit symptoms in 531 (23.4%) subjects, most often in Utrecht (37.0%) and least often in Reykjavik (6.3%). The majority were female (64.8%) and over 18 years of age (84.6%) (Table 1).

The most commonly reported symptoms were oral allergy symptoms in 426/531 (80.2%), subjects, of which 214 had no other symptoms. Symptoms of the upper airway, skin and digestive system were reported by respectively 33.3%, 32.0% and 23.2% of subjects. Fewer subjects reported lower airway (15.1%), cardiovascular (2.4%), or neurological (3.2%) symptoms. Anaphylaxis was reported by 15 subjects (2.8%).

Walnut sensitization patterns across Europe

SPT and ImmunoCAP with walnut extract were positive in 40.8% and 35.5% of subjects (Table 1). Positive serology to walnut extract was found in less than 30% of subjects reporting symptoms to walnut from Łódź, Strasbourg, Utrecht, and Zürich, but in more than 80% of subjects from Athens and Madrid. In subjects with positive serology to walnut extract, median IgE levels were lowest in Strasbourg, Sofia and Manchester, and highest in Milan, Łódź, Utrecht, Prague and Athens (Figure 1).

Sensitization by CRD was assessed in 202 subjects, and 79.4% of the 199 subjects with complete CRD results were found to be sensitized to at least one individual walnut component by ImmunoCAP. The distribution of IgE levels in subjects sensitized to a specific walnut component is shown in Figure 2. Median IgE levels for PR-10 protein Jug r 5 were highest.

Of the subjects with *negative* SPT and ImmunoCAP to walnut extract (N=237), in whom CRD with all walnut components was completed (N=79), 70.9% were sensitized to at least one component (N=56/79), most frequently to Jug r 5 (N=50/79, 63.3%) (Table S2).

For international comparison of walnut component sensitization patterns, only centres where CRD results were available for at least 10 subjects were taken into account (Table 1, Figure 3). Sensitization to PR-10 protein Jug r 5 was most prevalent everywhere except in Athens and Madrid. In Athens, sensitization to LTP Jug r 3 dominated. Besides Athens, LTP sensitization

TABLE I. CHARACTERISTICS OF SUBJECTS WITH SELF-REPORTED WALNUT ALLERGY ACROSS EUROPE

		European City					
	Total	**Athens**	**Madrid**	**Manchester**	**Milan**	**Łódź**	**Prague**
Self-reported walnut allergy, N	**531**	**44**	**25**	**30**	**39**	**74**	**19**
Age (y), mean ±SD	30.4 ±13.9	27.8 ±10.3	23.8 ±12.9	30.7 ±13.3	34.7 ±10.9	29.5 ±18.4	15.9 ±11.7
<18 y, n (%)	82 (15.4)	4 (9.1)	7 (28.0)	5 (16.7)	0 (0.0)	22 (29.7)	11 (57.9)
Female seks, n (%)	344 (64.8)	17 (38.6)	18 (72.0)	23 (76.7)	29 (74.4)	59 (79.7)	10 (52.6)
Symptom severity*							
Mild, n (%)	214 (40.3)	14 (31.8)	9 (36.0)	3 (10.0)	27 (69.2)	14 (18.9)	5 (26.3)
Moderate, n (%)	184 (34.7)	18 (40.9)	9 (36.0)	15 (50.0)	6 (15.4)	41 (55.4)	6 (31.6)
Severe, n (%)	133 (25.0)	12 (27.3)	7 (28.0)	12 (40.0)	6 (15.4)	19 (25.7)	8 (42.1)
Sensitizatvion to walnut†							
SPT walnut positive, n (%)	211 (40.8)	36 (81.8)	13 (54.2)	9 (30.0)	21 (53.8)	12 (16.9)	7 (38.9)
ImmunoCAP walnut positive	182 (35.5)	35 (81.4)	20 (87.0)	11 (39.3)	19 (48.7)	10 (13.9)	7 (43.8)
CRD walnut performed, N	**202**	**19**	**13**	**5**	**18**	**15**	**8**
CRD walnut positive‡	158 (79.4)	13 (68.4)	10 (76.9)	4 (80.0)	15 (83.3)	9 (64.3)	8 (100.0)
Jug r 1, n (%)	21 (10.4)	0 (0.0)	3 (23.1)	1 (20.0)	0 (0.0)	1 (6.7)	3 (37.5)
Jug r 2, n (%)	19 (9.6)	0 (0.0)	3 (23.1)	1 (20.0)	1 (5.6)	1 (7.1)	2 (25.0)
Jug r 2 LMW, n (%)	43 (22.1)	5 (26.3)	4 (30.8)	1 (20.0)	3 (16.7)	3 (23.1)	3 (37.5)
Jug r 3, n (%)	28 (13.9)	9 (47.4)	3 (23.1)	0 (0.0)	4 (22.2)	0 (0.0)	2 (25.0)
Jug r 4, n (%)	18 (9.2)	0 (0.0)	3 (23.1)	2 (40.0)	1 (5.6)	0 (0.0)	2 (25.0)
Jug r 5, n (%)	115 (58.1)	1 (5.3)	1 (7.7)	1 (20.0)	12 (66.7)	7 (50.0)	7 (87.5)
Jug r 6 , n (%)	12 (6.2)	0 (0.0)	1 (7.7)	0 (0.0)	0 (0.0)	0 (0.0)	2 (25.0)
Jug r 7, n (%)	47 (23.3)	4 (21.1)	4 (30.8)	0 (0.0)	7 (38.9)	1 (6.7)	1 (12.5)
Probable walnut allergy, n (%)	336 (65.8)	43 (97.7)	23 (95.8)	15 (53.6)	32 (82.1)	22 (31.4)	13 (81.3)

TABLE I. CHARACTERISTICS OF SUBJECTS WITH SELF-REPORTED WALNUT ALLERGY ACROSS EUROPE (CONTINUED)

	European City						
	Reykjavik	**Sofia**	**Strasbourg**	**Utrecht**	**Vilnius**	**Zürich**	***p*-value***
Self-reported walnut allergy; N	**9**	**10**	**50**	**74**	**50**	**107**	
Age (y), mean ±SD	36.4 ±17.6	23.2 ±14.3	33.8 ±12.8	31.2 ±11.3	27.9 ±14.0	33.8 ±12.8	<0.001
<18 y, n (%)	1 (11.1)	4 (40.0)	5 (10.0)	3 (4.1)	14 (28.0)	6 (5.6)	<0.001
Female seks, n (%)	6 (66.7)	7 (70.0)	34 (68.0)	54 (73.0)	22 (44.0)	65 (60.7)	<0.001
Symptom severity*							
Mild, n (%)	2 (22.2)	0 (0.0)	33 (66.0)	33 (44.6)	18 (36.0)	56 (52.3)	<0.001
Moderate, n (%)	2 (22.2)	7 (70.0)	9 (18.0)	20 (27.0)	22 (44.0)	29 (27.1)	
Severe, n (%)	5 (55.6)	3 (30.0)	8 (16.0)	21 (28.4)	10 (20.0)	22 (20.6)	
Sensitization to walnut†							
SPT walnut positive, n (%)	4 (44.4)	2 (20.0)	13 (26.5)	25 (37.3)	38 (77.6)	31 (29.0)	<0.001
ImmunoCAP walnut positive	3 (33.3)	3 (30.0)	13 (26.5)	19 (25.7)	15 (34.1)	27 (25.5)	<0.001
CRD walnut performed, N	**3**	**4**	**16**	**20**	**14**	**67**	
CRD walnut positive‡	1 (33.3)	1 (33.3)	15 (93.8)	19 (95.0)	11 (84.6)	52 (77.6)	0.065
Jug r 1, n (%)	1 (33.3)	0 (0.0)	1 (6.3)	7 (35.0)	0 (0.0)	4 (6.0)	0.001
Jug r 2, n (%)	1 (33.3)	1 (25.0)	1 (6.7)	6 (30.0)	1 (7.7)	1 (1.5)	0.007
Jug r 2 LMW, n (%)	1 (33.3)	1 (33.3)	3 (20.0)	8 (40.0)	3 (23.1)	8 (12.3)	0.527
Jug r 3, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	3 (15.0)	1 (7.1)	6 (9.0)	0.002
Jug r 4, n (%)	1 (33.3)	0 (0.0)	1 (6.7)	6 (30.0)	0 (0.0)	2 (3.1)	0.001
Jug r 5, n (%)	0 (0.0)	0 (0.0)	14 (93.3)	18 (90.0)	11 (84.6)	43 (62.5)	<0.001
Jug r 6 , n (%)	1 (33.3)	0 (0.0)	0 (0.0)	5 (25.0)	0 (0.0)	3 (4.6)	0.005
Jug r 7, n (%)	0 (0.0)	0 (0.0)	1 (6.3)	9 (45.0)	1 (7.1)	19 (28.4)	0.041

TABLE I. CHARACTERISTICS OF SUBJECTS WITH SELF-REPORTED WALNUT ALLERGY ACROSS EUROPE (CONTINUED)

	European City						
	Reykjavik	**Sofia**	**Strasbourg**	**Utrecht**	**Vilnius**	**Zürich**	***p*-value***
Probable walnut allergy, n (%)	5 (55.6)	5 (50.0)	29 (60.4)	37 (54.4)	42 (85.7)	70 (66.0)	<0.001

CRD: Component-resolved diagnostics; LMW: Low molecular weight; SPT: Skin prick test

*Symptom severity: mild = isolated oral allergy symptoms; moderate=symptoms of the skin, eyes, upper airway, or gastro-intestinal system; severe=dysphagia, dysphonia, cardiovascular, or neurological symptoms, or anaphylaxis.

†The results show the number and percentage of subjects with positive sensitization according to each test. SPT with walnut extract was performed in 517 subjects; ImmunoCAP with walnut extract in 513 subjects.

‡For some centres (Łódź, Sofia, Strasbourg, Vilnius, and Zürich), the results of 1 or 2 of the individual CRD tests were missing. The percentage given in brackets is the percentage of the total number of available CRD results.

p-values were determined for exploratory purposes (no correction for multiple testing) using the Pearson χ^2 test for categorical variables, and the ANOVA or Kruskall-Wallis test for continuous variables.

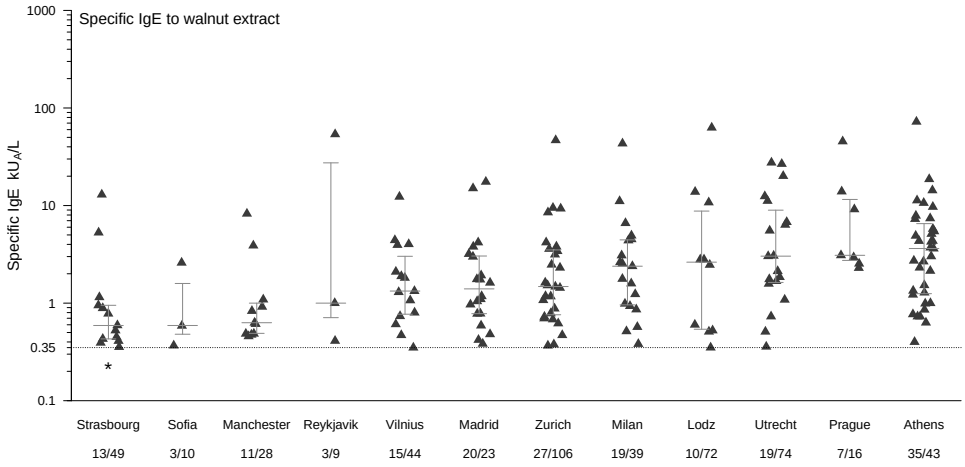


FIGURE I. IgE to walnut extract across Europe. Walnut specific IgE levels in subjects with positive serology to walnut extract in ImmunoCAP (≥ 0.35 kU_A/L). The triangles represent individual subjects, the lines indicate medians and interquartile ranges. n/N = number of subjects with positive serology/ number of subjects in whom ImmunoCAP with walnut extract was performed. *Significantly different from Prague, Athens and Utrecht.

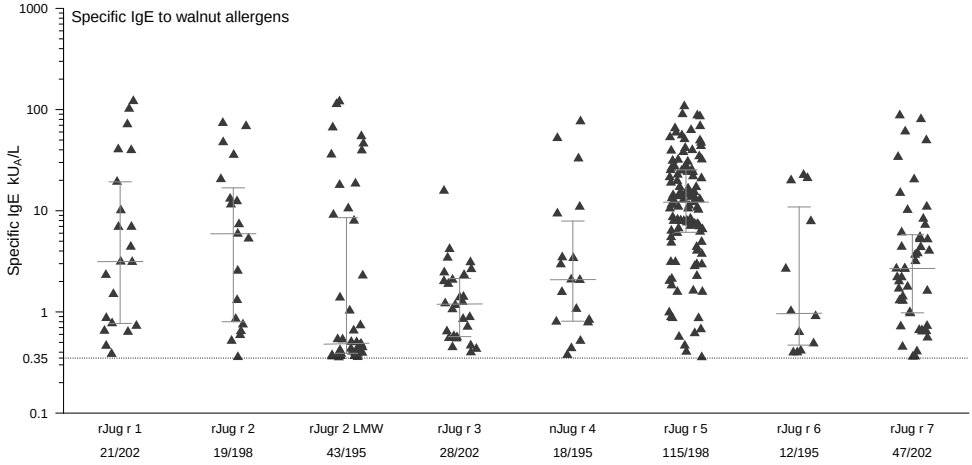


FIGURE II. IgE to walnut allergens. Walnut allergen specific IgE levels in subjects with positive serology to the respective walnut allergens in ImmunoCAP ≥ 0.35 kU_A/L). The triangles represent individual subjects, the lines indicate medians and interquartile ranges. n/N = number of subjects with positive serology/ number of subjects in whom ImmunoCAP with walnut extract was performed.

occurred most frequently in other Southern centres, Madrid and Milan. Sensitization to profilin Jug r 7 was most common after sensitization to Jug r 5, and was particularly recognized in Utrecht, Milan, Madrid, Zürich and Athens. Storage proteins Jug r 1, 2, 4 and 6 were recognized in up to 10% of subjects overall; all most frequently in Utrecht, followed by Madrid.

Relationship between IgE to walnut components and other allergens

Figure 4 and Supplemental Figure S1 reveal how IgE levels to walnut components correlated with IgE levels to pollen and other foods. Regarding pollen, the strongest correlation overall was between IgE to Jug r 5 and birch (Table S3, *Rho*=0.92). This positive correlation was prominent in all evaluated centres (*Rho* =0.75-0.97), except Madrid and Athens. In Madrid, the strongest correlation between a walnut component and pollen, was between Jug r 7 and grass pollen (*Rho* =0.70). In Athens, the correlations between Jug r 3 and mugwort, *Chenopodium*, and plane tree pollen (*Rho* =0.76-0.86), were most remarkable.

Regarding IgE levels to food extracts other than walnut, the overall strongest correlations were found between Jug r 5 and hazelnut (*Rho* =0.88), and between Jug r 3 and lentil (*Rho* =0.80).

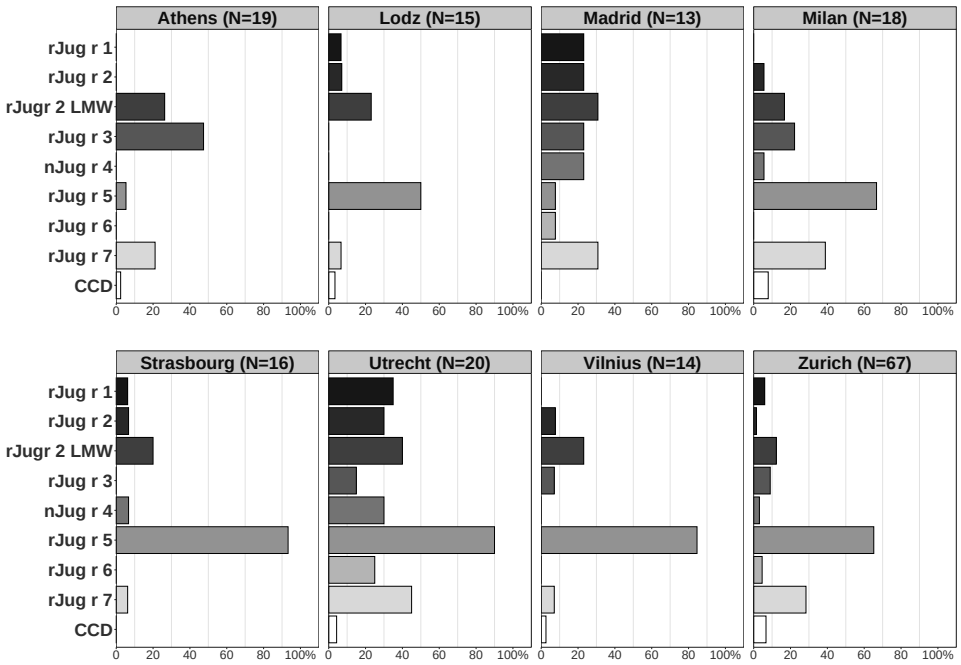


FIGURE III. Sensitization to walnut components stratified by city. N = the total number of subjects in whom CRD was performed. The number of subjects in whom CRD was positive, is visible for each centre in Table 1. Only centres where CRD was completed in at least 10 subjects, are shown. The length of the bars corresponds with the percentage of subjects with positive serology to each specific walnut allergen.

However, the walnut components most likely to show strong correlations with the various foods differed per centre (Table E4). For example, IgE levels to hazelnut correlated strongly with Jug r 5 IgE levels in most centres, but with Jug r 3 IgE levels in Athens. Lentil IgE levels were found to correlate strongly with different walnut components in each centre, but never with Jug r 5 or Jug r 7.

Predictors for severity of walnut allergy

Probable walnut allergy, where reported symptoms were supported by IgE sensitization, was identified in 336 subjects (Table 1). Of these 336 subjects, 246 (73.2%) had mild-to-moderate symptoms, and 90 (26.8%) had severe symptoms.

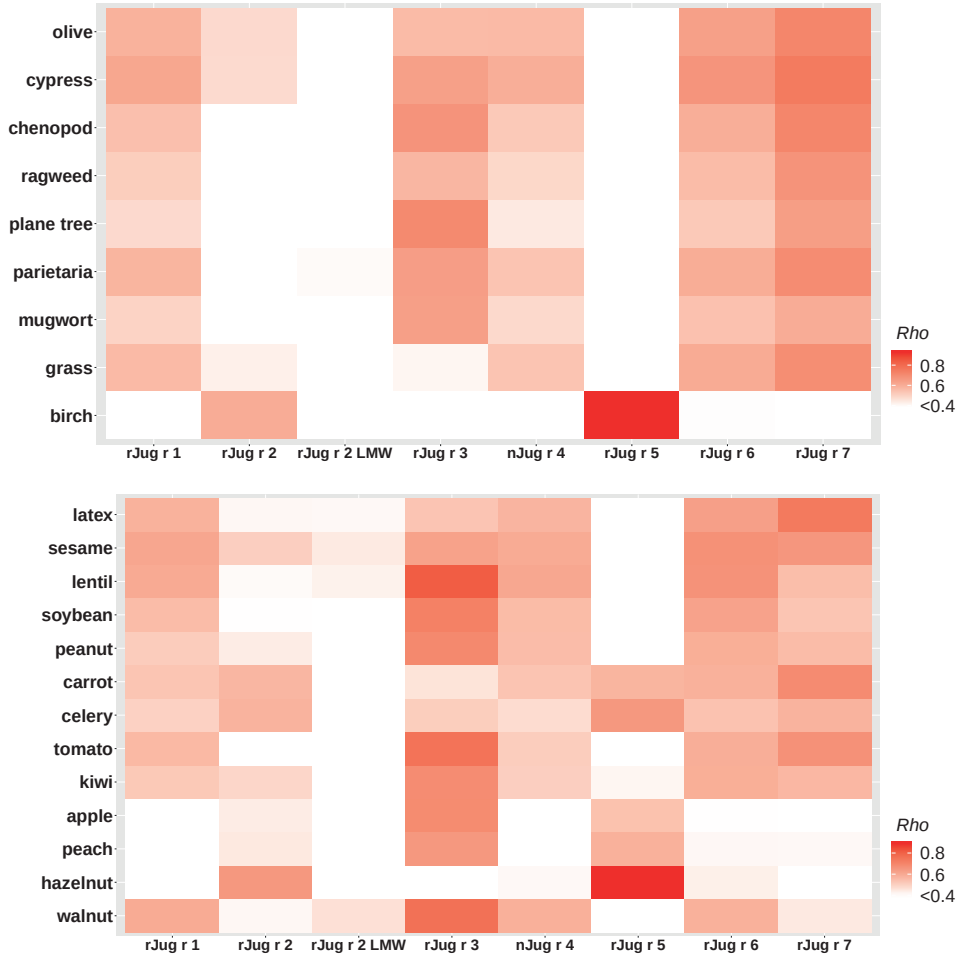


FIGURE IV. Correlation between IgE levels to walnut components and pollen and other foods. The numeric values of the Spearman rho correlation coefficients are available from Supplemental Table S3.

The results from univariable analyses are listed in Table 2. Regarding clinical history, subjects with severe walnut allergy were significantly more likely to have mugwort allergy, and significantly less likely to have birch pollen allergy or IgE sensitization to cat or dog, than subjects with mild-to-moderate walnut allergy. Although not statistically significant, severely allergic subjects were more often sensitized to walnut in SPT, and had higher median IgE levels to walnut extract in ImmunoCAP. No significant differences between severity groups were found regarding the percentage of subjects sensitized to specific walnut allergens, or median IgE levels, although trends amongst sensitized subjects suggested higher IgE levels to storage proteins and LTP in severely allergic and to PR-10 and profilin in mild-to-moderately allergic subjects (Table S5).

CRD was performed in 177 of 336 subjects with probable walnut allergy. These 177 subjects were included in the multivariable analyses for prediction of severity of walnut allergy. Table 3 shows the results of a Lasso regression analysis. Of all the demographics and clinical history variables included in model I, Lasso regression selected 'symptoms upon skin contact with walnut', 'family history of atopic disease', 'atopic dermatitis', and 'mugwort pollen allergy', which were positively associated with severe walnut allergy, and 'IgE sensitization to cat or dog', which was inversely associated with severe walnut allergy. In model II, all the variables selected in model I remained. Additionally, SPT positivity to walnut was selected as an extra predictor (positive association). Finally, in model III, IgE levels to Jug r 1, Jug r 5, Jug r 7, and Ana c 2 were found to further contribute to prediction of severity of walnut allergy.

Although walnut SPT positivity was selected as an additional predictor in model II, model accuracy remained similar to model I (AUC = 0.74 in both models). Addition of CRD in model III significantly increased the AUC to 0.81 (p_{DeLong}=0.002).

Additional analyses of the performance of individual tests, revealed that combinations of tests as defined in the Lasso regression models better predicted severity than SPT to walnut, ImmunoCAP to walnut extract, or ImmunoCAP to individual walnut allergens (evaluated separately or combined), for which AUC's ranged from 0.48 to 0.66 (Table S6).

Discussion

The current study is the largest European multi-centre study on walnut allergy to date. Clear geographical differences were observed in walnut component sensitization and co-sensitization patterns; and our predictive model combining demographic, clinical, and serological variables attained good accuracy with an AUC of 0.81 for distinguishing mild-to-moderate from severe walnut allergy.

TABLE II. CHARACTERISTICS OF SUBJECTS WITH PROBABLE WALNUT ALLERGY RELATED TO SEVERITY

	Severity Classification			
Characteristic	**Mild-to-moderate N=246**	**Severe N=90**	***p*-value**	**Univariable OR [95%-CI]**
Ag (y), mean ±SD	29.9 ± 13.0	28.4 ± 12.5	0.972	0.99 [0.97-1.01]
Female seks, n(%)	147 (59.8)	47 (52.2)	0.216	0.74 [0.45-1.98]
Clinical history, n(%)				
Age onset of symptoms < 14 years	97 (39.8)	38 (42.2)	0.683	1.11 [0.67-1.81]
Symptoms upon skin contact with walnut	9 (4.1)	7 (8.8)	0.117	2.23 [0.77-6.19]
Family history of atopic disease	152 (67.6)	60 (71.4)	0.514	1.20 [0.70-2.11]
Atopic dermatitis (ever)	68 (28.2)	32 (36.4)	0.155	1.45 [0.86-2.43]
Asthma (ever)	229 (97.0)	86 (96.6)	0.851	0.88 [0.24-4.14]
Birch pollen allergy	153 (64.6)	44 (51.8)	0.038	0.59 [0.36-0.97]
Grass pollen allergy	138 (58.5)	53 (62.4)	0.532	1.18 [0.71-1.97]
Mugwort pollen allergy	31 (13.3)	20 (23.0)	0.035	1.95 [1.03-3.62]
Planetree pollen allergy	17 (7.4)	8 (9.2)	0.595	1.27 [0.50-2.97]
House dust mite allergy	66 (28.1)	23 (26.7)	0.812	0.94 [0.53-1.61]
Latex allergy	12 (5.1)	5 (5.7)	0.813	1.14 [0.35-3.17]
Cat/dog sensitization	173 (73.6)	53 (60.9)	0.027	0.56 [0.33-0.94]
Sensitization to walnut*				
SPT walnut positive†	150 (61.5)	61 (68.5)	0.236	1.37 [0.82-2.31]
IgE level walnut extract	0.39 (0.05-1.70)	0.73 (0.15-3.63)	0.018	1.02 [0.99-1.05]
IgE level Jug r 1	0.01 (0.00-0.06)	0.01 (0.00-0.05)	0.719	1.00 [0.95-1.02]
IgE level Jug r 2	0.05 (0.02-0.13)	0.04 (0.01-0.08)	0.516	1.02 [0.98-1.06]
IgE level Jug r 2 LMW	0.24 (0.17-0.36)	0.23 (0.15-0.32)	0.571	1.01 [0.99-1.04]
IgE level Jug r 3	0.04 (0.01-0.17)	0.05 (0.01-0.12)	0.739	0.93 [0.54-1.21]
IgE level Jug r 4	0.03 (0.01-0.09)	0.02 (0.01-0.06)	0.215	1.00 [0.93-1.05]
IgE level Jug r 5	6.69 (0.03-16.83)	1.60 (0.02-9.11)	0.118	0.97 [0.94-1.00]
IgE level Jug r 6	0.03 (0.01-0.07)	0.02 (0.01-0.07)	0.399	1.04 [0.91-1.16]
IgE level Jug r 7	0.02 (0.00-0.65)	0.02 (0.00-0.18)	0.503	0.92 [0.75-1.00]

CI: Confidence interval; OR: Odds ratio; SPT: Skin prick test

All measurements are in n (%) or median (Q1-Q3) unless otherwise specified. All IgE levels were measured in kU_A/L on ImmunoCAP.

*For subjects with mild-to-moderate and severe probable walnut allergy

†SPT was performed in respectively 244 and 89 subjects; ImmunoCAP with walnut extract in 240 and 89 subjects; and CRD in 136 and 41 subjects.

Walnut allergy across Europe: Distribution of allergen (co-)sensitization patterns

The distribution of sensitization to walnut components across Europe was found to follow the same pattern as many other plant source foods, including other tree nuts[90]: sensitization to PR-10 proteins (Jug r 5) in Northern and Central Europe;[108] sensitization to profilin (Jug r 7) throughout Europe,[109] and sensitization to lipid transfer proteins (Jug r 3) in the Mediterranean.[110]

TABLE III. PREDICTION MODELS FOR WALNUT ALLERGY SEVERITY

	Model I:		Model II:		Model III:	
	Demographics & clinical history		Model 1 + sensitization to walnut extract		Model II + sensitization to walnut components	
	OR	95%-CI*	OR	95%-CI*	OR	95%-CI*
Symptoms upon skin contact with walnut	1.95	1.51-2.53	2.32	1.48-3.63	2.43	1.58-3.75
Family history atopic disease	1.65	1.49-1.82	1.97	1.74-2.23	2.69	2.35-3.07
Atopic dermatitis	1.89	1.64-2.19	2.12	1.82-2.48	2.68	2.26-3.18
Mugwort pollen allergy	1.96	1.66-2.32	2.28	1.93-2.69	3.75	3.18-4.42
Cat/dog sensitization	0.41	0.36-0.48	0.34	0.30-0.40	0.40	0.35-0.46
SPT walnut positive			1.06	0.94-1.18	1.07	0.96-1.20
IgE level Jug r 1					0.99	0.98-1.00
IgE level Jug r 5					0.97	0.97-0.97
IgE level Jug r 7					0.98	0.97-0.98
IgE level Ana c 2					0.63	0.55-0.73
Intercept	*-1.32*		*-1.45*		*-1.52*	
AUC (95%-CI)	**0.74 (0.65-0.83)**		**0.74 (0.65-0.83)**		**0.81 (0.73-0.89)**	

CI: Confidence interval; OR: Odds ratio

All IgE levels were measured in kU_A/L on ImmunoCAP. The 95% confidence intervals (CI) for each coefficient were calculated from standard errors obtained for each imputed datasets through bootstrapping, and pooled over the 10 imputed datasets using Rubin's rules.

Unselected variables model I: age, sex, age at onset of symptoms to walnut (<14 vs ≥ 14 years), asthma, birch/ grass/ plane tree pollen allergy, house dust mite allergy, latex allergy. Unselected variables model II: IgE level walnut extract. Unselected variables model III: IgE level Jug r 2, Jug r 3, Jugr4, and Jug r 6.

The highest overall sensitization rates were found for Jug r 5 and Jug r 7. Pollen exposure helps explain their geographical distribution, as sensitization to plant food PR10-proteins and profilins is induced by similar proteins in pollen.[5,99] Jug r 5 is homologous with Bet v 1, the major allergen of birch pollen, the dominating pollen in Northern and Central Europe.[108] Jug r 7 sensitization, on the other hand, could be secondary to sensitization to almost any type of pollen, as all pollen contains profilin. Our findings were consistent with these patterns of cross-reactivity (Figure 4 and Table S3): IgE to Jug r 5 showed strong correlations with IgE to birch pollen (*Rho*=0.92), and IgE to Jug r 7 moderate-to-strong correlations (*Rho* >0.60) with IgE to almost all pollen.

Sensitization to Jug r 3 is generally thought to occur through peach as primary sensitizer, [110-113] although plane tree and mugwort pollen have also been suggested as primary sources of sensitization to LTP.[7,8,33] Indeed, IgE to Jug r 3 correlated with IgE to peach, plane tree, and mugwort in our data (*Rho*>0.60), but also to other LTP-containing pollen (e.g. *Chenopodium, Parietaria,* cypress), fruits (tomato, apple, kiwi), and legumes (lentil, soybean, peanut).[110] Future studies with IgE inhibition assays could help further differentiate between independent co-sensitization and cross-reactivity, and identify primary sources of sensitization to Jug r 3 and other walnut components.

Similar distributions of Jug r 3 and Jug r 5 sensitization were observed by Ballmer-Weber *et al*, in Germany, Switzerland and Spain.[40] However, occurrence of sensitization to walnut storage proteins was more frequent in their data (48-57%) than in ours (7-10%). This is likely due to the diverse study populations, which in the study of Ballmer-Weber *et al.* included more severely allergic subjects, more paediatric subjects, and more subjects with onset of symptoms before the age of 14, all of which make primary sensitization more likely.

Notably, a high proportion of subjects sensitized to Jug r 5 tested negative to walnut extract (Table 1 and S2), as has also been observed previously.[95] This finding substantiates that the concentration of Jug r 5 is low in walnut extract, causing a low sensitivity of extract based tests for subjects with birch-pollen related walnut allergy.

Walnut allergy across Europe: Prediction of severity

A model combining symptoms upon skin contact with walnut, history of atopic dermatitis, family history of atopic disease, mugwort pollen allergy, sensitization to cat or dog, positive SPT for walnut, and IgE to Jug r 1, Jug r 5, Jug r 7 and CCD, was found to have the highest accuracy for predicting severity of walnut allergy (AUC 0.81 [95%-CI 0.73-0.89]).

Our findings suggest that sensitization via the cutaneous route may be associated with severity of walnut allergy. Several studies have established that atopic dermatitis predisposes to food sensitization and allergy, presumably as a result of skin barrier impairement.[12] In line with our findings, having atopic dermatitis was previously found to be associated with severe hazelnut allergy.[101] One could speculate that sensitization via the skin leads to primary (non-cross-reactive) food sensitization, which is thought to be associated with more severe reactions.[114]

In cross-reactive food allergy, pollen is generally the primary sensitizer, with sensitization most probably occurring through the respiratory tract. Symptomatic subjects generally present with mild symptoms.[5,108] As remarked previously, subjects with a birch-pollen related walnut allergy are poorly detected by diagnostic tests with walnut extract, explaining the positive association between SPT and severe walnut allergy.

Remarkably, mugwort pollen allergy almost quadrupled the odds of severe walnut allergy. LTP sensitization, which is associated with severe allergic reactions to plant source foods,[115] could be the link. It has been suggested that sensitization to mugwort LTP (Art v 3) can facilitate subsequent sensitization to LTP in plant source foods, and the other way around.[7,116] However, the observation that Jug r 3 IgE levels were not predictive of walnut allergy severity, makes this explanation less likely. Another plausible explanation is that other still uncharacterized mugwort allergens are associated with severe walnut allergy.

Addition of walnut component testing was found to considerably improve prediction of walnut allergy severity. Our expectations were that sensitization to PR-10 proteins and profilins would

be associated with mild-to-moderate walnut allergy, and to seed storage and lipid transfer proteins would predict severe walnut allergy.[40,99,101] The former associations were indeed confirmed in our data; IgE levels to Jug r 5 and 7 were predictive of mild-to-moderate walnut allergy. IgE to walnut storage proteins appears to be of lesser importance in prediction of walnut allergy severity in subjects from the general population, in whom such sensitization occurs infrequently. We have no clear explanation for why IgE to Jug r 1 was inversely associated with severity in our data.

Overall, the prediction models in this study provide insight into the clinical profile of subjects more likely to have mild-to-moderate or severe reactions to walnut, and suggest some particular focus areas during diagnostic work-up of walnut allergy. Besides obtaining information on allergic comorbidities and family atopy, as is standard in clinical history for food allergy, physicians assessing walnut allergy should find out if presenting patients are allergic to mugwort or have symptoms elicited by skin contact with walnut. Information on cross-reactive sensitization (Jug r 5, Jug r 7, CCD) contributes to prediction of a more mild phenotype. As Jug r 5 is underrepresented in walnut extract, diagnostic work-up in birch-endemic areas would benefit from additional testing of Jug r 5. After validation, the prediction of a mild-to-moderate phenotype using our final model could potentially translate into performance of fewer challenge tests in clinical practice (Table S6).

Strengths and limitations

All in all, this is the largest study to map walnut sensitization across Europe. The consistent and standardized approach to data collection makes our results particularly valuable. We did not include subjects with walnut allergy determined by food challenge, but all subjects presenting to an allergy clinic with symptoms to walnut within two hours of ingestion, and corresponding IgE sensitization. Through this approach, we likely captured more subjects with pollen-related walnut allergy, who form a significant proportion of walnut allergic subjects in Europe. We have also, for the first time, suggested a prediction model for assessing severity of walnut allergy, taking both clinical evaluation and serology testing into account. The main limitation of our study was that CRD was available for only 177 of 336 walnut allergic subjects. Multiple imputation and penalized regression were applied to appropriately deal with sparse data, and model I and II were also developed in the total population of 336 walnut-allergic subjects, revealing no relevant differences. However, it is important to realize that we could not adjust the multivariable analyses for centre due to sparsity of data. Although we do not expect the effect of predictors on severity to depend on centre, we do observe geographically varying baseline prevalence of severe walnut allergy (Table 1).

Conclusions

To conclude, we confirm that cross-reactivity with pollen is a major cause of walnut sensitization and allergy across Europe, leading to molecular recognition patterns similar to those of other

plant source foods. PR-10 protein and profilin sensitization occur frequently, and predict a mild-to-moderate walnut allergy phenotype. Sensitization to walnut storage proteins is less common. The information obtained from walnut CRD, in combination with results from extract-based testing and clinical background evaluation, allows for good discrimination between mild-to-moderate and severe walnut allergy. A prediction model combining this information performs significantly better than CRD, extract-based testing or clinical background alone.

Acknowledgements

We thank all the patients for their participation in the study. We would like to thank ALK Abello (Madrid, Spain) for their generous gift of SPT reagents. We acknowledge the support by the 6th and 7th Framework Programmes of the EU, for EuroPrevall (FP6-FOOD-CT-2005-514000) and iFAAM (Grant agreement no. 312147), respectively.

Supplemental files

Supplemental Methods

Skin Prick testing

SPT was performed with commercially available extracts (ALK-Abelló, Madrid, Spain) following guidelines of the European Academy of Allergology and Clinical Immunology.[89]

IgE testing

IgE levels in serum were measured by ImmunoCAP (ThermoFisher Scientific, Uppsala, Sweden). ImmunoCAP analyses with extracts were performed at the Paul-Ehrlich-Institut (Langen, Germany). ImmunoCAP analyses with walnut components were carried out at the Amsterdam University Medical Centre (Location AMC, Amsterdam, The Netherlands).

Jug r 2 LMW

The low-molecular-weight fraction of Jug r 2 consists of the N-terminal region of Jug r 2, which is removed during maturation. It does not contain any of the mature Jug r 2 cupin domains. In the nut, the N-terminal region is found as 6 individual peptides. Here they are expressed as one polypeptide chain. IgE to Jug r 2 LWM was not included as a candidate predictor for prediction of severity of walnut allergy, because a considerable number of walnut allergic subjects without sensitization to Jug r 2 were sensitized to Jug r 2 LMW at an IgE level below 1.0 kU_A/L, which in part may be due to an elevated background of this experimental assay.

Symptom severity classification

For classification of severe symptoms, *lower airway symptoms* included dyspnoea, wheezing, cough, or chest tightness; *cardiovascular symptoms* consisted of cardiac arrhythmia, myocardial ischaemia, or hypotension; *neurological symptoms* comprised disorientation/confusion, dizziness, seizures, incontinence, or loss of consciousness; and anaphylaxis included reactions with severe laryngeal oedema, severe bronchospasm, or hypotensive shock. For classification of mild-to-moderate symptoms, *skin symptoms* included urticaria, angioedema, erythema/flushing, or itching; *eye symptoms* comprised conjunctivitis; upper airway symptoms consisted of rhinitis, conjunctivitis, or tightness of throat; and gastro-intestinal symptoms comprised stomach pain, cramps, nausea, vomiting, diarrhoea.[103,104]

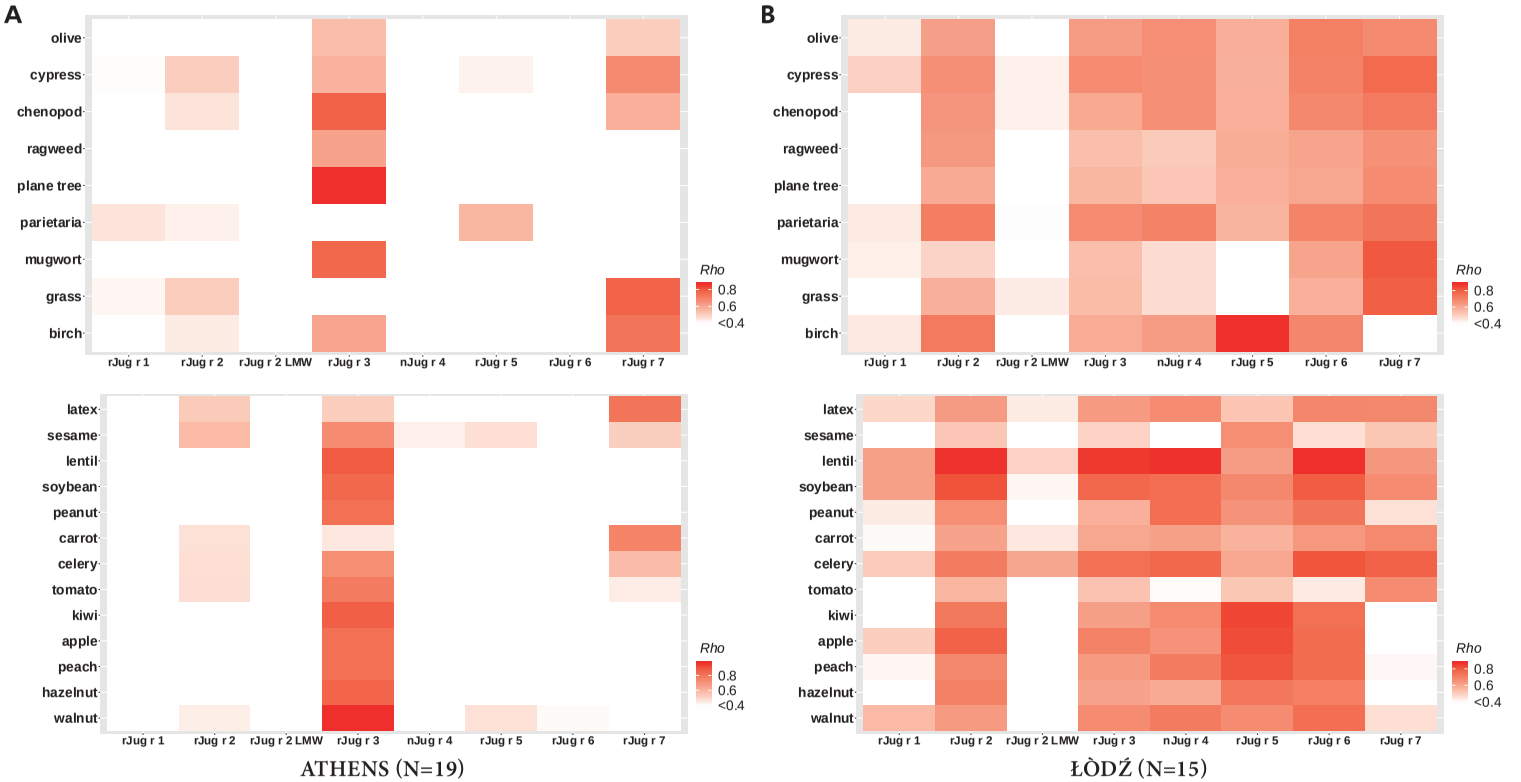
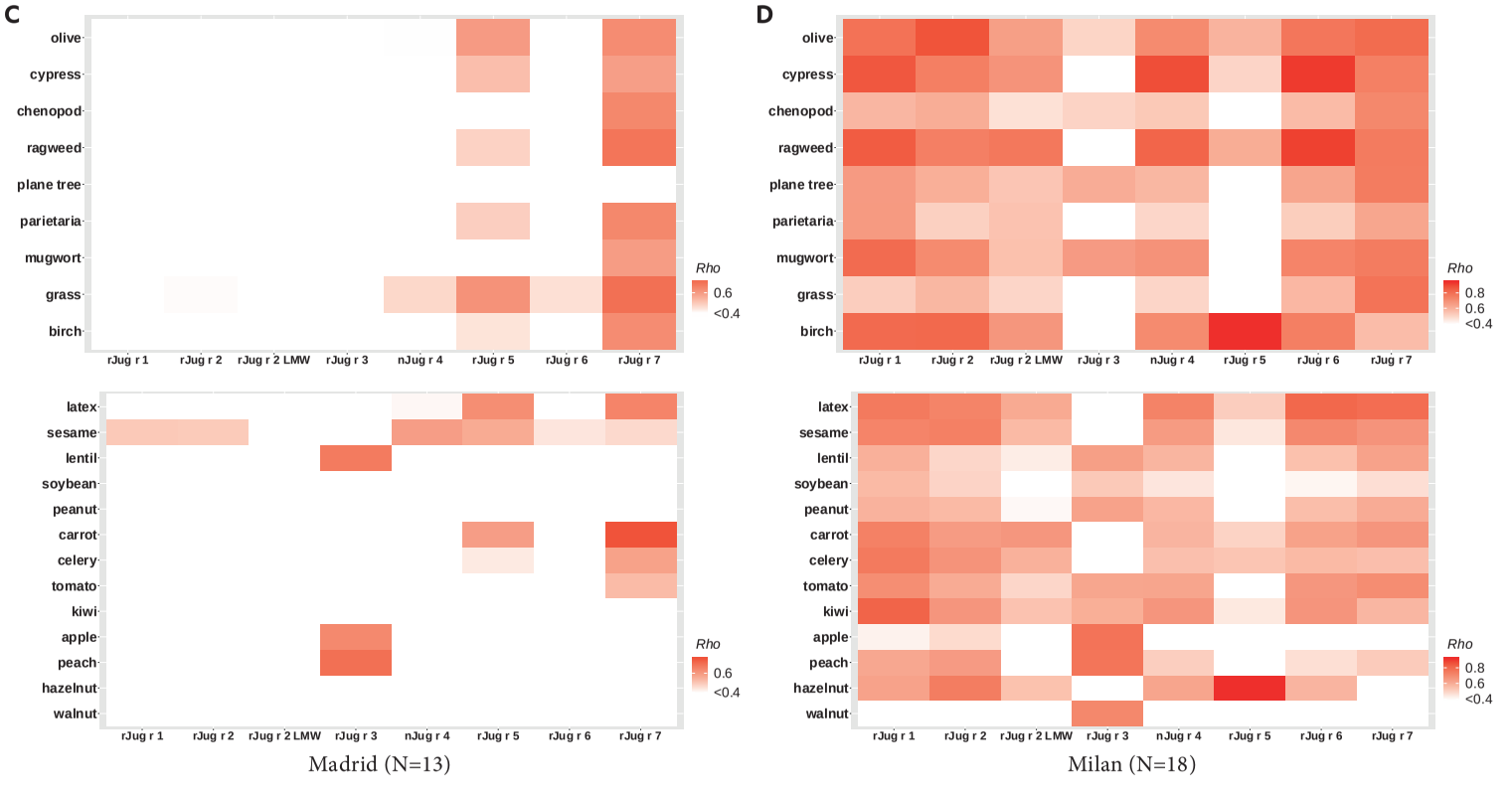


FIGURE S1. Correlation between IgE levels to walnut components and pollen and other foods per centre with at least 10 subjects completing CRD testing. Only centres with at least 10 subjects completing CRD were evaluated separately. Too few subjects completed CRD in Prague (N=8), Manchester (N=5), Reykjavik (N=3) and Sofia (N=4) to determine valid correlations.



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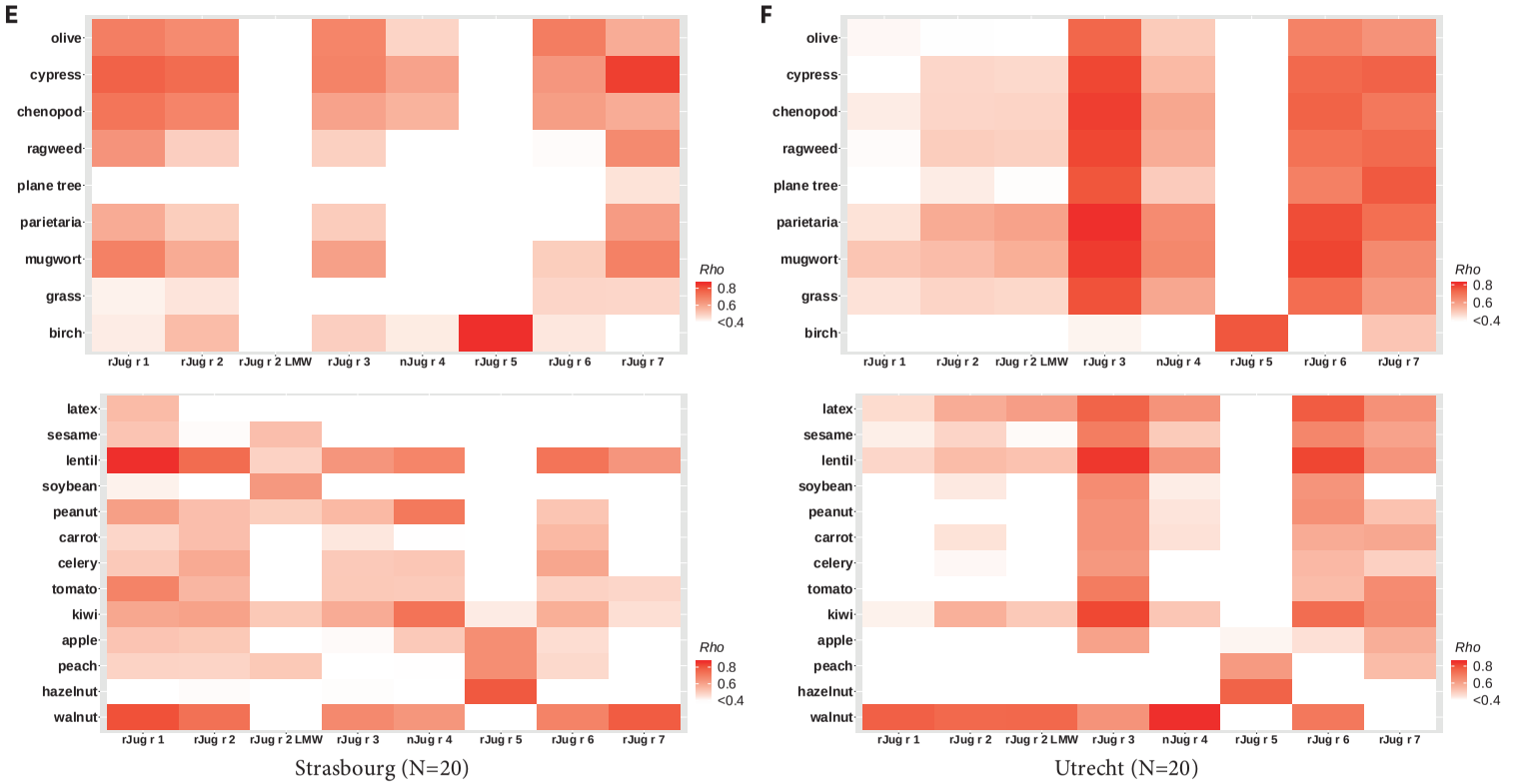

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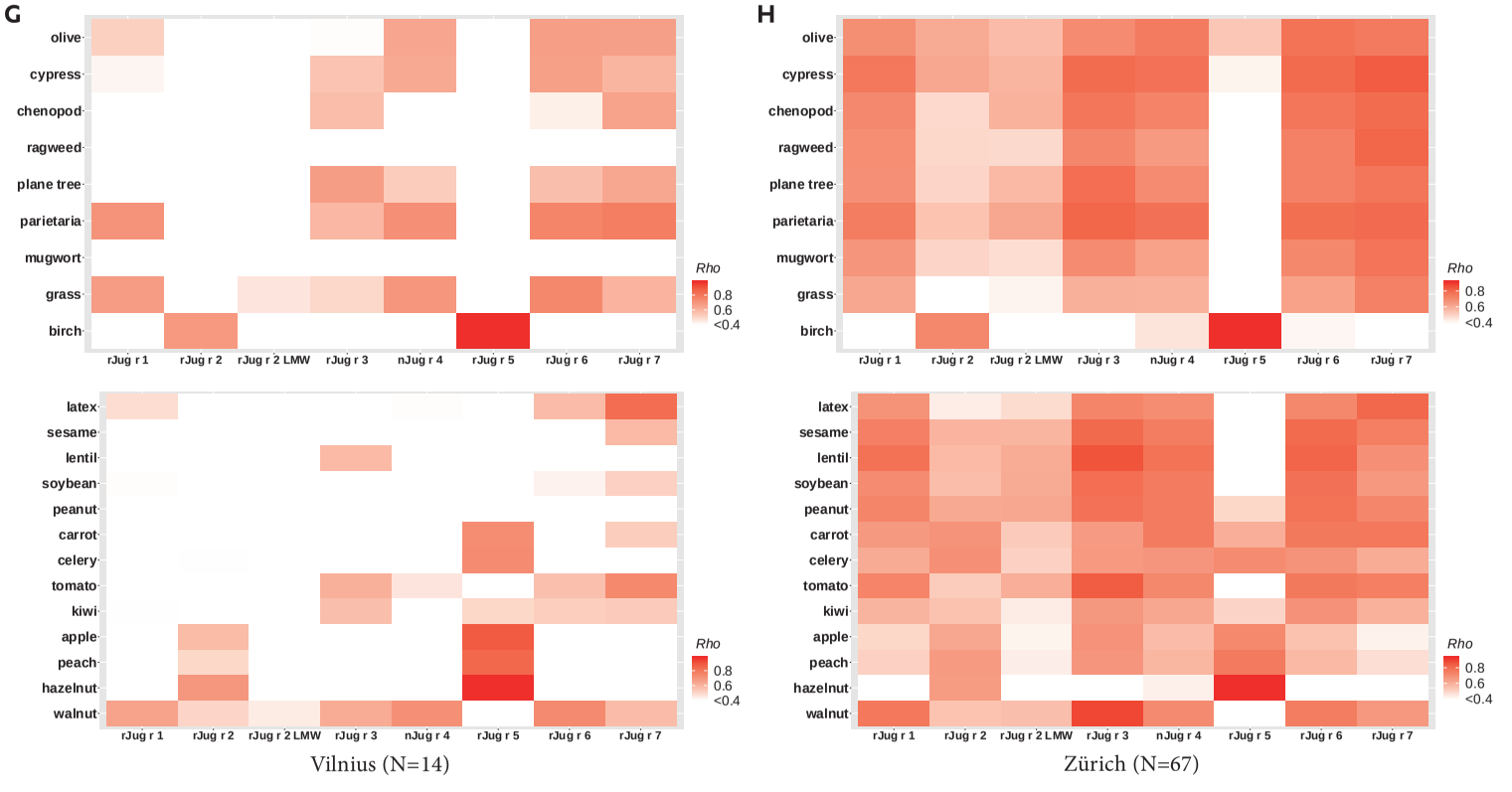

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TABLE S1. MISSING DATA IN VARIABLES INCLUDED FOR LASSO REGRESSION

Characteristic	Missing
Age	0
Female sex	0
Clinical history	0
Age onset symptoms	21
Symptoms upon skin contact with walnut	14
Family history of atopic disease	6
Atopic dermatitis	3
Asthma	2
Birch pollen allergy	5
Grass pollen allergy	7
Mugwort pollen allergy	4
Planetree pollen allergy	7
House dust mite allergy	6
Latex allergy	0
Cat/dog sensitization	0
SPT walnut positive	0
IgE level walnut extract	0
IgE level Jug r 1	0
IgE level Jug r 2	2
IgE level Jug r 2 LMW	4
IgE level Jug r 3	0
IgE level Jug r 4	4
IgE level Jug r 5	2
IgE level Jug r 6	4
IgE level Jug r 7	0

SPT: Skin prick test

Total = N177. Values for these missing data were estimated using multiple imputation procedures, for which all of the above determinants were included as covariates, along with severity of walnut allergy, IgE levels to other foods (hazelnut, peach, apple, kiwi, tomato, carrot, celery, peanut, soybean, lentils, sesame seed), and centre.

TABLE S2. IGE TO WALNUT COMPONENTS IN SUBJECTS WITH NEGATIVE WALNUT SPT AND IMMUNOCAP

	Negative ImmunoCAP			Negative SPT			Negative ImmunoCap and SPT		
		IgE level (kU_A/L)			IgE level (kU_A/L)			IgE level (kU_A/L)	
Total	**N**	**Median**	**IQR**	**N**	**Median**	**IQR**	**N**	**Median**	**IQR**
	331			306			237		
CRD performed	120			115			82		
CRD positive	88/117			85/112			56 /79		
Jug r 1	3/120	0.73	0.69-0.80	5/115	1.50	0.63-3.14	0/82	-	-
Jug r 2	3/117	0.75	0.70-0.80	5/112	0.59	0.52-0.65	1/79	0.65	-
Jug r 2 LMW	15/115	0.40	0.37-0.50	15/110	0.42	0.38-0.50	7/77	0.38	0.36-0.41
Jug r 3	1/120	0.89	-	9/115	0.89	0.55-1.41	1/82	0.89	-
Jug r 4	1/115	0.79	-	6/110	0.66	0.46-0.83	1/77	0.84	-
Jug r 5	79/117	11.46	5.20-23.46	67/112	10.75	5.76-20.57	50/79	9.44	4.90-19.13
Jug r 6	1/115	0.91	-	4/110	0.52	0.41-0.73	0/77	-	-
Jug r 7	21/120	1.62	0.72-4.02	29/115	3.91	1.31-6.39	16/82	1.91	0.72-5.01

CRD: Component-resolved diagnostics; IQR: Interquartile range; LMW: Low molecular weight; SPT: Skin prick test.

IgE levels were measured on ImmunoCAP in kU_A/L.

TABLE S3. CORRELATIONS BETWEEN IGE LEVELS TO WALNUT COMPONENTS AND POLLEN AND OTHER FOODS

	Walnut allergen							
	Jug r 1	**Jug r 2**	**Jugr 2 LMW**	**Jug r 3**	**Jug r 4**	**Jug r 5**	**Jug r 6**	**Jug r 7**
Birch	0.33	0.60	*0.18*	*0.22*	0.35	0.92	0.40	0.39
Grass	0.57	0.43	0.32	0.42	0.54	0.27	0.61	0.70
Mugwort	0.50	0.38	0.33	0.64	0.48	*0.21*	0.55	0.61
Parietaria	0.58	0.37	0.41	0.65	0.54	*0.19*	0.60	0.70
Plane tree	0.48	0.32	0.34	0.71	0.45	*0.18*	0.53	0.65
Ragweed	0.51	0.36	0.31	0.58	0.49	*0.24*	0.56	0.68
Chenopodium	0.55	0.36	0.38	0.68	0.53	*0.18*	0.60	0.72
Cypress	0.62	0.48	0.37	0.64	0.60	0.33	0.67	0.75
Olive	0.59	0.48	0.37	0.56	0.57	0.37	0.64	0.72
Latex	0.57	0.42	0.41	0.53	0.57	*0.20*	0.62	0.73
Sesame seed	0.61	0.50	0.44	0.61	0.59	0.27	0.67	0.65
Lentil	0.60	0.41	0.43	0.80	0.60	*0.14*	0.66	0.54
Soybean	0.55	0.40	0.40	0.71	0.55	*0.20*	0.61	0.53
Peanut	0.51	0.44	0.38	0.69	0.55	0.31	0.58	0.55
Carrot	0.53	0.56	0.33	0.45	0.53	0.57	0.58	0.68
Celery	0.50	0.57	0.30	0.51	0.47	0.65	0.53	0.57
Tomato	0.56	0.38	0.37	0.75	0.51	*0.20*	0.58	0.66
Kiwi	0.52	0.48	0.32	0.68	0.50	0.42	0.58	0.56
Apple	0.36	0.44	*0.21*	0.68	0.33	0.54	0.40	0.38
Peach	0.36	0.44	*0.23*	0.64	0.32	0.58	0.42	0.41
Hazelnut	0.37	0.64	*0.23*	0.28	0.41	0.88	0.43	0.29
Walnut	0.59	0.42	0.46	0.75	0.58	*0.01*	0.58	0.44

All correlations are Spearman's *Rho* correlations.

***Italics*: NOT statistically significant after Bonferroni correction (*p*-value < 0.007 for pollen and *p*-value < 0.00025 for food/latex).**

For all other correlations, the *p*-values were smaller than the Bonferroni corrected *p* -values

TABLE S4. FOOD EXTRACT IGE LEVELS CORRELATING STRONGLY WITH WALNUT COMPONENTS

Centre	Jug r 1	Jug r 2	Jug r 2 LMW	Jug r 3	Jug r 4	Jug r 5	Jug r 6	Jug r 7
Zürich	Tomato Peanut Lentil Sesame	-	-	**Tomato** Peanut **Lentil** Soy **Sesame**	Carrot Tomato Peanut Lentil Soy Sesame	HN Peach Apple Celery	Carrot Tomato Peanut **Lentil** Soy **Sesame**	Carrot Tomato Peanut Sesame
Madrid	-	-	-	Peach	-	-	-	Carrot
Athens	-	-	-	**HN** **Peach** **Apple** **Kiwi** Tomato Celery **Peanut** **Soy** **Lentil** Sesame	-	-	-	Carrot
Utrecht				**Kiwi** Tomato **Lentil** Sesame		HN	Kiwi **Lentil**	-
Łódź	-	HN Apple Kiwi Celery **Soy** **Lentil**	-	Celery **Lentil** Soy	Peach Celery Peanut Soy **Lentil**	HN **Peach** **Apple** **Kiwi**	HN Peach Apple Kiwi **Celery** Peanut **Soy** **Lentil**	Celery
Vilnius	-	-	-	-	-	**HN** **Peach** **Apple** Celery Carrot	-	Tomato
Milan	**Kiwi** Celery Carrot Sesame	HN Sesame	-	Peach Apple	-	**HN**	Sesame	-
Strasbourg	**Lentil**	Lentil	-	-	Kiwi Peanut	HN	Lentil	

This table shows the food extracts, other than walnut, of which the IgE levels correlated strongly with IgE levels to walnut components in each centre. Only those foods with *Rho* ≥ 0.7 and *Rho* ≥ 0.8 (**bold**) are shown. Only centres with at least 10 subjects completing CRD were evaluated.

TABLE S5. IGE LEVELS RELATED TO SEVERITY OF WALNUT ALLERGY IN SUBJECTS WITH POSITIVE SEROLOGY

	Mild-to-moderate probable walnut allergy N=246			Severe probable walnut allergy N=90			
	Tested N	Positive* N (%)	IgE level Median (IQR)	Tested N	Positive* N (%)	IgE level Median (IQR)	*p*-value
Walnut extract	240	127 (52.9)	1.34 (0.73-3.84)	89	55 (61.8)	2.31 (1.02-7.77)	0.049
Jug r 1	136	14(10.3)	3.13 (0.68-32.30)	41	7 (17.1)	4.40 (1.59-13.12)	0.765
Jug r 2	135	13 (9.6)	5.31 (0.75-13.15)	40	6 (15.0)	9.44 (2.48-29.62)	0.726
Jug r 2 LMW	134	35 (26.1)	0.46 (0.39-1.66)	39	8 (20.5)	5.97 (0.47-46.21)	0.126
Jug r 3	136	23 (16.9)	1.17 (0.56-2.05)	41	5 (12.2)	1.89 (1.06-2.65)	0.529
Jug r 4	134	14 (10.4)	1.57 (0.79-3.29)	39	4 (10.3)	6.42 (2.99-15.25)	0.167
Jug r 5	135	91 (67.4)	12.99 (6.63-27.59)	40	24 (60.0)	7.92 (2.63-27.59)	0.101
Jug r 6	134	9 (6.7)	0.91 (0.41-2.67)	39	3 (7.7)	7.88 (4.18-13.92)	0.518
Jug r 7	136	38 (27.9)	3.42 (1.07-6.97)	41	9 (22.0)	2.00 (0.55-2.68)	0.176

IQR: Interquartile range

*IgE ≥ 0.35 kU_A/L

IgE levels were measured on ImmunoCAP in kU_A/L. The *p* -value pertains to the difference in IgE levels between mild-to-moderate and severe probable walnut allergy.

TABLE S6. ACCURACY OF INDIVIDUAL DIAGNOSTIC TESTS AND MODELS FOR SEVERITY OF WALNUT ALLERGY

Individual test	AUC	Positivity threshold		Mild-to-moderate	Severe	Sensitivity	95%-CI	Specificity	95%-CI	PPV	95%-CI	NPV	95%-CI
Walnut SPT	0.54 (0.44-0.65)	0.50	<	77	20	51.2	35.1-67.1	56.6	47.9-65.1	26.3	17.0-37.3	79.4	70.0-86.9
			≥	59	21								
Walnut ImmunoCAP	0.54 (0.43-0.64)	0.35	<	75	20	51.2	35.1-67.1	55.2	46.4-63.7	25.6	16.6-36.4	79.0	69.4-86.6
			≥	61	21								
		0.002	<	8	5	**87.8**	**73.8-95.9**	5.9	2.3-11.3	22.0	15.9-29.1	61.5	31.6-86.1
			≥	128	36								
		12.46	<	130	39	4.9	0.6-16.5	**95.6**	**90.6-98.4**	25.0	3.2-65.1	76.9	69.8-83.1
			≥	6	2								
Jug r 1	0.52 (0.41-0.62)	0.35	<	122	34	17.1	7.2-32.1	89.7	83.3-94.3	33.3	14.6-57.0	78.2	70.9-84.4
			≥	14	7								
		0.002	<	38	12	**70.7**	**54.5-83.9**	27.9	20.6-36.3	22.8	15.9-31.1	76.0	61.8-86.9
			≥	98	29								
		3.14	<	130	37	9.8	2.7-23.1	**95.6**	**90.6-98.4**	40.0	12.2-73.8	77.8	70.8-83.9
r Jug r 2	0.53 (0.43-0.64)	0.35	≥	6	4								
			<	122	34	15.0	5.7-29.8	90.4	84.1-94.8	31.6	12.6-56.6	78.2	70.9-84.4
			≥	13	6								
		0.005	<	7	3	**92.5**	**79.6-98.4**	5.2	2.1-10.4	22.4	16.3-29.6	70.0	34.8-93.3
			≥	128	37								
		5.31	<	129	36	10.0	2.8-23.7	**95.6**	**90.6-98.4**	40.0	12.2-73.8	78.2	71.1-84.2
			≥	6	4								
r Jug r 2 LMW	0.53 (0.42-0.63)	0.35	<	99	31	20.5	9.3-36.5	73.9	65.6-81.1	18.6	8.4-33.4	76.2	67.2-83.2
			≥	35	8								
		0.11	<	13	3	**92.3**	**79.1-98.3**	9.7	5.3-16.0	22.9	16.6-30.3	81.3	54.4-96.0
			≥	121	36								
		9.12	<	128	35	10.3	2.9-24.2	**95.5**	**90.5-98.3**	40.0	12.2-73.8	78.5	71.4-84.6
			≥	6	4								

TABLE S6. ACCURACY OF INDIVIDUAL DIAGNOSTIC TESTS AND MODELS FOR SEVERITY OF WALNUT ALLERGY (CONTINUED)

Individual test	AUC	Positivity threshold		Mild-to-moderate	Severe	Sensitivity	95%-CI	Specificity	95%-CI	PPV	95%-CI	NPV	95%-CI
r Jug r 3	0.48 (0.38-0.58)	0.35	<	113	36	12.2	4.1-26.2	83.1	75.7-89.0	17.9	6.1-36.9	75.8	68.2-82.5
			≥	23	5								
		0.006	<	17	5	**87.8**	**73.8-95.9**	12.5	7.5-19.3	23.2	16.8-20.7	77.3	54.6-92.2
			≥	119	36								
		2.01	<	130	39	4.9	0.6-16.5	**95.6**	**90.6-98.4**	25.0	3.2-65.1	76.9	69.8-83.1
			≥	6	2								
n Jug r 4	0.57 (0.46-0.68)	0.35	<	120	35	10.3	2.9-24.2	89.6	83.1-94.2	22.2	6.4-47.6	77.4	70.0-83.7
			≥	14	4								
		0.003	<	4	3	**92.3**	**79.1-98.4**	3.0	0.8-7.5	21.7	15.7-28.7	57.1	18.4-90.1
			≥	130	36								
		2.07	<	128	36	7.7	1.6-20.9	**95.5**	**90.5-98.3**	33.3	7.5-70.2	78.0	70.9-84.1
			≥	6	3								
Jug r 5	0.58 (0.49-0.68)	0.35	<	44	16	60.0	43.3-75.1	32.6	24.8-41.2	20.9	13.9-29.4	73.3	60.3-83.9
			≥	91	24								
		0.003	<	6	3	**92.5**	**79.6-98.4**	4.4	1.7-9.4	22.3	16.2-29.4	66.7	29.9-92.5
			≥	129	37								
		62.73	<	129	40	0.0	0.0-8.8	**95.6**	**90.6-98.4**	0.0	0.0-45.9	76.3	69.2-82.5
			≥	6	0								
Jug r 6	0.54 (0.44-0.65)	0.35	<	125	36	7.7	1.6-20.9	93.3	87.6-96.9	25.0	5.5-57.2	77.6	70.4-83.8
			≥	9	3								
		0.005	<	8	3	**92.3**	**79.1-98.4**	6.0	2.6-11.4	22.2	16.1-29.4	72.7	39.0-94.0
			≥	126	36								
		0.41	<	128	36	7.7	1.6-20.7	**95.5**	**90.5-98.3**	33.3	7.5-70.1	78.0	70.9-84.1
			≥	6	3								

TABLE S6. ACCURACY OF INDIVIDUAL DIAGNOSTIC TESTS AND MODELS FOR SEVERITY OF WALNUT ALLERGY (CONTINUED)

Individual test	AUC	Positivity threshold		Mild-to-moderate	Severe	Sensitivity	95%-CI	Specificity	95%-CI	PPV	95%-CI	NPV	95%-CI
Jug r 7	0.53 (0.44-0.63)	0.35	<	98	32	22.0	10.6-37.6	72.1	63.7-79.4	19.1	9.2-33.3	75.4	67.1-82.5
			≥	38	9								
		0.004	<	42	12	**70.7**	**54.5-83.9**	30.9	23.2-39.4	23.6	16.4-32.1	77.8	64.4-88.0
			≥	94	29								
		15.00	<	130	41	0.0	0.0-8.6	**95.6**	**90.6-98.4**	0.0	0.00-45.9	76.0	68.9-82.2
			≥	6	0								
Prediction model	**AUC**	**Positivity threshold**		**Mild-to-moderate**	**Severe**	**Sensitivity**	**95%-CI**	**Specificity**	**95%-CI**	**PPV**	**95%-CI**	**NPV**	**95%-CI**
Model CRD only*	0.66 (0.57-0.75)	0.50	<	128	35	5.4	0.7-18.2	100.0	97.2-100.0	100.0	15.8-100.0	78.5	71.4-84.6
			≥	0	2								
		0.16	<	33	3	**91.9**	**78.1-98.3**	25.8	18.5-34.3	26.4	19.0-34.8	91.7	77.5-98.3
			≥	95	34								
		0.30	<	123	33	10.8	3.0-25.4	**96.1**	**91.1-98.7**	44.4	13.7-78.8	78.9	71.6-85.0
			≥	5	4								
Model I	0.74 (0.65-0.83)	0.50	<	103	29	9.4	2.0-25.0	100.0	96.5-100.0	100.0	29.2-100.0	78.0	70.0-84.8
			≥	0	3								
		0.17	<	56	8	**75.0**	**56.6-88.5**	54.4	44.3-64.2	33.8	23.0-46.1	87.5	76.85-94.45
			≥	47	24								
		0.34	<	99	23	28.1	13.8-46.8	**96.1**	**90.4-98.9**	69.2	38.6-90.9	81.2	73.1-87.7
			≥	4	9								
Model II	0.74 (0.65-0.83)	0.50	<	102	25	21.9	9.3-40.0	99.0	94.7-100.0	87.5	47.4-99.7	79.5	71.5-96.8
			≥	1	7								
		0.14	<	56	7	**78.1**	**60.0-90.7**	54.4	44.3-64.2	34.7	23.9-46.9	88.9	78.4-95.4
			≥	47	25								
		0.36	<	99	23	28.1	13.8-46.8	**96.1**	**90.4-98.9**	69.2	38.6-90.9	81.2	73.1-87.7
			≥	4	9								

TABLE S6. ACCURACY OF INDIVIDUAL DIAGNOSTIC TESTS AND MODELS FOR SEVERITY OF WALNUT ALLERGY (CONTINUED)

Prediction model	AUC	Positivity threshold		Mild-to-moderate	Severe	Sensitivity	95%-CI	Specificity	95%-CI	PPV	95%-CI	NPV	95%-CI
Model III	0.81 (0.73-0.89)	0.50	<	97	23	23.3	9.9-42.3	98.0	92.9-99.8	77.8	40.0-97.2	80.8	72.6-87.4
			≥	2	7								
		0.14	<	39	3	**90.0**	**73.5-97.9**	39.4	29.7-49.7	31.0	21.6-41.9	92.9	80.5-98.5
			≥	60	27								
		0.39	<	95	14	53.3	34.3-71.7	**96.0**	**90.0-99.9**	80.0	56.3-94.8	87.2	79.4-92.8
			≥	4	16								

CRD: Component-resolved diagnostics; LMW: Low molecular weight; SPT: Skin prick test;

Measures of accuracy were calculated for each of the individual diagnostic tests, and for the models on clinic background variables (model I), clinical background variables + sensitization to walnut extract in SPT or ImmunoCAP (model II), and clinical background variables + sensitization to walnut extract + sensitization to walnut components (model III). The three rows of threshold values given for each diagnostic test respectively indicate the cut-points generally used in clinical practice, corresponding with a high sensitivity (closest to 95%), and corresponding with a high specificity (closest to 95%). **Bold** indicates the sensitivity and specificity estimates closest to 95%.

* Model including Jug r 1, 2, 3, 4, 5, 6, 7 and Ana c 2 (not Jug r 2 LMW)

5 Estimating the risk of severe peanut allergy using clinical background and IgE sensitization profiles

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Frontiers in Allergy 2021;2:19
DIO: 10.3389/falgy.2021.670789

Abstract

Background: It is not well understood why symptom severity varies between patients with peanut allergy (PA).

Objective: To gain insight into the clinical profile of subjects with mild-to-moderate and severe PA, and investigate individual and collective predictive accuracy of clinical background and IgE to peanut extract and components for PA severity.

Methods: Data on demographics, patient history and sensitization at extract and component level of 393 patients with probable PA (symptoms ≤ 2 hours + IgE sensitization) from 12 EuroPrevall centres were analyzed. Univariable and penalized multivariable regression analyses were used to evaluate risk factors and biomarkers for severity.

Results: Female sex, age at onset of PA, symptoms elicited by skin contact with peanut, family atopy, atopic dermatitis, house dust mite and latex allergy were independently associated with severe PA; birch pollen allergy with mild-to-moderate PA. The cross-validated AUC of all clinical background determinants combined (0.74) was significantly larger than the AUC of tests for sensitization to extract (0.63) or peanut components (0.54-0.64). Although larger skin prick test wheal size, and higher IgE to peanut extract, Ara h 1 and Ara h 2/6, were associated with severe PA, and higher IgE to Ara h 8 with mild-to-moderate PA, addition of these measurements of sensitization to the clinical background model did not significantly improve the AUC.

Conclusions: Models combining clinical characteristics and IgE sensitization patterns can help establish the risk of severe reactions for peanut allergic patients, but clinical background determinants are most valuable for predicting severity of probable PA in an individual patient.

Key messages

- Combining clinical background determinants and IgE sensitization patterns helps estimating the severity of an allergic reaction to peanuts
- Information from a patients clinical background are most valuable for predicting severity of peanut allergy

Introduction

Patients with peanut allergy (PA) often require strict elimination diets to prevent potentially severe allergic reactions. Beyond levels of exposure, it is not well understood why symptom severity varies between patients.[117]

To gain insight into severity of PA in a particular patient, accurate clinical evaluation is essential. Besides patient history, routine diagnostic tests include extract-based skin prick testing (SPT) and serum IgE measurements. There is conflicting evidence on the usefulness of SPT and IgE levels for predicting severity of PA.[118-121] In recent years, serum IgE testing using whole food extracts has been complemented with allergen component testing. For peanut, IgE to Ara h 2 has been demonstrated to better distinguish PA from tolerance than IgE to peanut extract.[22–25,27,36,42,122,123] Some studies have reported a relationship between IgE levels to Ara h 2 and severity of PA,[23,35–37,123] whereas other studies report no clear difference.[24,41–43] Food challenge, preferably double-blind placebo-controlled food challenge (DBPCFC), is the reference standard for confirming presence and severity of PA. However, due to the burdensome and resource-intensive nature of food challenge, daily practice diagnosis is often based on a suggestive patient history in combination with IgE sensitization (i.e. probable PA).[124]

Peanut and tree nuts are reportedly the most common causes of food-induced anaphylaxis.[117] In recent papers on hazelnut allergy[101] and walnut allergy[125], we set out to develop prediction models in which a patient's demographic and clinical background is combined with results from routine extract-based tests and from component-resolved diagnostics (CRD). For both tree nuts, models combining clinical background information with measures of IgE-sensitization were shown to improve the accuracy of predicting severe reactions significantly compared with clinical variables, IgE to extract, or IgE to allergen components alone. Although several previous studies have evaluated the predictive accuracy of combined clinical and serological information for predicting PA,[23,24,126,127] the focus is rarely on prediction of *severity*. Petterson et al. developed a model for severe PA based on clinical characteristics and serum IgE against peanut extract, but did not assess contribution of CRD, and included only children.[126]

In the present study, we evaluated data collected from predominantly adult patients reporting PA during the EuroPrevall outpatient clinic surveys in 12 different European cities,[35] using an approach comparable to that in previous evaluations for hazelnut and walnut. In a subset of these patients that underwent DBPCFC, Ballmer-Weber and colleagues previously reported that systemic reactions occurred significantly more frequently in subjects sensitized to peanut extract (IgE ≥ 0.35 kU_A/L) or to Ara h 2 (IgE ≥ 1.0 kU_A/L).[35] Our aim was to further investigate the association of demographics, clinical background, and markers of peanut sensitization, with the severity of PA, and to subsequently develop prediction models using all this information to improve discriminatory ability for estimating the risk of severe reactions.

Methods

Study design and population

Twelve European allergy centres in Athens (Greece), Łódź (Poland), Madrid (Spain), Manchester (United Kingdom), Milan (Italy), Prague (Czech Republic), Reykjavik (Iceland), Sofia, (Bulgaria), Strasbourg (France), Utrecht (The Netherlands), Vilnius (Lithuania) and Zürich (Switzerland), enrolled patients with a history of food allergy (FA) in the EuroPrevall outpatient clinic study. Each local ethical committee approved the study. Recruitment took place between 2006 and 2009. Informed consent was documented for all patients before enrollment in the study. For the current study, we included all patients reporting adverse reactions within 2 hours of ingestion of peanut.

Clinical evaluation

The methodology of the EuroPrevall outpatients study has been described in detail elsewhere.[59] All patients underwent an extensive a questionnaire, which focused on reaction characteristics and allergic comorbidities, and was administered and interpreted by trained physicians. Skin prick test (SPT) reactivity to peanut extract was assessed using a commercially available extract (ALK-Abelló, Madrid, Spain). Serum samples were collected locally in each centre, and analyzed by ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden) at the Paul-Ehrlich Institute (Langen, Germany). All available sera were tested for sensitization to peanut extract, as well as to other food and inhalant allergens.[59] A custom-made microarray chip, technically resembling the ImmunoCAP ISAC test, was used to test for sensitization to food allergen components, amongst which were peanut allergens nAra h 1 (7S globulin), nAra h 2/6 (2S albumin), nAra h 3 (11S globulin), and rAra h 8 (Pathogenesis-related protein family 10 [PR-10] protein).[59,128] DBPCFC was carried out in all consenting subjects by trained clinicians as described previously.[93]

Definitions

Patients who, along with symptoms within 2 hours of peanut ingestion, had IgE sensitization to peanut, as measured by positive SPT, ImmunoCAP or microarray, were defined as having *probable PA*. SPT allergen/histamine wheal ratios were considered positive at a ratio ≥ 0.5, IgE in ImmunoCAP at levels ≥ 0.35 kU_A/L, and IgE in microarray at levels ≥ 0.3 ISU/L.

Severity of symptoms, as determined from the physician-administered questionnaire, was classified into 2 groups: *Mild-to-moderate* if isolated oral allergy symptoms, symptoms of the skin, eyes, upper airway and/or gastrointestinal system occurred; *severe* in case of symptoms of the lower airway, cardiovascular or neurological system.[103,104] S*kin symptoms* included urticaria, angioedema, erythema/flushing, or itching; *eye symptoms* pertained to conjunctivitis; *upper airway symptoms* consisted of rhinitis, conjunctivitis, or tightness of throat; and *gastro-intestinal symptoms* included stomach pain, cramps, nausea, vomiting, diarrhoea. *Lower airway symptoms* consisted of dyspnoea, wheezing, cough, or chest tightness; *cardiovascular symptoms*

included cardiac arrhythmia, myocardial ischaemia, or hypotension; *neurological symptoms* comprised disorientation/confusion, dizziness, seizures, incontinence, or loss of consciousness. Severity was based on each participant's most severe reaction to peanut.

Patients with proven sensitization in SPT or ImmunoCAP matching their reported rhinoconjunctivitis or asthma symptoms to birch, grass, mugwort, house dust mite (HDM) or latex were considered to be allergic to the respective allergen sources.

Statistical analysis

All analyses were performed in subjects with probable PA. In univariable analysis, differences in demographic factors and clinical background (age, sex, age at onset of PA [<14 years *vs.* ≥ 14 years], symptoms upon skin contact with peanuts, first degree family members with atopy, AD [ever], allergy to pollen, HDM or latex, and sensitization to cats or dogs), results from extract-based testing (SPT and ImmunoCAP with peanut extract), and results from CRD (microarray Ara h 1, 2/6, 3 and 8), were evaluated using chi-square tests, independent sample t-tests, or Mann-Whitney U tests where appropriate. Bonferroni corrections were used to correct for multiple testing.

Multivariable analyses were performed to identify the most relevant set of predictors for severity of probable PA. To limit overfitting and improve generalizability, the Least Absolute Shrinkage and Selection Operator (Lasso) regression approach was chosen. This method selects only the most discriminative combination of variables, and applies cross-validation to shrink regression coefficients.[105] To ensure use of all data, missing data were imputed ten-fold using the *mice* package in R software version 3.2.4. Details on missing data and included covariates are available from Table S1. Lasso regression was repeated on each of the 10 datasets. Predictor variables selected in at least 7/10 imputed datasets were included. Bootstrapping was used to estimate 95% confidence intervals (CI) for each coefficient. Results were pooled using Rubin's rules.

A stepwise approach to model building was taken, and the Lasso selection process was applied in each step. In model I, all variables on demographics and clinical background were entered. In model II, peanut extract-based test results (SPT [wheal ratios] and ImmunoCAP [IgE levels]) were added to the selected model I variables. In model III, peanut CRD results were entered, along with the variables selected in model II. Finally, to explore if knowledge of IgE levels to plant source food extracts and components other than peanut could improve prediction of PA severity, ImmunoCAP and CRD results related to sensitization to soybean, lentil, hazelnut, walnut, sesame seed, peach, apple, kiwi, tomato, carrot, and celery, were entered in a final step, after fixing the variables selected in model III. The discriminatory ability of the resulting regression models to distinguish between mild-to-moderate and severe probable PA was quantified by area under the receiving operating curve (AUC) estimators. AUCs were compared using DeLong's test.[107]

For comparative purposes, Lasso regression analyses were repeated in a subgroup consisting of only subjects with clinically determined symptom severity based on DBPCFC and subjects excluded from DBPCFC because of a convincing history of severe life-threatening anaphylaxis. The latter subjects were defined as having had a reaction involving hypotension, severe bronchospasm or laryngeal edema within 2 hours of peanut ingestion, leading to emergency treatment.[127] The principal investigators in Madrid, Utrecht and Zurich reviewed these severe reactions and all agreed upon exclusion of these subjects from DBPCFC, making these patients history particularly reliable. Subjects with a negative DBPCFC outcome and placebo-reactors were grouped with the mild-to-moderate DBPCFC reactors for this subgroup analysis.

Analyses were conducted with R version 3.4.1.

Results

Of the 517 subjects reporting symptoms within 2 hours of ingestion of peanut, 393 (76%) had probable PA. Overall, 216 (55%) had mild-to-moderate and 177 (45%) had severe probable PA (Table 1, Supplemental Figure S1). Of the subjects with mild-to-moderate probable PA, 89/216 (41%) had isolated oral allergy symptoms (OAS).

Demographic and clinical characteristics associated with severity of probable PA

Frequencies of demographic and clinical background characteristics of patients with mild-to-moderate and those with severe probable PA are presented in Table 1 and Figure 1. Subjects with the severe phenotype were younger than those with the mild-to-moderate phenotype, and manifestation of probable PA more often occurred before the age of 14 years. Subjects with the severe phenotype were more likely to have symptoms elicited by skin contact with peanut, AD, HDM allergy, latex allergy or sensitization to cats and/or dogs, but less likely to be allergic to birch pollen.

Measures of IgE sensitization associated with severity of probable PA

Of subjects with probable PA, 320/387 (83%) had a positive SPT and 284/376 (76%) had a positive ImmunoCAP test to peanut extract (Table 1), and 240/370 (65%) tested positive to both tests. The allergen/histamine wheal ratios and levels of IgE to peanut extract were significantly higher in patients with severe symptoms than in patients with mild-to-moderate symptoms (Table 1 and Figure 1).

Microarray was performed in 322 of 391 (82%) subjects with probable PA, and 230/322 (71%) were sensitized to at least one peanut component. All 27 component-sensitized subjects who were not sensitized to peanut extract in SPT or ImmunoCAP, were sensitized to Ara h 8 (Table S2). Overall, sensitization to Ara h 8 was most common, and associated with mild-to-moderate probable PA (although not significantly after Bonferroni correction). Sensitization to

TABLE I. CHARACTERISTICS OF SUBJECTS WITH PROBABLE PA

	Severity classification		
	Mild-to-moderate (N=216)	**Severe (N=177)**	***p*-value**
Demographics			
Age at visit (y), mean *(±SD)*	28.2 (±14.3)	24.8 (±13.7)	**0.019**
<14 years (y), n/N(%)	30/216 (13.9)	39/177 (22.0)	**0.048**
Female sex, n/N(%)	126/216 (58.3)	106/177 (59.9)	0.835
Clinical background, n/N(%)			
Age at onset of symptoms < 14 years	86/211 (40.8)	113/174 (64.9)	**<0.001***
Symptoms upon skin contact with peanut	10/192 (5.2)	48/146 (32.9)	**<0.001***
Family history of atopic disease	131/210 (62.4)	123/176 (69.9)	0.150
Atopic dermatitis	62/212 (29.2)	89/175 (50.9)	**<0.001***
Birch pollen allergy‡	124/213 (58.2)	81/172 (47.1)	**0.038**
Grass pollen allergy‡	124/213 (58.2)	109/172 (63.4)	0.355
Mugwort pollen allergy‡	42/213 (19.7)	23/172 (13.4)	0.130
House dust mite allergy‡	98/201 (48.8)	106/160 (66.2)	**0.001**
Latex allergy‡	10/195 (5.1)	23/165 (13.9)	**0.007**
Cat/dog sensitisation ‡	146/215 (67.9)	137/175 (78.3)	**0.030**
Peanut sensitisation§			
SPT peanut extract			
Positive, n/N(%)	176/212 (83.0)	144/175 (82.3)	0.956
Allergen/histamine wheal ratio, median (IQR)	0.78 (0.57-1.00)	1.07 (0.64-1.80)	**<0.001***
ImmunoCAP peanut extract			
Positive, n/N(%)	144/209 (68.9)	140/167 (83.8)	**0.001***
IgE level, median (IQR)	0.95 (0.22-3.23)	2.21 (0.75-12.84)	**<0.001***
Microarray peanut allergens†			
Ara h 1			
Positive, n/N(%)	26/176 (14.8)	54/144 (37.5)	**<0.001***
IgE level, median (IQR)	0.00 (0.00-0.00)	0.00 (0.00-0.83)	**0.004**
Ara h 2/6			
Positive, n/N(%)	19/176 (10.8)	56/144 (38.9)	**<0.001***
IgE level, median (IQR)	0.00 (0.00-0.00)	0.00 (0.00-6.89)	**<0.001***
Ara h 3/3.02			
Positive, n/N(%)	10/176 (5.7)	43/144 (29.9)	**<0.001***
IgE level, median (IQR)	0.00 (0.00-0.00)	0.00 (0.00-0.49)	**0.001**
Ara h 8			
Positive, n/N(%)	112/176 (63.6)	67/144 (46.5)	**0.003**
IgE level, median (IQR)	0.44 (0.00-1.21)	0.12 (0.00-0.82)	0.096

IQR, interquartile range; SPT, skin prick test.

p-values indicate difference between patients with mild-to-moderate and patients with severe allergic symptoms to peanut.

Bold indicates $p < 0.05$. *Differences remained significant after Bonferroni correction.

‡Reported symptoms + matching sensitisation by SPT or ImmunoCAP.

§Not all patients had complete testing for peanut sensitisation.

†Allergen components measured by microarray in 322 patients.

Ara h 1, Ara h 2/6 or Ara h 3 was associated with severe probable PA, and IgE levels to these components were significantly higher in those with severe symptoms (Table 1 and Figure 1). Of the 179 subjects with IgE to Ara h 8 (Table 1), 48 (27%) also tested positive to Ara h 1, Ara h 2/6 or Ara h 3. Co-sensitization to storage proteins in those sensitized to Ara h 8 was associated with a more severe phenotype ($p = 0.009$).

Regarding foods other than peanut, IgE levels to extract from other legumes, soybean and lentil, were higher in subjects with severe probable PA than in those with mild-to-moderate probable PA (Supplemental Table S3). At a molecular level, subjects with severe probable PA were significantly more often sensitized to soybean Gly m 5 (7S globulin) and Gly m 6 (11S globulin), hazelnut Cor a 11 (7S globulin), walnut Jug r 2 (7S globulin), and sesame Ses i 1 (2S albumin) (Table S4). IgE levels to peach, apple and celery extract were higher in subjects with mild-to-moderate probable PA than in subjects with severe probable PA. The mild-to-moderately peanut allergic subjects were more often sensitized to PR10 proteins Gly m 4 (soybean), Cor a 1 (hazelnut), and Mal d 1 (apple).

Discriminating between mild-to-moderate and severe probable PA

The AUCs of single tests (SPT peanut extract, ImmunoCAP peanut extract, microarray peanut components) for discriminating between patients with mild-to-moderate and severe probable PA ranged from 0.54 to 0.64 (Table S5). The accuracy of SPT wheal ratio and of peanut extract and component IgE levels at specific cutpoints, are shown in Supplemental Table S6. The most discriminative model combining microarray results comprised IgE levels to Ara h 2/6 and Ara h 8, with an AUC of 0.65 (95% CI 0.63-0.66). The AUCs of our three models taking demographic and clinical factors as starting point, and combining those with markers for peanut extract and component sensitization, were significantly larger than the AUCs of the single peanut sensitization tests ($P_{\text{De Long's test}} < 0.001$) (Table 2 and Table S5).

In the first model, female sex, age at onset of PA < 14 years, symptoms elicited by skin contact with peanut, family atopy, AD, birch pollen allergy, HDM allergy, and latex allergy, were selected by Lasso regression. All determinants, except for birch pollen allergy, were associated with severe probable PA. This combination of clinical and demographic factors resulted in an AUC of 0.74 (95% CI 0.72-0.75). Lasso regression selected SPT wheal size ratio and ImmunoCAP IgE level to peanut extract (both associated with severe PA) as additionally contributing variables in model II, and IgE to Ara h 1 and Ara h 2/6 (severe) and Ara h 8 (mild-to-moderate) in model III, although AUC showed only a limited increase (Table 2). After model III, no IgE levels to foods and food components other than peanut were additionally selected to help discriminate between mild-to-moderate and severe PA.

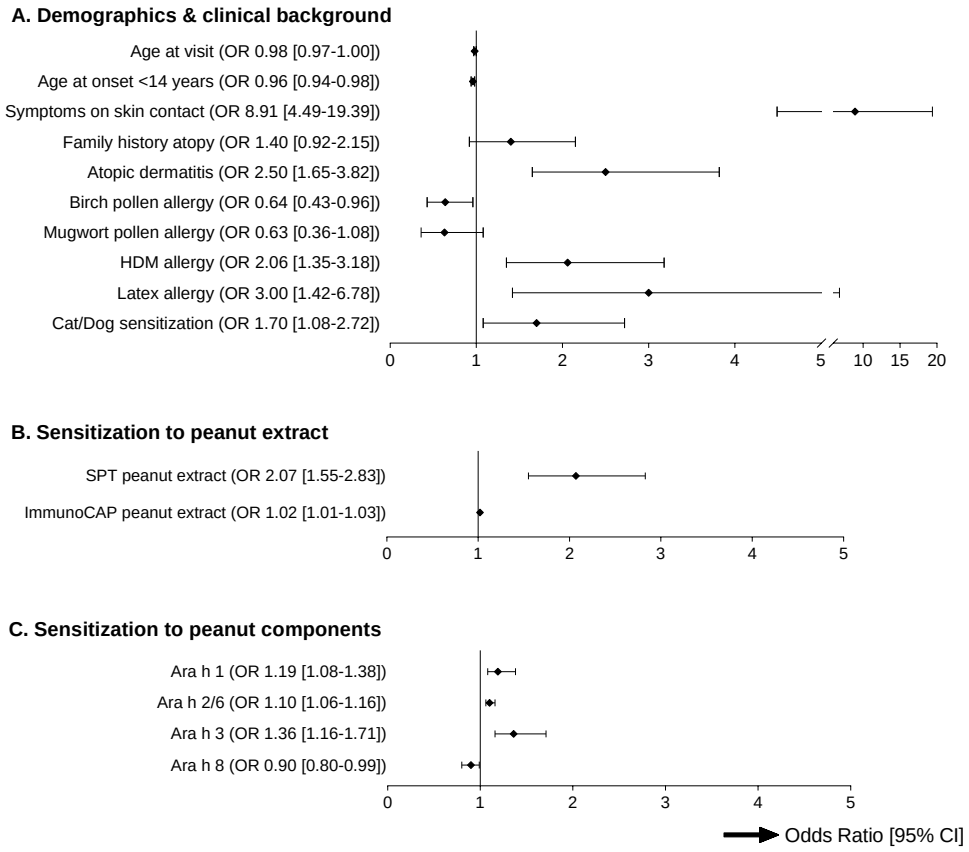

FIGURE I. Univariable Odds Ratios for prediction of severity of probable PA This forest plot shows the ORs and their respective confidence intervals from univariable analyses of all predictors for severity of probable peanut allergy with $p < 0.2$ (Table 1). All variables under B and C, and 'age at visit' were entered as continuous variables. All other variables were dichotomous.

Discriminating between mild-to-moderate and severe symptoms to peanut in subjects who underwent DBPCFC, or experienced severe life-threatening anaphylaxis

Overall, 52/393 subjects with probable PA agreed to undergo DBPCFC, of which 4 were excluded from analyses because of incomplete data. A total of 91 subjects were included in the subgroup analysis: 47 subjects with no or mild-to-moderate symptoms during DBPCFC (18 subjects with no symptoms, 22 with mild-to-moderate symptoms, 7 placebo-reactors), and 44 subjects with severe symptoms during DBPCFC (N=1) or a convincing history of severe life-threatening anaphylaxis, leading to exclusion from DBPCFC (N=43). Details on demographics, clinical variables, SPT and IgE results are available from Table S7.

Just like for probable PA, symptoms elicited by skin contact with peanut (associated with severe PA), female sex (severe), family atopy (severe), birch pollen allergy (mild-to-moderate)

TABLE 2. PREDICTION MODELS FOR SEVERITY OF PROBABLE PA

	Model I: Demographics & clinical background		Model II: Model I + sensitisation to peanut extract		Model III: Model II + sensitisation to peanut components	
	OR	**95%-CI**	**OR**	**95%-CI**	**OR**	**95%-CI**
Age at onset <14 years	1.34	0.84-2.13	1.16	0.77-1.77	1.15	0.77-1.70
Female sex	1.27	0.82-1.97	1.30	0.83-2.04	1.29	0.84-1.99
Family atopy	1.35	0.85-2.15	1.35	0.85-2.16	1.31	0.85-2.01
Atopic dermatitis	1.51	0.93-2.44	1.43	0.90-2.27	1.46	0.91-2.35
Symptoms skin contact	5.71	2.98-10.93	4.78	2.47-9.25	4.57	2.33-8.89
Birch pollen allergy	0.61	0.37-1.01	0.63	0.38-1.04	0.57	0.44-1.15
HDM allergy	1.58	0.98-2.56	1.47	0.91-2.36	1.43	0.91-2.25
Latex allergy	1.71	0.73-4.00	1.73	0.78-3.86	1.67	0.74-1.58
SPT peanut extract			1.26	0.98-1.61	1.22	0.94-1.58
IgE level peanut extract			1.01	1.00-1.01	1.00	1.00-1.01
IgE level Ara h 1					1.02	0.95-1.05
IgE level Ara h 2/6					1.01	0.98-1.04
IgE level Ara h 8					0.95	0.87-1.03
Intercept	*-1.25*		*-1.40*		*-1.36*	
AUC (95%-CI)	0.74 (0.72-0.75)		0.74 (0.73-0.76)		0.75 (0.74-0.77)	

HDM: House dust mite; SPT: Skin prick test

The area under the curve (AUC) indicates the ability of the model to discriminate between patients with mild-to-moderate and patients with severe allergic symptoms to peanuts.

and HDM allergy (severe) were selected as demographic and clinical predictors for PA in the DBPCFC/anaphylaxis subgroup, with additionally lower age at visit (mild-to-moderate) and grass pollen allergy (mild-to-moderate). IgE to peanut extract (severe) was selected in model II, but no longer in model III, where IgE to Ara h 1 (severe) and Ara h 8 (severe) were favoured. The AUC of these models ranged from 0.68 to 0.72 for discriminating between mild-to-moderate and severe PA as determined in the DBPCFC/anaphylaxis subgroup, and did not differ significantly from the AUCs of individual extract- and allergen-based tests (Supplemental Table S5).

Discussion

The current study provides insight into the clinical profiles of subjects with mild-to-moderate and severe probable PA, and quantifies the relative importance of information obtained during diagnostic work-up of PA for prediction of severity. Sex, age at onset of PA, symptoms elicited by skin contact with peanut, family atopy, AD (ever), birch pollen allergy, HDM allergy, latex allergy, peanut extract SPT wheal ratio, and IgE levels to peanut extract, Ara h 1, 2/6 and 8, were found to be independently associated with severity, of which only birch pollen allergy and

IgE to Ara h 8 were associated with a mild-to-moderate phenotype. A model combining these determinants led to optimal discrimination between mild-to-moderate and severe probable PA (cross-validated AUC 0.75), but measures of peanut sensitization contributed only limited predictive value in addition to clinical background determinants alone.

It was intriguing that some of the strongest independent predictors from clinical background associated with severe probable PA were skin-related: having symptoms elicited by skin contact with peanut, AD (ever), or latex allergy (Figure 1). Exposure to food allergens in early life via the skin has been proposed to play an important role in allergic sensitization.[52] Loss-of-function mutations in genes encoding the skin component filaggrin are related to a disrupted skin barrier, are often seen in children with AD, and are associated with IgE sensitization and allergy to foods in general, [11,12] and peanut specifically.[11,54,55,129] Little has been reported on the relationship between AD and severity of food allergic reactions, but in agreement with our findings, Van der Leek et al. also found that peanut allergic children reporting skin contact reactions to peanut were more likely to experience severe peanut allergic reactions. [130] Similarly, our prediction models developed for hazelnut and walnut allergy also contained AD (hazelnut and walnut), latex allergy (hazelnut), and symptoms elicited by skin contact (walnut) as predictors for severe reactivity.[101,125] Altogether, cutaneous sensitivity may be a marker for severe food allergy.

The only independent determinants to be associated with mild-to-moderate probable PA, were birch pollen allergy and sensitization to Ara h 8, a PR-10 protein homologous to major birch pollen allergen Bet v 1. Birch pollen-related FA is one of the most common types of plant source FA in adults in (especially Northern and Central) Europe and generally presents with mild (often isolated oral allergy) symptoms. [108,117] The frequent occurrence of this condition is reflected in our study population - 41% of subjects with mild-to-moderate PA had isolated OAS, of which 73% were sensitized to Ara h 8, making birch pollen-related PA plausible.

Interestingly, all subjects with probable PA who were not sensitized to peanut extract in SPT or ImmunoCAP, were found to be sensitized to Ara h 8 (Table S2). The peanut PR-10 protein is apparently underrepresented in peanut extract. This suggests that subjects with birch pollen related PA are not well detected with peanut extract, which partly explains why SPT wheal size and IgE level to peanut extract are associated with severe probable PA. Our findings were similar for walnut allergy, where the majority of subjects with negative extract-based tests were sensitized to walnut PR-10 protein Jug r 5.[21] In contrast, sensitization to hazelnut extract, which is spiked with hazelnut PR-10 protein Cor a 1, is more common in subjects with mild-to-moderate hazelnut allergy.[101] In the awareness that the association between extract-based testing and severity of PA was limited, these observations still underline the importance of understanding the allergen composition of food extracts for clinical interpretation of extract-based test results.

Our data showed that levels of IgE to peanut storage proteins Ara h 1, 2/6 and 3 (and also to other legumes', tree nuts' and seeds' storage proteins) were significantly higher in subjects with severe

TABLE III. PREDICTION MODELS FOR SEVERITY OF PA ACCORDING TO DBPCFC OR HISTORY OF ANAPHYLAXIS

	Model I: Demographics & clinical background		Model II: Model I + sensitisation to peanut extract		Model III: Model II + sensitisation to peanut components	
	OR	**95%-CI**	**OR**	**95%-CI**	**OR**	**95%-CI**
Age at visit	0.95	0.90-1.01	0.96	0.91-1.02	0.96	0.90-1.03
Female sex	2.37	0.69-8.14	2.43	0.62-9.57	2.64	0.34-20.77
Family atopy	5.53	1.45-21.06	4.97	1.27-19.45	5.16	1.15-23.14
Symptoms skin contact	9.93	2.22-44.39	9.00	1.83-44.33	8.69	0.97-77.97
Birch pollen allergy	0.64	0.19-2.14	0.61	0.18-2.14	0.57	0.12-2.65
Grass pollen allergy	0.39	0.09-1.63	0.40	0.09-1.76	0.43	0.08-2.28
HDM allergy	3.11	0.75-12.84	2.96	0.67-12.99	2.85	0.64-12.59
IgE level peanut extract			1.01	0.99-1.03		
IgE level Ara h 1					1.08	0.71-1.63
IgE level Ara h 8					1.06	0.75-1.48
Intercept	*-1.33*		*-1.60*		*-1.74*	
AUC (95%-CI)	0.74 (0.72-0.75)		0.74 (0.73-0.76)		0.75 (0.74-0.77)	

HDM: House dust mite; SPT: Skin prick test.
The area under the curve (AUC) indicates the ability of the model to discriminate between patients with mild-to-moderate and patients with severe allergic symptoms to peanuts.

probable PA, in accordance with several previous studies in primarily adult populations.[23,35,131,132] Of the individual tests for IgE sensitization to peanut extract or components, IgE to Ara h 2/6 had the strongest ability to discriminate between mild-to-moderate and severe probable PA, but the AUC only reached 0.64 (Table S5). This observation indicated that, although IgE levels to Ara h 1, 2/6 and 3 correlated significantly with severity, they could not be used independently to predict severity of probable PA in an individual patient. These findings were in support of those previously reported by Klemans et al, who also found that IgE to Ara h 2 was associated with severity of PA in their adult population, but could not discriminate well between mild and severe PA in individual patients, with comparable AUCs of 0.58 for severity based on patient history and 0.65 for severity based on DBPCFC.[23]

In the current study, IgE to peanut extract (in both SPT and ImmunoCAP) and to peanut storage proteins Ara h 1 and Ara h2/6, were found to contribute to an increased risk of severe probable PA in multivariable analyses. However, the negligible increase of the AUC after addition of measures of peanut IgE sensitization (in model II and III) to information from clinical background (model I), implies that clinical background is most useful for predicting severity of probable PA in an individual patient, and patient history can detect most of the variation explained by differences in IgE levels. To our knowledge, only one previous study, by Petterson

et al, assessed prediction of *severity* of PA using a combination of variables from clinical background and measures of IgE sensitization (only peanut extract), but in a pediatric population and using linear regression.[126] They conclude that reaction severity is largely unpredictable, but the differences in methodological approach prevent in-depth comparison to our study results. Some studies suggest that other laboratory predictors than taken into account in our study may also contribute to prediction of severe PA, such as epitope diversity (combined rather than isolated recognition of Ara h 1, 2 and 3),[77,133] sIgE/sIgG$_4$ ratios,[37,134] or results from the basophil activation test (BAT).[37,135] Especially the BAT has recently been explored independently and as part of multivariable approaches for prediction of PA severity in several studies. The promising results, albeit in primarily paediatric populations, suggest that the BAT may have the potential to truly enhance prediction of PA severity in the coming years.[134-138]

Other recommendations for improving prediction of severity of PA in future research, building on the findings in the current study, would be to use ImmunoCAP rather than the less sensitive microarray for measurement of component-specific IgE, and to include other potentially relevant peanut components, like profilin Ara h 5, 2S albumin Ara h 7 and lipid transfer protein (LTP) Ara h 9.[30,35,124,139] The latter is a major peanut allergen in Southern Europe and may contribute to higher predictive accuracy in those regions.[30,35]

In our population, approximately 16% of subjects with probable PA were sensitized to peach LTP Pru p 3 (see Supplemental Table S4), which is considered the primary source of LTP sensitisation.[33,110,140] A previous EuroPrevall study revealed that 73% of peanut allergic subjects with Pru p 3 sensitisation were sensitised to Ara h 9,[35] which suggests that up to 12% of the subjects with probable PA in our population may have Ara h 9 sensitisation. That said, it remains unclear whether knowledge of Ara h 9 sensitisation would contribute to prediction of PA severity, as LTP sensitisation has been linked to both mild and severe food allergy phenotypes,[141] and was not associated with systemic reactions to peanut by Ballmer-Weber et al. In accordance, we also found that sensitisation to Pru p 3 was not significantly associated with mild-to-moderate or severe PA in our population (Supplemental Table S4), nor did IgE levels to pru p 3 improve prediction of PA severity in the multivariable model. The results from the current studies are, for the largest part, based on subjects from birch-endemic areas. It is important to realize that we made the conscious decision to include subjects with likely birch-pollen related PA in our population, even though pollen-related food allergy is considered a separate clinical entity by some. Exclusion of these patients would make the clinical relevance of our findings much more limited for the average presenting outpatient population in most countries in this study. In future research, further specification of the study population to only include subjects from regions with similar pollen exposure, or only children or adults, could further refine prediction and clinical applicability of findings.

One might consider the main limitation of our study that the primary outcome measure was based on self-reported symptoms rather than symptoms during challenge testing. For this

reason, we made sure only subjects with IgE sensitization to peanut extract or components were included, and additionally explored the results of our analyses in the subgroup of subjects who underwent challenge testing or were excluded from challenge testing because of a history of severe anaphylaxis. We found it reassuring that there was considerable overlap in independent predictors. It was surprising that Ara h 8 tended to be associated with a more severe phenotype of PA in the DBPCFC/anaphylaxis group, for which we have no clear explanation other than that the subgroup may not accurately represent an unselected population of subjects with PA. We also point out that reaction severity based on self-reported symptoms may better reflect real life than reaction severity estimated by challenge, because of exclusion and stopping criteria, and the disinclination of patients who experience severe reactions to undergo or complete a burdensome challenge. As a result of the latter, dietary avoidance advice and medical prescriptions in daily practice are often decided based on clinical history and measurements of IgE sensitization, making models predicting severity of probable PA particularly interesting. We used penalized regression to prevent overfitting of our models to the population in which they were developed, but as with all prediction models, the models should still be validated in an external population.

To our knowledge, this is the first study to evaluate the individual and combined contribution of clinical background, extract-based tests, and CRD, for prediction of PA severity in a primarily adult population. The penalized regression method increases the generalizability of results, and the standardized approach facilitates comparison to similar models designed for tree nuts. Although not superimposable, clinical profiles for hazelnut and walnut displayed clear similarities. However, it was interesting to observe that measurements of IgE sensitization only contributed minimally to prediction of severity of probable PA, in contrast to the models for severity of hazelnut or walnut allergy. Clinical background determinants were clearly most valuable for predicting severity of probable PA in an individual patient. It will be interesting to validate and further expand these models in other populations to increase predictive accuracy, and to develop models according to the same approach in other food groups for comparative purposes.

Acknowledgements

We would like to thank all the patients for their participation in the study. We would like to thank ALK Abelló (Madrid, Spain) for their generous gift of SPT reagents. We acknowledge the support by the 6th and 7th Framework Programmes of the EU, for EuroPrevall (FP6-FOOD-CT-2005-514000) and iFAAM (Grant agreement no. 312147), respectively.

Supplemental files

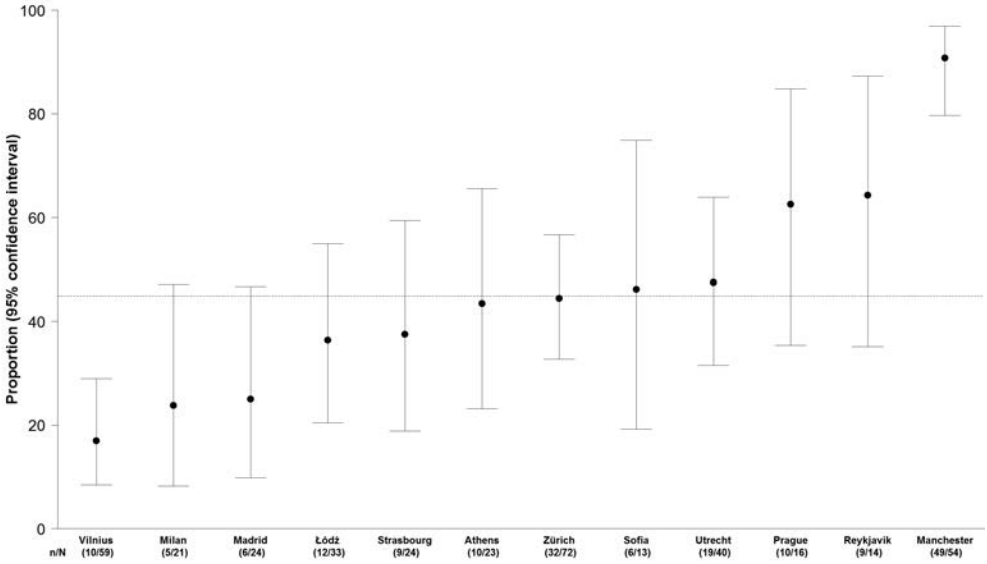

FIGURE S1. Occurrence of severe probable peanut allergy in European cities.

TABLE S1. MISSING DATA IN VARIABLES INCLUDED FOR LASSO REGRESSION

Variable	Number of missings
Age at visit	0
Sex	0
Age at onset of symptoms peanut allergy	8
Symptoms upon skin contact peanut	55
Family history of atopic disease	7
Atopic dermatitis (ever)	6
Birch pollen allergy (reported)	8
IgE birch extract	20
SPT birch extract	22
Grass pollen allergy (reported)	8
IgE grass extract	20
SPT grass extract	22
Mugwort pollen allergy (reported)	8
IgE mugwort extract	20
SPT mugwort extract	25
House dust mite allergy (reported)	32
IgE house dust mite extract	20
SPT house dust mite extract	21
Latex allergy (reported)	33
IgE latex	42
IgE cat	20
IgE dog	42
SPT peanut extract	6
IgE peanut extract (ImmunoCAP)	17
Ara h 1 (microarray)	73
Ara h 2/6 (microarray)	73
Ara h 3/3.02 (microarray)	73
Ara h 8 (microarray)	73

SPT: Skin prick test.

Total N = 393. Values for these missing data were estimated using multiple imputation procedures, for which all of the above determinants were included as covariates, along with reported symptoms (0 missings), centre, and reported allergy, SPT, ImmunoCAP and microarray results for foods other than peanut.

TABLE S2. IGE TO PEANUT COMPONENTS IN SUBJECTS WITH NEGATIVE PEANUT SPT AND IMMUNOCAP

	Negative SPT and ImmunoCAP peanut extract (N=27)	
	N microarray positive*	**IgE level, median (IQR)**
Ara h 1	2/27	0.30; 0.31
Ara h 2/6	0/27	NA
Ara h 3	0/27	NA
Ara h 8	27/27	0.51 (0.68-4.10)

NA: Not applicable, because 0 subjects sensitized

* IgE ≥ 0.3 ISU/L.

TABLE S3. SENSITIZATION TO FOOD EXTRACTS OTHER THAN PEANUT IN SUBJECTS WITH MILD-TO-MODERATE AND SEVERE PROBABLE PA

Food extract	Measurement	Mild-to-moderate (N=216)		Severe (N=177)		*p*-value
Soybean	n positive/N total, %	94/209	45.0	83/167	49.7	0.419
	IgE level median, IQR	0.25	0.05-0.79	0.35	0.07-1.54	**0.022**
Lentil	n positive/N total, %	89/208	42.8	83/168	49.4	0.240
	IgE level median, IQR	0.24	0.05-0.90	0.33	0.06-1.95	**0.020**
Hazelnut	n positive/N total, %	168/208	80.8	135/168	80.4	1.000
	IgE level median, IQR	4.74	0.73-24.89	3.55	0.64-19.53	0.065
Walnut	n positive/N total, %	91/208	43.8	76/168	45.2	0.854
	IgE level median, IQR	0.25	0.04-1.36	0.27	0.06-1.09	0.512
Sesame seed	n positive/N total, %	111/208	53.4	106/167	63.5	0.062
	IgE level median, IQR	0.40	0.15-1.40	0.71	0.21-2.08	0.461
Peach	n positive/N total, %	168/207	81.2	107/168	63.7	**<0.001***
	IgE level median, IQR	2.59	0.59-7.20	1.24	0.18-4.35	**0.006**
Apple	n positive/N total, %	147/207	71.0	104/168	61.9	0.079
	IgE level median, IQR	1.39	0.27-4.97	0.77	0.16-2.86	**0.038**
Kiwi	n positive/N total, %	109/207	52.7	95/168	56.5	0.517
	IgE level median, IQR	0.40	0.10-1.68	0.42	0.08-1.27	0.074
Tomato	n positive/N total, %	111/208	53.4	87/168	51.8	0.841
	IgE level median, IQR	0.41	0.10-1.91	0.38	0.09-1.52	0.128
Carrot	n positive/N total, %	121/208	58.2	95/168	56.5	0.832
	IgE level median, IQR	0.51	0.11-2.54	0.55	0.08-1.76	0.064
Celery	n positive/N total, %	122/208	58.7	97/168	57.7	0.941
	IgE level median, IQR	0.68	0.14-2.73	0.54	0.08-1.98	**0.043**

Sensitization was considered positive at IgE levels ≥ 0.35 kU_A/L. The *p*-value indicates the difference between mild-to-moderate and severe probable peanut allergy subjects. **Bold** indicates $p < 0.05$.

*Differences remained significant after Bonferroni correction.

TABLE S4. SENSITIZATION TO FOOD ALLERGENS OTHER THAN PEANUT ALLERGENS IN SUBJECTS WITH MILD-TO-MODERATE AND SEVERE PROBABLE PA

		Mild-to-moderate (N=216)		Severe (N=177)		
Food source	**Allergen**	**n positive/N**	**%**	**n positive/N**	**%**	***p*-value**
Soybean	Gly m 4	95/176	54.0	45/144	31.2	**<0.001***
	Gly m 5	7/176	4.0	24/144	16.7	**<0.001***
	Gly m 6	6/176	3.4	25/144	17.4	**<0.001***
Hazelnut	Cor a 1.0401	115/176	65.3	71/144	49.3	**0.005**
	Cor a 2	32/176	18.2	17/144	11.8	0.156
	Cor a 8	26/176	14.8	23/144	16.0	0.888
	Cor a 9	30/176	17.0	23/144	16.0	0.916
	Cor a 11	2/176	1.1	17/144	11.8	**<0.001***
Walnut	Jug r 2	10/176	5.7	20/144	13.9	**0.021**
	Jug r 4	2/176	1.1	7/144	4.9	0.084
Sesame seed	Ses i 1	0/176	0.0	5/144	3.5	**0.018**
	Ses i 2	1/176	0.005	5/144	3.5	0.094
	Ses i 3	35/176	19.9	24/144	16.7	0.553
Peach	Pru p 1	108/176	61.4	74/144	51.4	0.093
	Pru p 3	34/176	19.3	16/144	11.1	0.063
Apple	Mal d 1	82/176	46.6	49/144	34.0	**0.031**
	Mald 2	2/176	1.1	2/144	1.4	1.000
	Mal d 3	30/176	17.0	21/144	14.6	0.656
	Mal d 4	34/176	19.3	29/144	20.1	0.966
Kiwi	Act d 1	4/176	2.3	10/144	6.9	0.054
Tomato	Lyc e 3	22/176	12.5	15/144	10.4	0.686
Carrot	Dau c 1.0201	24/176	13.6	24/144	16.7	0.550
	Dau c 1.0103	9/176	5.1	8/144	5.6	1.000
	Dauc 4	38/176	21.6	32/144	22.2	1.000
Celery	Api g 1.01	36/176	20.5	24/144	16.7	0.472
	Api g 4	46/176	26.1	39/144	27.1	0.949
	Api g 5	37/176	21.0	26/144	18.1	0.601

Sensitization was considered positive at IgE levels ≥ 0.3 ISU/L.

The *p*-value indicates the difference between mild-to-moderate and severe probable PA subjects. **Bold** indicates $p < 0.05$.

*Differences remained significant after Bonferroni correction.

TABLE S5. AREA UNDER THE ROC-CURVE OF INDIVIDUAL AND COMBINED TESTS FOR PREDICTION OF SEVERITY OF PA

	Probable peanut allergy		DBPCFC/anaphylaxis	
Test	**AUC**	**95%-CI**	**AUC**	**95%-CI**
Peanut extract				
SPT	0.63	0.61-0.65	0.63	0.60-0.67
ImmunoCAP	0.63	0.62-0.65	0.72	0.69-0.75
Peanut allergens (microarray)				
Ara h 1	0.62	0.59-0.64	0.70	0.66-0.75
Ara h 2/6	0.64	0.61-0.66	0.70	0.60-0.81
Ara h 3/3.02	0.60	0.58-0.63	0.69	0.64-0.73
Ara h 8	0.54	0.50-0.61	0.47	0.43-0.51
CRD only*				
Ara h 2/6 & Ara h 8*	0.65	0.63-0.66	-	-
Ara h 1 & Ara h 2/6*	-	-	0.70	0.66-0.75
Models**				
Model I	0.74†	0.72-0.75†	0.68	0.65-0.72
Model II	0.74†	0.73-0.76†	0.72	0.68-0.75
Model III	0.75†	0.74-0.77†	0.71	0.67-0.74

CI: Confidence interval; DBPCFC: Double-blind placebo-controlled food challenge; SPT: Skin prick test.

The areas under the curve (AUC) and the 95% confidence intervals (95%-CI) indicate the ability to discriminate between patients with mild-to-moderate and patients with severe allergic symptoms to peanuts. AUCs for SPT, peanut extract and allergen components by microarray were averaged over the 10 imputed datasets.

*Allergens selected by Lasso regression when combining peanut allergens measured by microarray. For probable peanut allergy, the model included Ara h 2/6 and Ara h 8. For the DBPCFC group, the model included Ara h 1 and Ara h 2/6.

**As shown in Table 3.

† Significantly larger ($p < 0.001$) than the AUC of individual extract-based and allergen-based tests (De Long's test).

TABLE S6. ACCURACY OF INDIVIDUAL DIAGNOSTIC TESTS AND MODELS FOR SEVERITY OF PEANUT ALLERGY

Individual test	Positivity threshold		Mild-to-moderate	Severe	Sensitivity	95%-CI	Specificity	95%-CI	PPV	95%-CI	NPV	95%-CI
Peanut Extract SPT	**0.50**	≥	173	143	81.7	75.2-87.1	18.4	13.4-24.3	45.2	39.7-50.9	54.9	42.7-66.8
		<	39	32								
	0.29	≥	181	150	**85.1**	**79.6-90.5**	14.6	10.2-20.1	45.3	39.8-50.8	55.4	41.5-68.7
		<	31	25								
	2	≤	11	34	19.4	13.8-26.1	**94.8**	**90.9-97.4**	75.6	60.5-87.1	58.8	53.4-64.0
		>	201	141								
Peanut Extract ImmunoCAP	**0.35**	≥	144	140	83.8	77.4-89.1	31.1	24.9-37.9	49.3	43.3-55.3	70.7	60.2-79.7
		<	65	27								
	0.007	≥	182	158	**94.6**	**90.0-97.5**	12.9	8.7-18.2	46.5	41.1-51.9	75	57.8-87.9
		<	27	9								
	25.7	≥	10	33	19.8	14.0-26.6	**95.2**	**91.4-97.7**	76.7	61.4-88.2	59.8	54.3-65.1
		<	199	134								
Microarray Ara h 1	**0.30**	≥	26	54	37.5	29.6-46.0	87.5	81.7-92.0	71.0	59.5-80.9	63.1	56.7-69.2
		<	150	90								
	0.10	≥	34	63	**43.8**	**35.5-52.3**	80.7	74.1-86.2	65.0	54.6-74.4	63.7	5.0-70.0
		<	142	81								
	0.85	≥	8	36	25.0	18.2-32.9	**95.5**	**91.3-98.0**	81.8	67.3-91.8	**60.9**	**54.8-66.7**
		<	168	108								
Microarray Ara h 2/6	**0.30**	≥	19	56	38.9	30.9-47.4	89.2	83.7-93.4	74.7	63.3-84.0	64.1	57.7-70.1
		<	157	88								
	0.11	≥	19	59	**41.0**	**32.9-49.8**	89.2	83.7-93.4	75.6	64.6-84.7	64.9	58.5-70.9
		<	157	85								
	8.27	≥	8	34	23.6	16.9-31.4	**95.5**	**91.2-98.0**	81.0	65.9-91.4	60.4	54.4-66.2
		<	168	110								

TABLE S6. ACCURACY OF INDIVIDUAL DIAGNOSTIC TESTS AND MODELS FOR SEVERITY OF PEANUT ALLERGY (CONTINUED)

Individual test	Positivity threshold		Mild-to-moderate	Severe	Sensitivity	95%-CI	Specificity	95%-CI	PPV	95%-CI	NPV	95%-CI
Microarray Ara h 3	**0.30**	≥	10	43	29.9	22.5-38.0	94.3	89.8-97.2	81.1	68.0-90.6	62.2	56.1-68.0
		<	166	101								
	0.19	≥	12	47	**32.6**	**25.1-40.9**	93.2	88.4-96.4	79.7	67.2-89.0	62.8	56.7-68.7
		<	164	97								
	0.26	≥	8	33	22.9	16.3-30.6	**95.5**	**91.2-98.0**	80.5	65.1-91.2	60.2	54.2-66.0
		<	168	111								
Microarray Ara h 8	**0.30**	≥	112	67	46.5	38.2-55.0	36.4	29.3-43.9	37.4	30.3-45.0	45.4	37.0-54.0
		<	64	77								
	0.10	≥	119	86	**59.7**	**51.2-67.8**	32.4	25.5-39.8	42.0	35.1-49.0	49.6	40.1-59.0
		<	57	58								
	8.53	≥	8	1	0.7	0.3-8.0	**95.5**	**91.2-98.0**	11.1	0.3-48.2	54.0	48.3-59.7
		<	168	143								
Model	**Positivity threshold**		**Mild-to-moderate**	**Severe**	**Sensitivity**	**95%-CI**	**Specificity**	**95%-CI**	**PPV**	**95%-CI**	**NPV**	**95%-CI**
Model I	**0.23**	≥	135	110	**94.8**	**89.1-98.1**	15.1	9.9-21.6	44.9	38.6-51.4	80.0	61.4-92.3
		<	24	6								
	0.61	≥	8	41	35.3	26.7-44.8	**95.0**	**90.3-97.8**	83.7	70.3-92.7	66.8	60.3-72.9
		<	151	75								
Model II	**0.24**	≥	128	104	**95.4**	**89.6-98.5**	16.9	11.3-23.8	44.8	38.3-51.5	83.9	66.3-94.6
		<	26	5								
	0.64	≥	7	41	37.6	28.5-47.4	**95.5**	**90.7-95.2**	85.4	72.3-93.9	68.4	61.7-74.5
		<	147	68								

TABLE S6. ACCURACY OF INDIVIDUAL DIAGNOSTIC TESTS AND MODELS FOR SEVERITY OF PEANUT ALLERGY (CONTINUED)

Model	Positivity threshold		Mild-to-moderate	Severe	Sensitivity	95%-CI	Specificity	95%-CI	PPV	95%-CI	NPV	95%-CI
Model III	**0.25**	≥	132	104	**95.4**	**89.6-98.5**	14.3	9.2-20.8	44.1	37.6-50.7	81.5	61.9-93.7
		<	22	5								
	0.63	≥	7	4	36.7	27.7-46.5	**95.5**	**90.8-98.2**	85.1	71.7-93.8	68.1	61.4-74.2
		<	147	69								

CI, confidence interval; CRD, component-resolved diagnostics; NPV, negative predictive value; PPV, positive predictive value; SPT, skin prick test.

Measures of accuracy were calculated for each of the individual diagnostic tests, and for the models on clinical background variables (model I), clinical background variables + sensitization to peanut extract in SPT or ImmunoCAP (model II), and clinical background variables + sensitization to peanut extract + sensitization to peanut components (model III). The three rows of threshold values given for each diagnostic test respectively indicate the cut-offs generally used in clinical practice, corresponding with a high sensitivity (closest to 95%), and corresponding with a high specificity (closest to 95%). **Bold** indicates the sensitivity and specificity estimates closest to 95%.

TABLE S7. CHARACTERISTICS OF SUBJECTS WHO UNDERWENT DBPCFC OR HAD SEVERE ANAPHYLAXIS TO PEANUT

	No or mild-to-moderate symptoms (N=47)	Severe symptoms (N=44)	*p*-value
Demographics			
Age in years, *mean (±SD)*	26.0 (±9.9)	20.6 (±9.8)	0.013
Age < 14 years, n/N (%)	6/47 (12.8)	11/44 (25.0)	0.180
Female sex	26/47 (55.3)	28/44 (63.6)	0.553
Clinical background			
Age at onset of symptoms < 14 years	25/47 (53.2)	35/44 (79.5)	**0.015**
Symptoms upon skin contact with peanut	7/45 (15.6)	17/30 (56.7)	**<0.001***
Family history of atopic disease	22/47 (46.8)	35/44 (79.5)	**0.002**
Atopic dermatitis	15/47 (31.9)	25/44 (56.8)	0.029
Birch pollen allergy‡	16/46 (34.8)	12/43 (27.9)	0.639
Grass pollen allergy‡	27/46 (58.7)	26/43 (60.5)	1.000
Mugwort pollen allergy‡	2/46 (4.3)	6/43 (14.0)	0.149
House dust mite allergy‡	21/43 (48.8)	30/43 (69.8)	0.079
Latex allergy‡	3/43 (7.0)	4/42 (9.5)	0.713
Cat/dog sensitisation ‡	31/47 (66.0)	34/43 (79.1)	0.249
Peanut sensitisation§			
SPT peanut extract			
Positive	37/46 (80.4)	36/42 (85.7)	0.708
Allergen/histamine wheal ratio, *median (IQR)*	0.92 (0.58-1.55)	1.28 (0.92-2.13)	0.238
ImmunoCAP peanut extract			
Positive	37/47 (78.7)	39/41 (95.1)	**0.031**
IgE level, *median (IQR)*	1.33 (0.51-6.17)	5.67 (1.54-57.47)	**0.031**
Microarray peanut allergens			
Ara h 1			
Positive	11/39 (28.2)	24/40 (60.0)	**0.009**
IgE level, *median (IQR)*	0.00 (0.00-0.32)	0.60 (0.00-5.6)	0.059
Ara h 2/6			
Positive	10/39 (25.6)	25/40 (62.5)	**0.002**
IgE level, *median (IQR)*	0.00 (0.00-0.24)	6.28 (0.00-19.34)	**0.014**
Ara h 3/3.02			
Positive	6/39 (15.4)	22/40 (55.0)	**<0.001***
IgE level, *median (IQR)*	0.00 (0.00-0.00)	0.44 (0.00-2.68)	0.088
Ara h 8			
Positive	18/39 (46.2)	10/40 (25.0)	0.084
IgE level, *median (IQR)*	0.00 (0.00-0.40)	0.00 (0.00-0.29)	0.243

IQR: Interquartile range; SPT: Skin prick test.

Subjects with severe symptoms during DBPCFC (N=1) or life-threatening anaphylaxis based on patient history (N=43) were classified as severe. All measurements are in n/N (%) unless otherwise specified. *P*-values indicate difference between patients with no or mild-to-moderate and patients with severe symptoms to peanut. **Bold** indicates $p < 0.05$.

*Differences remained significant after Bonferroni correction.

‡Reported symptoms + matching sensitisation by SPT or ImmunoCAP. §Not all patients had complete testing for peanut sensitisation.

6 Ratios of specific IgG$_4$ over IgE antibodies do not improve prediction of peanut allergy nor of its severity compared to specific IgE alone

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Clinical and Experimental Allergy 2019; 49(2):216-226
DOI: 10.1111/cea.13286

Abstract

Background: $IgG_{(4)}$ antibodies have been suggested to play a protective role in the translation of peanut sensitization into peanut allergy. Whether they have added value as diagnostic read-out has not yet been reported.

Objective: To evaluate whether (1) peanut specific IgG, IgG_4 and/or IgA antibodies are associated with tolerance and/or less severe reactions, and (2) they can improve IgE-based diagnostic tests.

Methods: Sera of 137 patients with challenge-proven peanut allergy and of 25 subjects that tolerated peanut, both with known IgE profiles to peanut extract and five individual peanut allergens, were analyzed for specific IgG and IgG_4. Antibody levels and ratios thereof were associated with challenge outcome including symptom severity grades. For comparison of the discriminative performance, receiver operating characteristic curve (ROC) analysis was used.

Results: IgE against Ara h 2 was significantly higher in allergic than in tolerant patients and associated with severity of reactions ($p < 0.001$) with substantial diagnostic capability (AUC 0.91, 95%CI 0.87-0.96 and 0.80, 95%CI 0.73-0.87, respectively). IgG and IgG_4 were also positively associated albeit significantly weaker (AUCs from 0.65 to 0.72). On the other hand, ratios of IgG and IgG_4 over IgE were greater in patients that were tolerant or had mild symptoms as compared to severe patients but they did not predict challenge outcomes better than IgE alone (AUCs from 0.54-0.89).

Conclusion: IgE against Ara h 2 is the best biomarker for predicting peanut challenge outcomes including severity and IgG and IgG_4 antibody ratios over IgE do not improve these outcomes.

Key messages

- Peanut- and in particular Ara h 2-specific IgE, IgG and IgG_4 but not IgA antibody levels are higher in peanut allergic than in tolerant subjects and increase with severity of peanut allergy.
- The ratio of IgG and IgG_4 over IgE is lower in allergic than in tolerant subjects and decreases with severity of peanut allergy.
- IgG and IgG_4 antibody levels and their respective ratios over IgE did not improve distinction between allergic and tolerant or prediction of severity; IgE against Ara h 2 is the best biomarker in both cases.

Introduction

Allergic symptoms to peanut are mediated by IgE antibodies against specific components of peanut, of which Ara h 1, 2, 3 and 6 are generally considered to be the major allergens. Other components are Ara h 8 (Bet v 1 homologue) and Ara h 9 (lipid transfer protein), but sensitization to these molecules is well-established to be indirect (cross-reactivity). However, specific IgE against peanut allergens is also found in serum of subjects that tolerate peanuts. Although in tolerant but sensitized subjects IgE levels are usually lower than in peanut allergic patients, they show large overlap between both groups. Why similar IgE levels sometimes translate into tolerance and sometimes into clinical allergy is still not fully understood. In addition, it is also not clear why symptom severity varies between patients.[117]

Altogether, this limits the prognostic value of serum IgE tests and their contribution to the diagnosis of peanut allergy. Traditionally, serum IgE tests like ImmunoCAP measure IgE against whole peanut extract. With the advent of component-resolved diagnosis (CRD), the potential of serum IgE testing to distinguish between tolerance and allergy, and beyond that, to better assess the risk for severe reactions, has significantly increased. In multiple studies, IgE to Ara h 2 has been reported to perform better than extract in discriminating peanut allergic patients from tolerant sensitized subjects, both in children[22,24,25,27,36,42,142] and adults.[23] More recently, IgE against Ara h 6 has been reported to perform similarly well as Ara h 2 as biomarker for peanut allergy.[122,143-145] This is not surprising knowing that both allergens are closely related 2S albumins sharing (cross-reactive) IgE epitopes.[146] An association of IgE against Ara h 2 with symptom severity has also been reported, both in children and adults[23,35-37] as well as it being a good discriminator between mild and severe symptoms[145-147], but there are also conflicting reports.[24,41-43]

Not only IgE against peanut extract but also against Ara h 2 can be found in peanut-tolerant subjects. What tips the balance towards tolerance or (severe life-threatening) allergy? One hypothesis is that other antibody isotypes, such as IgG (or more specifically IgG_4) and possibly IgA play a protective role by functionally acting as blocking antibodies. Several mechanisms have been proposed for the protective role of blocking antibodies, the most important being the blocking of IgE-facilitated antigen presentation to T-cells by CD23-carrying antigen presenting cells (B-cells) and the blocking of allergen-induced mast cell/basophil triggering through mixed IgE/IgG_4-receptor cross-linking[46] Whether identical epitopes for IgE and IgG_4 are a prerequisite for blocking activity is still not fully understood.[148,149] Patients that outgrow a food allergy or successfully undergo immunotherapy have been shown to have increased specific IgG_4 levels.[150,151] Early introduction of peanut in children at high risk of developing food allergy showed that a lower ratio of IgG_4/IgE against peanut was associated with peanut allergy, suggesting a protective role for blocking antibodies.[47] Santos *et al.*[135] also reported that the ratio of IgG_4/IgE was significantly higher in sensitized but tolerant subjects than in those

sensitized with allergic symptoms. Song *et al*.[37] found a similar association with the outcome of a food challenge, but ratios did not correlate with symptom severity.

Altogether, these reports suggest that antibody isotypes like $IgG_{(4)}$ and possibly IgA functionally act as blocking antibodies, counteracting the symptom-inducing role of IgE antibodies. However, it has not been evaluated whether measurement of these antibodies may complement serum IgE testing to improve allergy diagnosis, on top of the improvements already achieved by the introduction of CRD.

The aim of this study was therefore to [1] explore associations between peanut extract- and component-specific IgE, IgG, IgG_4 and IgA antibodies and the outcome of peanut challenges including symptom severity grades; [2] evaluate the diagnostic accuracy of observed antibody levels and ratios thereof to discriminate between tolerant but sensitized and allergic patients as well as between patients with a mild peanut allergy and a more severe phenotype. To this end, sera of peanut sensitized tolerant and allergic subjects (n=162) were analyzed by ImmunoCAP for different isotype antibody reactivities against peanut extract, Ara h 1, Ara h 2, Ara h 3, Ara h 8 and Ara h 9.

Methods

Patient selection, peanut challenges and classification of severity (reference standard)

Data and serum from children and adults with a history of peanut allergy visiting the Allergy Center at Odense University Hospital, Denmark were consecutively collected between March 2003 and March 2009 and stored for later analyses. All subjects (or their legal representatives) signed an informed consent form. The project was approved by the Danish Data Inspectorate Board, license no. 2012-58-0018.

We included 162 sensitized subjects that had undergone a food challenge to confirm or exclude peanut allergy, as previously described[25], and of whom a blood sample was available that had been taken and stored within a year from the challenge. Twenty-five of the 162 patients were negative during their first challenges and of the remaining 137 positive, 42 were followed longitudinally with one or multiple re-challenges and matched blood samples. Six of these 42 patients later developed tolerance to peanut verified by a negative challenge. All children younger than 4 years of age and patients with compliance problems underwent OFCs (n=122). All other patients had a DBPCFC (n=40). In total, 212 challenges were performed of which 181(85.4%) were positive.

Details of the challenges and threshold doses were published elsewhere.[50] Patients were challenged with whole roasted unsalted peanuts under guidance of trained staff following the European Academy of Allergy and Clinical Immunology(EAACI) guidelines.[21] Allergic reactions during the challenge were graded according to Sampson et al.[104] as follows: oral

symptoms only (I), angioedema, generalized urticaria and/or emesis (II), rhinorrhea and/or repetitive vomiting (III), diarrhea and asthma (IV). None of the patients showed any loss of bowel control, respiratory arrest or severe bradycardia and /or hypotension (V). Primary outcomes of this study were being tolerant or have a mild positive reaction to the challenge (grade I-II) and having (more) severe symptoms (grade III and IV).

Sensitization measurements (index tests)

Blood samples were stored at -25° C for later analysis; specific and total IgE was measured by ImmunoCAP at Odense University Hospital, whereas specific IgG, IgG_4 and IgA were tested by ImmunoCAP at the Academic Medical Centre, Amsterdam, according to the manufacturer's instructions (Thermo Fisher Scientific, Uppsala, Sweden). Serum was tested for specific IgE, IgG, IgG_4 and IgA antibodies against whole peanut (extract) and peanut components rAra h 1 (7S globulin), rAra h 2 (2S albumin), rAra h 3(11S globulin), rAra h 8(Bet v 1 homologue) and rAra h 9 (lipid transfer protein).

Statistical analysis

Differences in patient characteristics and antibody serum levels were compared between tolerant and allergic subjects and between the severity of the allergic reactions (tolerant, grade I, II, III or IV). We used generalized linear mixed-effect models to adjust for patients with measurements on multiple time points. Ratios were calculated for IgG/IgE, IgG_4/IgE and IgA/IgE. All values were converted from kilo units per liter IgE (kU_A/L), micrograms per liter IgG_4 (μg/L) and milligram per liter IgG and IgA (mg/L) to nanogram per milliliter (ng/ml). Because correlation analyses were comparable when using random effect models to adjust for multiple testing, Spearman's rank correlation coefficients (*Rho*) are reported for correlations between IgE, IgG, IgG_4 and IgA antibodies and the challenge cumulative dose. *P*-values were adjusted using Bonferroni correction for multiple testing.

For comparison of the discriminative performance of all antibody isotypes and the ratio's receiver operating characteristic curve (ROC) analysis was used. We calculated the area under the ROC curve (AUC) for discriminating between tolerant and allergic patients and for discriminating between patients with mild-to-moderate (tolerant, grade I-II) and patients with severe (grade III and IV) symptoms. We compared AUCs of the different antibodies isotypes/subclasses using DeLong tests. Of the patients with multiple challenges, only the initial challenge was included in the ROC analysis.

Finally, we selected the markers that performed best according to the ROC analysis. Optimal cutoff values corresponding to the best sensitivity and specificity are data-driven and consequently prone to bias.[152] Therefore cutoff values were drawn from both a sensitivity and a specificity of 95%, respectively, or if not attainable closest to 95%. From these cutoffs the corresponding specificity or sensitivity, and positive predictive values (PPV) and negative predictive values (NPV) were calculated. We used R software version 3.2.4 for all statistical analyses.

Results

Patient characteristics

The age of the 162 patients ranged from 0.6 – 26.6 years, with a mean age of 6.5 (SD 4.4). The majority was younger than 18 years of age (157/162, 96.9%). Of the 181 positive challenges, the symptoms of 7 patients (3.9%) were classified as grade I, 56 (30.9%) as grade II, 92 (51.8%) as grade III and 26 (14.4%) as grade IV (Table 1). Overall, Ara h 2 was the most frequently recognized peanut allergen (82.1%), mainly in patients with grade II symptoms or higher (84-100%, see also Table E1 in the online repository). Of the tolerant subjects and grade I patients 35.5% and 28.6% respectively had IgE against Ara h 2 but with very low levels, i.e. geometric mean of 0.09 and 0.16 kU_A/L, respectively.

Associations of antibody isotype levels with tolerance and different severity grades

IgE levels to peanut extract were significantly higher in allergic than tolerant subjects (Figure 1A and Supplemental Table S2) and increased significantly with severity (see Figure 1B and Table S2). The same was observed for IgE against Ara h 1- 3, but not against Ara h 8 and Ara h 9. Overall, IgE responses against Ara h 2 were clearly the highest except in tolerant subjects and grade I patients (Table 1 and Figure 1). IgG antibody levels against peanut extract, Ara h 1 and Ara h 2, and IgG_4 against Ara h 2 were also significantly higher in allergic patients than tolerant subjects (Figure 2) and increased with severity (Figure 1 and Table S2). For IgA no significant associations with tolerance or symptom severity were found (Table S2). Analyses were also performed for ratios of IgG, IgG_4, IgA and total IgE over specific IgE (Figure 2, Figure 3 and Supplemental Table S3). In all four cases ratios were significantly higher in tolerant than allergic subjects for peanut extract, Ara h 1-3 but not for Ara h 8 and Ara h 9. For the same allergens, all four ratios decreased along with increasing severity of symptoms.

Finally, we analyzed whether thresholds and/or cumulative dose for objective reactions during challenge were associated with severity. Although the threshold dose for objective symptoms was not associated, there was a negative association of severity with the cumulative dose, independent from sIgE levels to peanut (Table 1). Only IgE against Ara h 2 showed significant but a weak negative correlation after Bonferroni correction with the cumulative dose (*Rho*= -0.252, $p = 0.001$).

Correlations between IgE and non-IgE antibody levels

Significant correlations of non-IgE isotypes with IgE were found for all allergens in case of IgG and IgG_4, and for peanut extract, Ara h 1- 3 for IgA (Supplemental Figures S1 and S2). The highest correlation coefficients ($p < 0.002$) were found for IgE and IgG_4 against Ara h 1 (*Rho* = 0.728), Ara h 8 (*Rho* = 0.651) and Ara h 2(*Rho* = 0.625), and for IgE and IgG against whole peanut (*Rho* = 0.683), Ara h 1 (*Rho* = 0.582) and Ara h 2 (*Rho* = 0.531).

TABLE I. PATIENT CHARACTERISTICS AND SPECIFIC IGE RESPONSES ACCORDING TO SEVERITY

	Severity					
Characteristic	**Tolerant N=31**	**Grade I N=7**	**Grade II N=56**	**Grade III N=92**	**Grade IV N=26**	***p*-value**
Sex, males, no (%)	20(64.5)	2(28.6)	32(57.1)	67(72.8)	15(57.7)	0.890
Age, mean (sd)	6.3(5.2)	10.3(2.1)	6.1(3.2)	7.4(4.3)	7.0(4.7)	0.806
LOAEL (g) median(IQR)	-	0. 1(0.1-0.1)	0.1(0.0-0.5)	0.4(0.3-2.2)	1(0.3-2.4)	0.100
Cumulative Dose (g) median (IQR)	9.90 (9.90-9.90)	11.6 (6.20-19.90)	2.0 (0.30-19.90)	0.37 (0.30-11.92)	1.01 (0.33-11.1)	**0.005**
Peanut Specific IgE (geometric mean and 95%CI, kU_A/L)						
Peanut	1.38 (0.97-1.96)	3.18 (1.66-6.1)	8.15 (4.87-13.62)	22.3 (14.7-33.85)	26.56 (13.06-54.02)	**<0.001**
Ara h 1	0.10 (0.04-0.24)	0.03 (0.00-0.32)	0.43 (0.19-0.98)	1.07 (0.51-2.22)	1.72 (0.41-7.28)	**0.002**
Ara h 2	0.09 (0.04-0.19)	0.16 (0.05-0.54)	2.90 (1.65-5.08)	11.28 (7.52-16.92)	15.28 (8.05-29.01)	**<0.001**
Ara h 3	0.04 (0.02-0.08)	0.02 (0.00-0.09)	0.10 (0.05-0.2)	0.31 (0.18-0.56)	0.41 (0.13-1.3)	**<0.001**
Ara h 8	0.10 (0.03-0.36)	0.21 (0.01-5.70)	0.11 (0.04-0.28)	0.18 (0.09-0.34)	0.14 (0.04-0.51)	0.891
Ara h 9	0.02 (0.01-0.06)	0.01 (0.00-0.07)	0.02 (0.01-0.04)	0.03 (0.02-0.04)	0.02 (0.01-0.04)	0.985
Total IgE	281.21 (180.01-439.31)	224.32 (90.75-554.52)	347.53 (237.95-507.57)	508.59 (403.24-641.46)	411.08 (258.39-653.97)	0.198

IQR: interquartile range. LOAEL: lowest observed adverse effect level for objective symptoms

Doses are expressed in gram/ peanut protein. The total number of each group represent the total number of challenges resulting in that symptom grade. All 162 patients had at least on challenge. The 42 patients with more than one challenge can be in more than on group or more than one time in the same group. *P*-values were calculated by generalized and linear mixed effect models to account for data from the same patient at different time points. Significant values are indicated in **bold**.

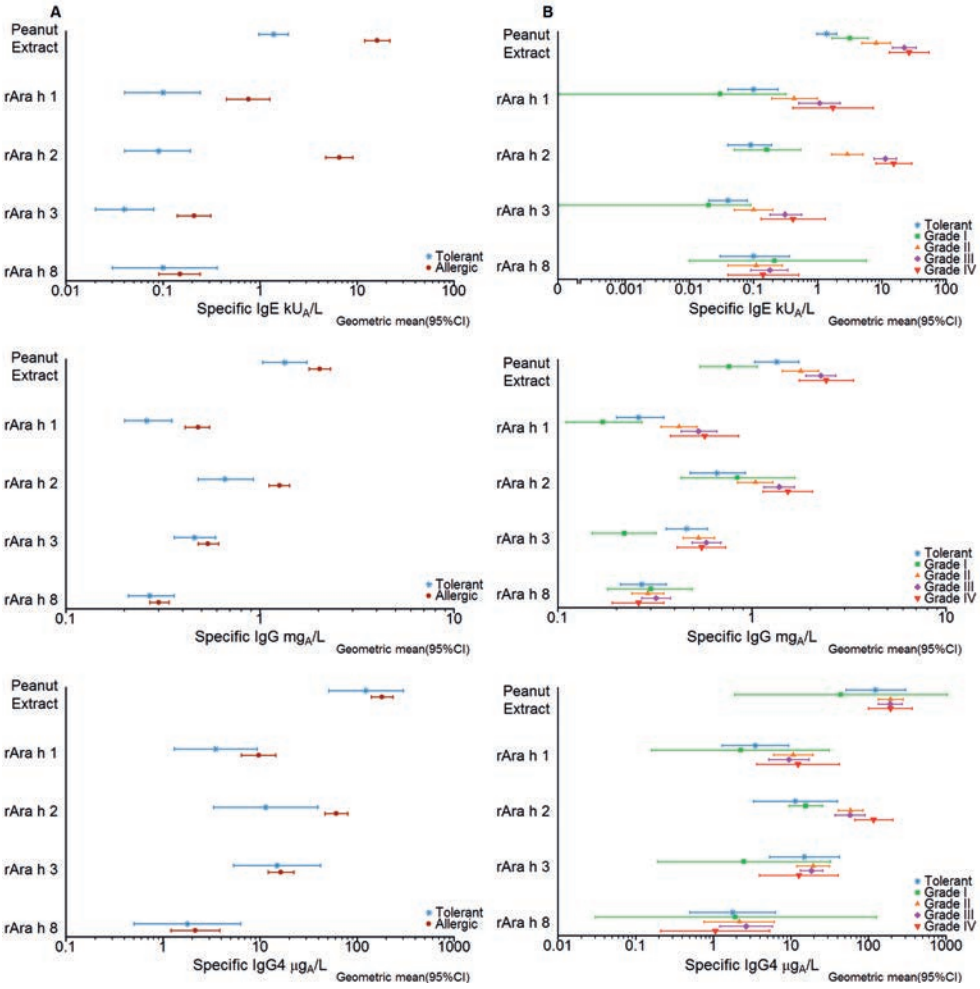

FIGURE I. Peanut specific antibody levels. Antibody levels are summarized for (A) tolerant versus allergic peanut-sensitized patients and (B) stratified for the severity of allergic reactions. The x-axis represents the serum antibody levels. The symbols and the lines indicate the geometric mean and the 95% confidence interval (CI) around that mean.

Identification of peanut allergic patients and severity of peanut allergy

To evaluate the diagnostic potential of the different allergen-specific antibody isotypes and their ratios, ROC analysis was performed. The complete results of all ROC analysis are shown in Supplemental Tables S4 and S5. To distinguish tolerant from allergic subjects, peanut-specific (AUC 0.86, 95% CI 0.79-0.92) and Ara h 2-specific IgE (AUC 0.91, 95% CI 0.87-0.96) performed significantly ($p < 0.001$) better than IgG, IgG_4 and IgA (AUC between 0.52 and 0.72) (Figure 4A and Table S4).

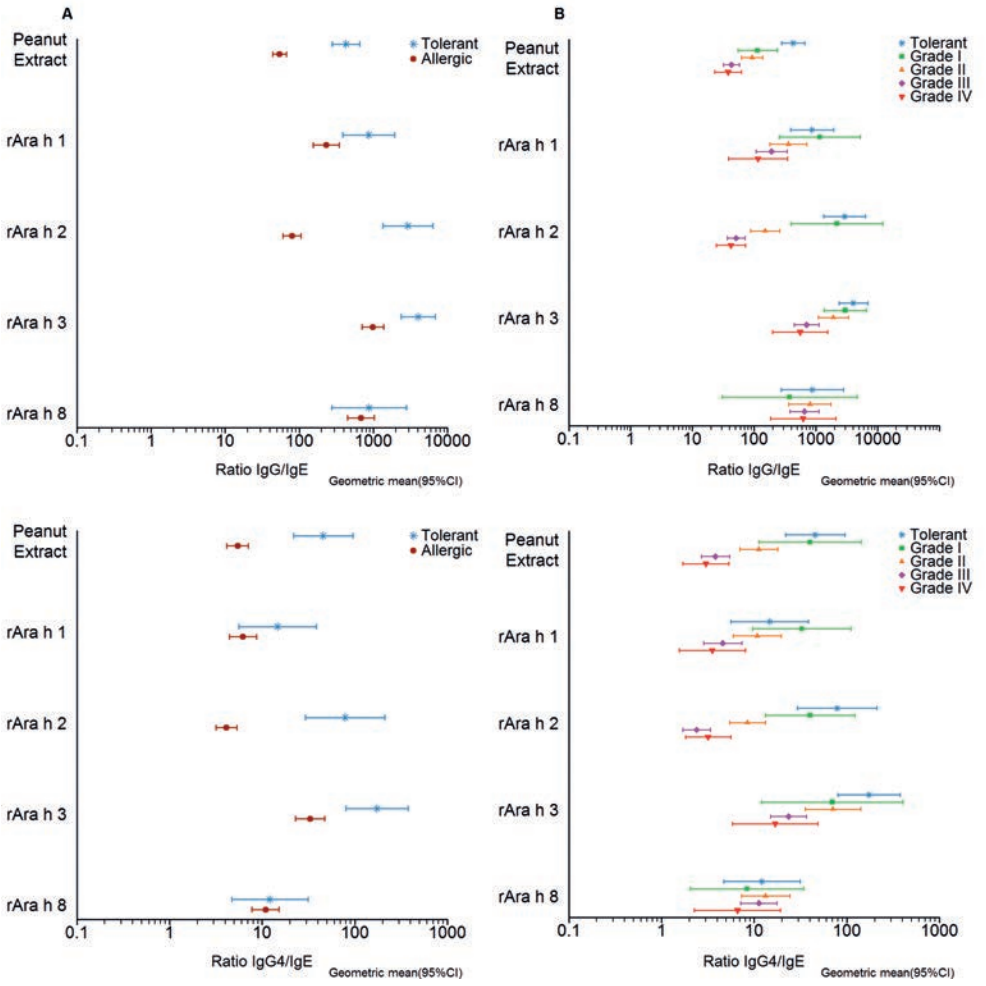

FIGURE II. Differences in peanut specific IgG/IgE and IgG4/IgE ratios. Serum $IgG_{(4)}$ antibody ratios relative to IgE in (A) tolerant versus allergic peanut-sensitized patients and (B) stratified for the severity of allergic reactions. The symbols and the lines indicate the geometric mean and the 95% confidence interval (CI) around that mean.

Similar results were found when discriminating patients with a severe peanut allergy (grades III/ IV) from those having mild to moderate symptoms (Grade I/II) or being tolerant (Figure 4B and Table S4). The AUCs were highest for IgE against Ara h 2 (0.80, 95%CI 0.73-0.87) and peanut (0.74, 95%CI 0.66-0.81). All other AUCs were ≤ 0.70. Antibody ratios did not provide a better diagnostic prognostic value compared to IgE alone (Figure 5 and Table S5). AUCs were the same or slightly lower than of IgE alone.

Thresholds for IgE and for the ratios of IgG and IgG_4 over IgE to achieve either optimal sensitivity (~95%) or optimal specificity (~95%) are summarized in Supplemental Tables S6

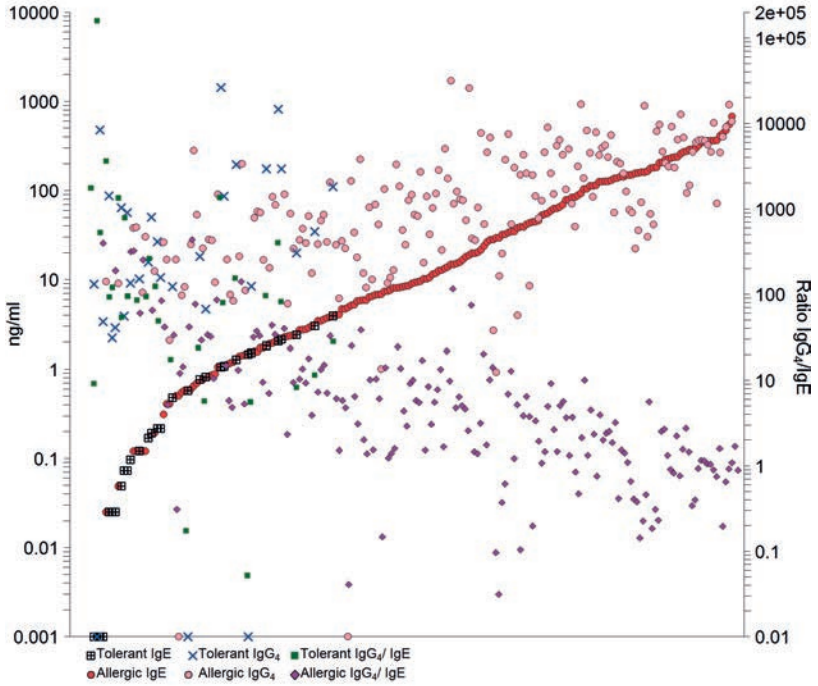

FIGURE III. Variation in IgE and IgG4 levels to Ara h 2 and in the IgG4 /IgE ratio. IgE and IgG_4 levels to Ara h 2 are displayed on the left y-axis for each patient. All results were converted to ng/ml. On the right y-axis the IgG_4/IgE ratios are given. Patients were ordered on the x-axis from those with low levels to high levels of specific IgE against Ara h 2. The red dots represent allergic subjects and black crosses tolerant. The IgG4 levels to Ara h 2 for that same patient (same location on the x-axis) are indicated as pink dots (allergic) and blue crosses (tolerant). The IgG_4/IgE ratios are indicated as green squares (tolerant) and purple diamonds(allergic).

and S7. Thresholds for peanut extract and for Ara h 2 corresponding to 95% sensitivity for discriminating between tolerance subjects and allergy patients, the specificities were low (24-52%) for IgE as well for IgG, IgG_4 and IgA ratios over IgE. At the highest attainable specificity of 92% (IgE ≥ 1.3), the sensitivity for IgE against Ara h 2 was highest (76%) and corresponded to a PPV and NPV of 98% and 41%, respectively. For the classification of severe patients, specificities were also low at a high sensitivity (~95%) and sensitivities were low (18-35%) at a high specificity (~95%).

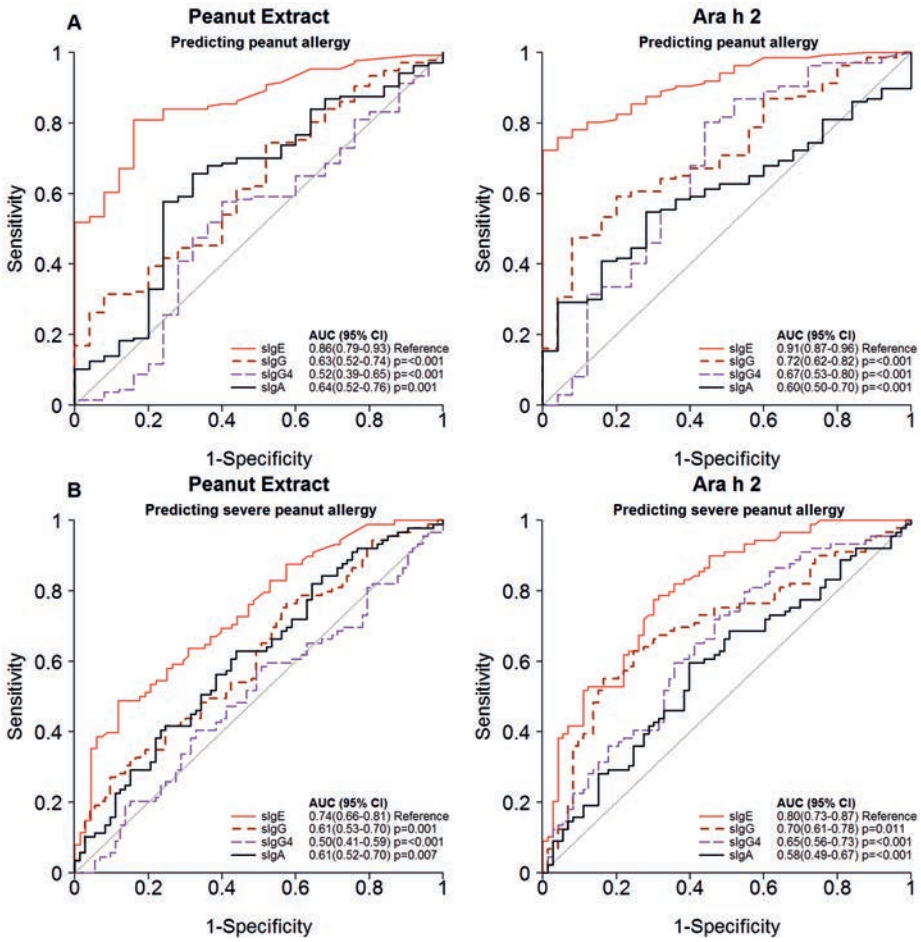

FIGURE IV. Receiver operating characteristic (ROC) curves for specific antibody levels against peanut extract based test and Ara h 2. A: Predicting the outcome of a positive peanut challenge. B: Predicting outcome of a severe peanut allergy. The *P*-values indicate the difference in performance of IgG, IgG_4 and IgA as compared to IgE.

Discussion

It has been reported earlier[37,135] and now confirmed in the present study that the ratio of peanut-specific, and in particular of Ara h 2-specific IgG_4 over IgE antibody levels is higher in subjects that tolerate peanuts than in those that are allergic to peanuts. In several studies Ara h 2-specific IgE has been demonstrated to be a better diagnostic marker to predict a positive challenge than IgE against peanut extract.[22–25,27,36,42,142,145]

We were interested to know whether ratios of specific IgG_4 over IgE could further improve diagnostic performance. By comparing a large group of patients with challenge-proven peanut

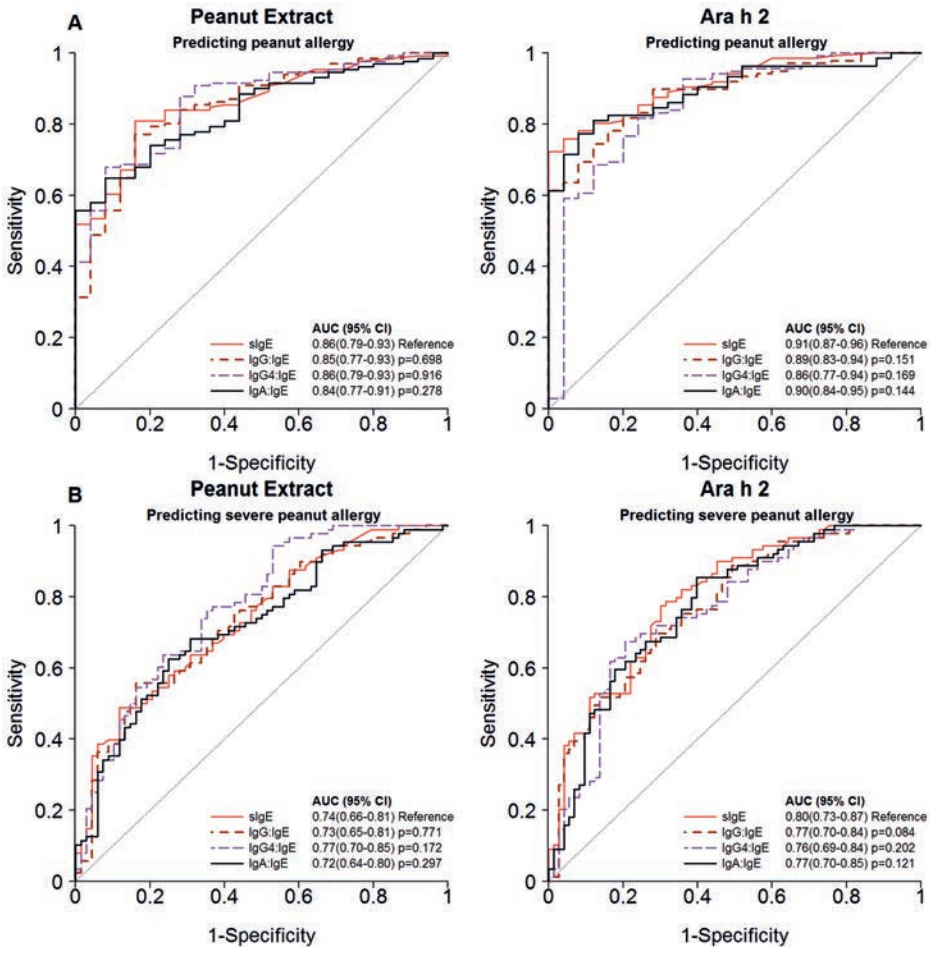

FIGURE V. Receiver operating characteristic (ROC) curves for specific ratios against peanut extract and Ara h 2. A: Predicting the outcome of a positive peanut challenge. B: Predicting outcome of a severe peanut allergy. The *p*-values indicate the difference in performance of IgG, IgG_4 and IgA as compared to IgE.

allergy to tolerant peanut-sensitized subjects, we have now demonstrated that this is not the case. In the present study, the established dominant role of Ara h 2 for peanut allergy was confirmed in group of 162 peanut sensitized allergic and tolerant children and adolescents: by adding Ara h 2-specific IgG_4 into the equation and use ratios over Ara h 2-specific IgE, the diagnostic prognostic value compared to specific IgE alone did not improve.

In line with some earlier publications[23,35–37,145,147] but opposite to some others,[24,41–43] our study found clear support for an association between sensitization to Ara h 2 and symptom severity during challenge. Conflicting results in very similarly designed studies such as

the study by Blumchen et al.[41] and the present study may perhaps be explained by differences in stop-criteria during challenge. Here we extended the present and published observations in support of an association between Ara h2-specific IgE and symptom severity to demonstrate that it is a good diagnostic discriminator between mild and severe symptoms during challenge (AUC 0.80, 95% CI 0.73-0.87).

IgE against peanut allergens is overall higher in patients reacting to peanuts than those tolerating peanuts, especially in patients with more severe symptoms, but a large overlap between groups makes it difficult to accurately discriminate them from each other. The aim of the present study was to investigate whether specific IgG, IgG_4 and/or IgA levels are related to challenge outcomes, and whether their measurement may help to improve on the predictive potential of serum IgE testing. Although still a matter of some debate, $IgG_{(4)}$ antibodies are generally thought to be (part of) the working mechanism of immunotherapy.[46] Also natural exposure to environmental or dietary allergens induces IgG_4 antibodies.[153] Recently, the LEAP intervention study[47] showed that in young children in the early introduction intervention group exposed to peanut protein, decreased development of peanut allergy was associated with increased IgG_4 levels and IgG_4/IgE ratios. The classical hypothesis is that specific $IgG_{(4)}$ antibodies play a protective role in allergic disease by blocking IgE binding to allergens. This would inhibit IgE-facilitated antigen presentation and activation of effector cells and could thus explain why some sensitized subjects do not have allergic symptoms to peanut. We observed that in patients with peanut allergy, similar to IgE, specific IgG and $IgG_{(4)}$ levels against peanut Ara h 2 were higher in allergic than tolerant subjects and increased with symptom severity. Although apparently contradicting with a protective role, higher levels of IgG4 against Ara h 2 in allergic patients have been previously described by Glaumann *et al.*[154] Both IgE and IgG_4 are part of a Th2-skewed immune response, and their production is therefore closely intertwined.25 When however expressed as ratio over IgE, a clear inverse association was observed with challenge-proven allergy and severity of symptoms. This supports a protective role of IgG_4 as was also proposed earlier in reports by Du Toit et al.[47] and Santos et al.[135]

How to explain the apparent discrepancy between a positive association of IgG_4 and allergy and symptom severity, and its proposed protective role? Overall, absolute quantities of IgG_4 are significantly higher than of IgE, both in tolerant subjects and allergic patients. However, our data show that in patients with IgE levels <100 ng/ml (< 40 kU_A/L) the IgG_4 levels are comparable. The range of IgE levels showed an approximately 50.000 fold difference between highest and lowest, this was around 5000 fold for IgG_4. This explains why the ratio of IgG_4/IgE decreased with severity while at the same time IgE and IgG_4 levels both increased with the severity of allergic reactions. The differences in ratios is greatly affected by the increase of specific IgE, which is much steeper compared to IgG_4 .

Can differences in $IgG_{(4)}$ antibodies improve the predictive accuracy compared to IgE against peanut and in particular Ara h 2? The accuracy of the IgG_4/IgE ratio in predicting the outcome of peanut challenges, with an AUC of 0.86 (95% CI 0.77-0.94), was comparable to IgE alone (0.90, 95% CI 0.87-0.96). Also for the severity of symptoms, its predictive accuracy was comparable to that of IgE alone (AUC 0.76, 95% CI 0.69-0.84 vs 0.80, 95% CI 0.73-0.87). Overall, it is clear that, although IgG_4/IgE is significantly associated with protection in a peanut challenge, in the equation specific IgE on its own is the decisive risk factor for allergy and severity. Using a cutoff of Ara h 2 > 0.6 kU_A/L to identify severe patients, we found a sensitivity of 95% and a NPV of 86.1%, thus ruling out severe peanut allergy with high certainty. On the other hand, a cutoff of 47 kU_A/L corresponded to a specificity of 94% and PPV of 90%. High specificity indicates a low false positive rate (rule in severe reactions) but the consequence is that ~50% have a negative test and need additional evaluation.

An important aspect of this study is that these results reflect the situation in a highly specialized hospital with selected patients with high likelihood of having true peanut allergy. This consequently affects the PPV and NPV, since they are highly related to the prevalence of the outcome measure. All patients that are included have positive IgE against peanut extract and this will tend to overestimate the discriminatory accuracy of peanut extract but also of the other markers.

Conclusion

In conclusion, specific IgG and IgG_4 antibody levels are higher in peanut allergic than in sensitized but tolerant subjects and levels increase with the severity of challenge-associated symptoms. Although their ratios over specific IgE are inversely associated with a positive challenge and with symptom severity, these ratios do not translate into a better predictive accuracy than with specific IgE alone. Specific IgE against Ara h 2 is the best biomarker in peanut allergy diagnosis, both to distinguish allergic from tolerant sensitized subjects and to estimate the risk for severe reactions.

Acknowledgements

We would like to thank all the patients for their participation in the study.

Supplemental files

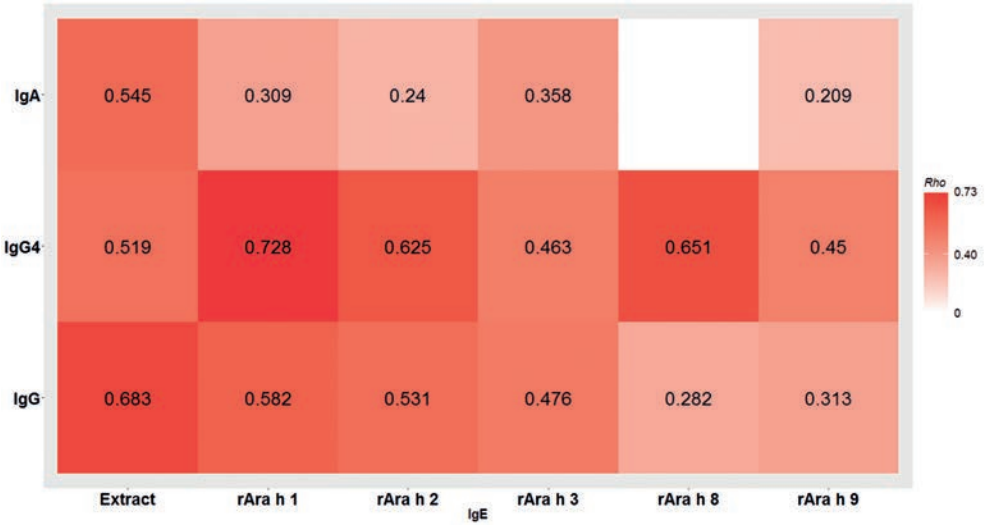

FIGURE S1. Correlations between specific IgE levels and specific levels of IgG, IgG_4 and IgA. Correlations that remained significant after bonferroni correction are displayed. The x-axis indicate IgE levels. The color indicates the strength in correlation.

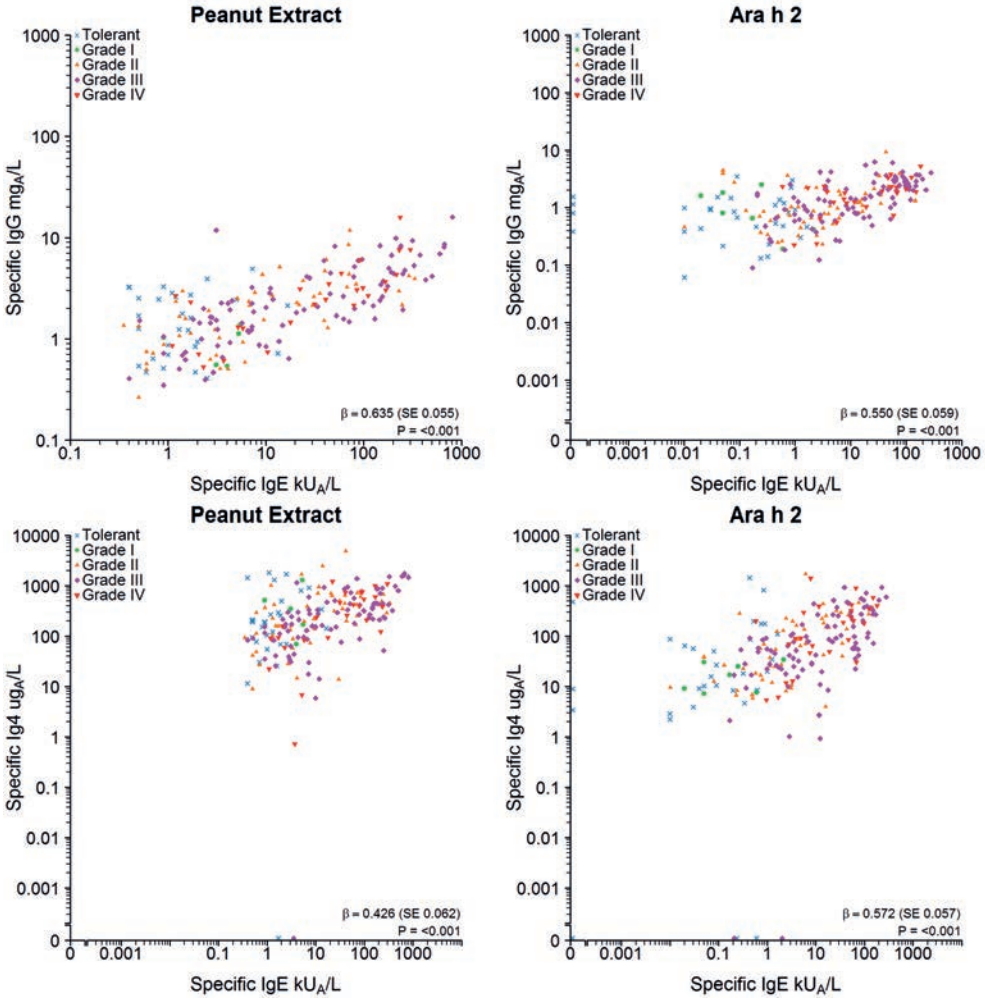

FIGURE S2. Correlations between specific IgE levels and specific levels of IgG and IgG_4 against peanut extract and Ara h 2. All values are converted to nl/ml.

TABLE S1. FREQUENCY OF PEANUT SENSITIZATION

							Severity allergy§								
Challenge results	**All**		**Tolerant**		**Allergic**		**Grade I**		**Grade II**		**Grade III**		**Grade IV**		
Peanut Sensitization*	**n/N**	**(%)**	**n/N**	**(%)**	**n/N**	**(%)**	**n/N**	**(%)**	**n/N**	**(%)**	**n/N**	**(%)**	**n/N**	**(%)**	***p*-value**
Peanut	202/202	(100)	29/29	(100)	173/173	(100)	5/5	(100)	51/51	(100)	91/91	(100)	26/26	(100)	1
Ara h 1	118/212	(55.7)	12/31	(38.7)	106/181	(58.6)	2/7	(28.6)	27/56	(48.2)	58/92	(63.0)	19/26	(73.1)	**0.038**
Ara h 2	174/212	(82.1)	11/31	(35.5)*	163/181	(90.1)	2/7	(28.6)	47/56	(83.9)	88/92	(95.7)	26/26	(100)	**<0.001**
Ara h 3	77/212	(36.3)	3/31	(9.7)*	74/181	(40.9)	0/7	(0.0)	17/56	(30.4)	41/92	(44.6)	16/26	(61.5)	**<0.001**
Ara h 8	82/212	(38.7)	12/31	(38.7)	70/181	(38.7)	3/7	(42.9)	21/56	(37.5)	36/92	(39.1)	10/26	(38.5)	0.398
Ara h 9	23/210	(11.0)	5/31	(16.1)	18/179	(10.1)	0/7	(0.0)	7/55	(12.7)	10/91	(11.0)	1/26	(3.8)	0.471

P-values show an overall difference between the tolerant patients and the 4 severity subgroups. All analyzes were adjusted for patients with follow-up data using generalized linear mixed models. Significant difference between allergic and tolerant patients: *p <0.001. Significant values are indicated in **bold**.

*IgE cutoff $\geq 0.35\ kU_A/L$

§ Missing data 1 patient

TABLE S2. SERUM ANTIBODY LEVELS

Challenge results	Tolerant N=31	Allergic N=181	Severity				*p*-value
			Grade I N=7	Grade II N=55	Grade III N=92	Grade IV N=26	
IgE (kU_A/L)							
Peanut	1.38 (0.97-1.96)***	16.08 (11.94-21.66)	3.18 (1.66-6.1)	8.15 (4.87-13.62)	22.3 (14.7-33.85)	26.56 (13.06-54.02)	**<0.001**
Ara h 1	0.10 (0.04-0.24)**	0.76 (0.45-1.26)	0.03 (0.00-0.32)	0.43 (0.19-0.98)	1.07 (0.51-2.22)	1.72 (0.41-7.28)	**0.002**
Ara h 2	0.09 (0.04-0.19)***	6.57 (4.77-9.05)	0.16 (0.05-0.54)	2.90 (1.65-5.08)	11.28 (7.52-16.92)	15.28 (8.05-29.01)	**<0.001**
Ara h 3	0.04 (0.02-0.08)***	0.21 (0.14-0.31)	0.02 (0.00-0.09)	0.10 (0.05-0.2)	0.31 (0.18-0.56)	0.41 (0.13-1.3)	**<0.001**
Ara h 8	0.10 (0.03-0.36)	0.15 (0.09-0.24)	0.21 (0.01-5.70)	0.11 (0.04-0.28)	0.18 (0.09-0.34)	0.14 (0.04-0.51)	0.891
Ara h 9	0.02 (0.01-0.06)	0.02 (0.01-0.03)	0.01 (0.00-0.07)	0.02 (0.01-0.04)	0.03 (0.02-0.04)	0.02 (0.01-0.04)	0.985
Total	281.21 (180.01-439.31)	424.79 (353.53-510.42)	224.32 (90.75-554.52)	347.53 (237.95-507.57)	508.59 (403.24-641.46)	411.08 (258.39-653.97)	0.198
IgG (mg/L)							
Peanut	1.34 (1.03-1.75)**	2.03 (1.79-2.30)	0.76 (0.54-1.06)	1.78 (1.44-2.21)	2.26(1.9-2.7)	2.42(1.75-3.33)	**0.004**
Ara h 1	0.26 (0.20-0.35)**	0.48 (0.41-0.55)	0.17 (0.11-0.27)	0.42 (0.34-0.52)	0.53(0.43-0.67)	0.57(0.38-0.85)	**0.007**
Ara h 2	0.66 (0.48-0.92)**	1.26 (1.11-1.42)	0.84 (0.43-1.66)	1.04 (0.85-1.28)	1.38(1.15-1.65)	1.53(1.14-2.05)	**0.001**
Ara h 3	0.46 (0.36-0.59)	0.54 (0.48-0.61)	0.22 (0.16-0.32)	0.53 (0.45-0.64)	0.58(0.49-0.69)	0.55(0.41-0.73)	0.107
Ara h 8	0.27 (0.21-0.36)	0.30 (0.27-0.34)	0.30 (0.18-0.49)	0.29 (0.24-0.35)	0.32(0.27-0.38)	0.26(0.19-0.35)	0.923
Ara h 9	0.38 (0.26-0.56)	0.37 (0.32-0.43)	0.33 (0.23-0.47)	0.39 (0.29-0.51)	0.39(0.32-0.48)	0.27(0.19-0.39)	0.596
IgG4 (µg/L)							
Peanut	123.55 (51.03-299.14)	181.18 (139.55-235.23)	45.42 (2.04-1009.42)	192.57 (132.99-278.85)	190.84 (133.52-272.77)	191.88 (100.37-366.82)	0.987
Ara h 1	3.48 (1.30-9.32)	9.67 (6.44-14.52)	2.26 (0.16-31.22)	10.72 (5.95-19.31)	9.46(5.20-17.19)	12.37 (3.64-42.1)	0.055
Ara h 2	11.43 (3.31-39.49)*	61.05 (46.58-80.01)	15.49 (9.45-25.39)	58.62 (40.65-84.53)	57.85 (37.08-90.26)	116.58 (66.05-205.78)	**0.022**
Ara h 3	14.96 (5.35-41.86)	16.42 (12.17-22.15)	2.46 (0.19-32.19)	19.36 (11.91-31.48)	18.45 (13.26-25.67)	12.70 (3.95-40.78)	0.476
Ara h 8	1.78 (0.50-6.29)	2.15 (1.21-3.85)	1.93 (0.03-126.78)	2.16 (0.76-6.14)	2.65 (1.21-5.79)	1.06 (0.21-5.34)	0.624
Ara h 9	2.98 (0.59-15.11)	1.81 (0.92-3.54)	2.57 (0.04-165.78)	2.86 (0.90-9.05)	1.84 (0.70-4.83)	0.57 (0.10-3.33)	0.108

TABLE S2. SERUM ANTIBODY LEVELS (CONTINUED)

			Severity				
Challenge results	**Tolerant N=31**	**Allergic N=181**	**Grade I N=7**	**Grade II N=55**	**Grade III N=92**	**Grade IV N=26**	***p*-value**
			IgA (mg/L)				
Peanut	0.26 (0.17-0.40)	0.40 (0.36-0.45)	0.32 (0.16-0.64)	0.34 (0.29-0.40)	0.44 (0.36-0.53)	0.46(0.35-0.61)	0.113
Ara h 1	0.15 (0.1-0.22)	0.18 (0.16-0.20)	0.20 (0.09-0.46)	0.16 (0.13-0.18)	0.19 (0.16-0.23)	0.18(0.15-0.22)	0.158
Ara h 2	0.24 (0.16-0.38)	0.37 (0.33-0.43)	0.59 (0.29-1.21)	0.31 (0.25-0.39)	0.39 (0.32-0.48)	0.41(0.29-0.57)	0.178
Ara h 3	0.16 (0.11-0.25)	0.20 (0.18-0.22)	0.20 (0.09-0.45)	0.18 (0.16-0.21)	0.21 (0.18-0.24)	0.2(0.17-0.24)	0.125
Ara h 8	0.15 (0.1-0.23)	0.18 (0.16-0.20)	0.27 (0.12-0.58)	0.17 (0.14-0.21)	0.18 (0.15-0.21)	0.17(0.13-0.21)	0.787
Ara h 9	0.15 (0.1-0.22)	0.17 (0.15-0.19)	0.23 (0.11-0.5)	0.16 (0.13-0.19)	0.17 (0.15-0.2)	0.16(0.13-0.21)	0.948

P-values show an overall difference between tolerant and 4 severity challenged-based outcomes. All analyzes were adjusted for patients with follow-up data using multilevel non-parametric correlation coefficients.

The * symbols indicate a significant difference between allergic and tolerant patients: *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$. Significant values are indicated in **bold**.

TABLE S3. ANTIBODY RATIOS BY SEVERITY

Challenge results	Tolerant N=31	Allergic N=181	Severity[§] Grade I N=7	Grade II N=55	Grade III N=92	Grade IV N=26	*p*-value
Peanut							
IgG:IgE	427.29 (277.98-656.8)***	54.23 (43.55-67.53)	112.95 (54.33-234.85)	92.89 (62.51-138.03)	42.68 (31.49-57.84)	37.88 (22.93-62.56)	**<0.001**
IgG4:IgE	45.47 (21.71-95.21)***	5.42 (4.15-7.06)	39.96 (11.24-142.13)	11.23 (7.02-17.96)	3.81 (2.68-5.43)	3.01 (1.7-5.33)	**<0.001**
IgA:IgE	83.69 (55.85-125.41)***	10.78 (8.34-13.94)	51.23 (17.21-152.54)	17.43 (11.16-27.21)	8.49 (5.88-12.25)	7.19 (4.09-12.64)	**<0.001**
tIGE:IgE	217.26 (137.8-342.53)***	28.86 (22.45-37.11)	97.2 (30.97-305.03)	49.87 (31.57-78.79)	23.74 (16.85-33.45)	15.48 (8.66-27.68)	**<0.001**
Ara h 1							
IgG:IgE	864.69 (387.76-1928.22)*	231.34 (154.37-346.68)	1152.07 (253.69-5231.8)	359.05 (180.18-715.48)	190.52 (106.75-340.04)	115.78 (38.46-348.52)	**0.009**
IgG4:IgE	14.7 (5.61-38.55)**	6.19 (4.42-8.67)	32.54 (9.58-110.52)	10.75 (5.92-19.55)	4.57 (2.84-7.37)	3.52 (1.55-8)	**0.006**
IgA:IgE	556.19 (255.56-1210.48)*	88.86 (56.11-140.73)	1392.07 (210.66-9198.89)	132.9 (63.35-278.81)	72.14 (37.14-140.12)	37.23 (10.6-130.8)	**0.003**
tIGE:IgE	2242.48 (1156.33-4348.85)**	494.02 (322.36-757.09)	3691.28 (471.76-28882.37)	713.9 (355.82-1432.31)	436.6 (235.95-807.89)	201.42 (63.25-641.4)	**0.001**

TABLE S3. ANTIBODY RATIOS BY SEVERITY (CONTINUED)

			Severity[§]				
Challenge results	**Tolerant N=31**	**Allergic N=181**	**Grade I N=7**	**Grade II N=55**	**Grade III N=92**	**Grade IV N=26**	***p*-value**
Ara h 2							
IgG:IgE	2913.25 (1337.69-6344.54)***	79.87 (60.21-105.95)	2183.58 (397.22-12003.44)	149.92 (87.45-257.02)	50.87 (36.65-70.61)	41.67 (24.23-71.65)	**<0.001**
IgG4:IgE	78.66 (29.32-211.03)***	4.11 (3.16-5.33)	40.13 (13.27-121.34)	8.46 (5.41-13.23)	2.39 (1.7-3.37)	3.18 (1.82-5.57)	**<0.001**
IgA:IgE	1234.32 (568.09-2681.85)***	24.07 (17.4-33.29)	1533.55 (252.36-9319.1)	44.92 (24.76-81.49)	14.93 (10.13-21.99)	11.11 (5.93-20.82)	**<0.001**
tIGE:IgE	2967.66 (1388.39-6343.33)***	64.79 (48.76-86.09)	1395.06 (249.85-7789.29)	120.38 (70.24-206.31)	45.1 (32.09-63.38)	26.91 (15.8-45.84)	**<0.001**
Ara h 3							
IgG:IgE	4015.53 (2351.38-6857.43)**	978.87 (699.38-1370.03)	2965.67 (1350.06-6514.67)	1911.77 (1083.34-3373.69)	703.44 (444.2-1113.98)	553 (196.94-1552.84)	**<0.001**
IgG4:IgE	173.06 (79.95-374.6)***	32.84 (22.94-47.01)	69.56 (12.06-401.09)	70.79 (35.48-141.22)	23.47 (15.09-36.51)	16.82 (5.8-48.73)	**<0.001**
IgA:IgE	1604.69 (900.79-2858.65)**	369.65 (256.52-532.67)	2694.26 (798.27-9093.47)	652.47 (376.44-1130.9)	265.22 (157.14-447.66)	206.16 (67.41-630.54)	**0.001**
tIGE:IgE	5867.9 (3358.61-10251.92)**	1850.71 (1360.56-2517.44)	7202.89 (3920.42-13233.68)	2996.59 (1877.28-4783.28)	1481.36 (948.13-2314.46)	999.46 (390.86-2555.68)	**<0.001**
Ara h 8							**<0.001**
IgG:IgE	872.7 (272.59-2793.9)	673.67 (444.51-1020.95)	373.47 (30.18-4621.31)	795.69 (363.12-1743.59)	652.3 (378.1-1125.36)	618.34 (182.96-2089.78)	0.829
IgG4:IgE	12.09 (4.68-31.26)	10.84 (7.75-15.16)	8.37 (2.05-34.17)	13.27 (7.26-24.25)	11.24 (7.17-17.62)	6.6 (2.26-19.22)	0.493

TABLE S3. ANTIBODY RATIOS BY SEVERITY (CONTINUED)

			Severity[§]				
Challenge results	**Tolerant N=31**	**Allergic N=181**	**Grade I N=7**	**Grade II N=55**	**Grade III N=92**	**Grade IV N=26**	***p*-value**
IgA:IgE	510.7 (145.46-1793.09)	406.78 (262.91-629.38)	333.13 (28.73-3862.58)	471.56 (212.93-1044.36)	379.98 (207.12-697.11)	397.39 (121.17-1303.28)	0.815
tIGE:IgE	2167.8 (812.97-5780.48)	2294.93 (1579.48-3334.45)	674.24 (83.09-5471.39)	2291.37 (1187.44-4421.62)	2499.56 (1470.99-4247.35)	2366.92 (880.1-6365.54)	0.964
Ara h 9							
IgG:IgE	4328.3 (2096.56-8935.71)	5150.85 (3947.22-6721.5)	5221.05 (2051.74-13285.96)	6383.69 (3724.18-10942.42)	4674.15 (3302.67-6615.16)	4579.01 (2185.85-9592.33)	0.955
IgG4:IgE	73.32 (27.72-193.95)	73.7 (50.42-107.73)	171.89 (35.41-834.31)	103.6 (52.99-202.56)	68.64 (40.65-115.9)	36.61 (11.99-111.81)	0.164
IgA:IgE	1810.75 (734-4467.08)	2381.63 (1774.44-3196.59)	3640.07 (1279.93-10352.2)	2569.44 (1341.01-4923.16)	2122.76 (1462.22-3081.68)	2706.79 (1360.53-5385.18)	0.781
tIGE:IgE	7671.86 (3331.82-17665.26)	14537.8 (11509.41-18363.03)	8509.26 (4577.97-15816.52)	13978.27 (8222.56-23762.91)	14933.15 (11116.48-20060.22)	16611.03 (10074.07-27389.75)	0.237

Values indicate the geometric mean and the 95% confidence interval around that mean. All analyzes were adjusted for patients with follow-up data using multilevel non-parametric correlation coefficients. *P*-values show an overall difference between tolerant and 4 severity challenged-based outcomes.

[§] Missing data 1 challenge.

The * symbols indicate a significant difference between allergic and tolerant challenges: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Significant values are indicated in **bold**.

TABLE S4. AREA UNDER THE CURVES FOR SPECIFIC ANTIBODIES IN PREDICTING ALLERGY AND SEVERITY

	Allergy			Severity		
	AUC	**(95% CI)**	***p*-value**	**AUC**	**(95% CI)**	***p*-value**
Peanut						
IgE	0.86	(0.79-0.93)	-	0.74	(0.66-0.81)	-
IgG	0.63	(0.52-0.74)	**<0.001**	0.61	(0.53-0.70)	**0.001**
IgG4	0.52	(0.39-0.65)	**<0.001**	0.50	(0.41-0.59)	**<0.001**
IgA	0.64	(0.52-0.76)	**0.001**	0.61	(0.52-0.70)	**0.007**
Ara h 1						
IgE	0.70	(0.61-0.78)	-	0.65	(0.57-0.74)	-
IgG	0.68	(0.57-0.79)	0.819	0.62	(0.54-0.71)	0.484
IgG4	0.66	(0.54-0.77)	0.520	0.58	(0.49-0.67)	0.047
IgA	0.58	(0.47-0.70)	0.120	0.60	(0.51-0.69)	0.347
Ara h 2						
IgE	0.91	(0.87-0.96)	-	0.80	(0.73-0.87)	-
IgG	0.72	(0.62-0.82)	**<0.001**	0.70	(0.61-0.78)	**0.011**
IgG4	0.67	(0.53-0.80)	**<0.001**	0.65	(0.56-0.73)	**<0.001**
IgA	0.60	(0.50-0.70)	**<0.001**	0.58	(0.49-0.67)	**<0.001**
Ara h 3						
IgE	0.67	(0.58-0.77)	-	0.66	(0.57-0.74)	-
IgG	0.56	(0.44-0.68)	0.097	0.57	(0.48-0.65)	0.056
IgG4	0.54	(0.41-0.68)	0.180	0.48	(0.39-0.57)	**<0.001**
IgA	0.60	(0.47-0.73)	0.289	0.58	(0.49-0.68)	0.157
Ara h 8						
IgE	0.54	(0.40-0.68)	-	0.56	(0.47-0.65)	-
IgG	0.50	(0.37-0.62)	0.665	0.52	(0.43-0.61)	0.515
IgG4	0.54	(0.43-0.65)	0.957	0.58	(0.49-0.67)	0.969
IgA	0.58	(0.45-0.07)	0.719	0.48	(0.39-0.58)	0.195
Ara h 9						
IgE	0.53	(0.40-0.66)	-	0.50	(0.41-0.59)	-
IgG	0.50	(0.37-0.63)	0.687	0.49	(0.40-0.58)	0.988
IgG4	0.54	(0.42-0.66)	0.901	0.54	(0.45-0.63)	0.403
IgA	0.46	(0.34-0.58)	0.407	0.54	(0.45-0.63)	0.568

Area Under the Curves (AUC) and the 95% confidence intervals (95% CI) that indicate the ability to discriminate between tolerant and allergic patients and between tolerant/mild and severe patient. *P*-values show the diffence between the diagnostic performance of the antibodies. Significant values are indicated in bold. The antibody with the highest AUC was used as the reference and compared with the AUCs of the other antibodies.

TABLE S5. AREA UNDER THE CURVES FOR ANTIBODY RATIOS IN PREDICTING ALLERGY AND SEVERITY

	Allergy			Severity		
	AUC	**(95% CI)**	***p*-value**	**AUC**	**(95% CI)**	***p*-value**
Peanut						
IgE	0.86	(0.79-0.93)	-	0.74	(0.66-0.81)	-
IgG: IgE	0.85	(0.77-0.93)	0.698	0.73	(0.65-0.81)	0.771
IgG4: IgE	0.86	(0.79-0.93)	0.916	0.77	(0.70-0.85)	0.172
IgA: IgE	0.84	(0.77-0.91)	0.278	0.72	(0.64-0.8)	0.297
Ara h 1						
IgE	0.70	(0.61-0.78)	-	0.65	(0.57-0.74)	-
IgG: IgE	0.66	(0.56-0.76)	**0.056**	0.63	(0.54-0.71)	0.089
IgG4: IgE	0.61	(0.48-0.74)	0.105	0.64	(0.55-0.73)	0.709
IgA: IgE	0.70	(0.61-0.78)	0.850	0.64	(0.55-0.73)	0.272
Ara h 2						
IgE	0.91	(0.87-0.96)	-	0.80	(0.73-0.87)	-
IgG: IgE	0.89	(0.83-0.94)	0.151	0.77	(0.70-0.84)	0.084
IgG4: IgE	0.86	(0.77-0.94)	0.169	0.76	(0.69-0.84)	0.202
IgA: IgE	0.90	(0.84-0.95)	0.144	0.77	(0.70-0.85)	0.121
Ara h 3						
IgE	0.67	(0.58-0.77)	-	0.66	(0.57-0.74)	-
IgG: IgE	0.67	(0.58-0.77)	0.182	0.65	(0.57-0.73)	0.722
IgG4: IgE	0.74	(0.64-0.84)	-	0.69	(0.61-0.77)	0.207
IgA: IgE	0.66	(0.56-0.76)	0.133	0.64	(0.56-0.72)	0.211
Ara h 8						
IgE	0.54	(0.40-0.68)	-	0.56	(0.47-0.65)	-
IgG: IgE	0.55	(0.41-0.69)	0.549	0.56	(0.47-0.65)	0.830
IgG4: IgE	0.54	(0.41-0.67)	0.923	0.52	(0.43-0.62)	0.390
IgA: IgE	0.54	(0.39-0.68)	0.859	0.56	(0.47-0.65)	0.958
Ara h 9						
IgE	0.53	(0.40-0.66)	-	0.50	(0.41-0.59)	-
IgG: IgE	0.56	(0.42-0.69)	0.419	0.49	(0.40-0.58)	0.797
IgG4: IgE	0.50	(0.38-0.62)	0.688	0.55	(0.46-0.64)	0.489
IgA: IgE	0.46	(0.33-0.6)	0.602	0.5	(0.41-0.59)	0.964

Area Under the Curves (AUC) and the 95% confidence intervals (95% CI) that indicate the ability to discriminate between tolerant and allergic patients and between tolerant/mild and severe patient. *P*-values show the diffence between the diagnostic performance of the antibodies. Significant *p*-values are indicated in bold. The antibody with the highest AUC was used as the reference and compared with the AUCs of the other antibodies.

TABLE S6. SENSITIVITY, SPECIFICITY, PPV AND NPV IN DISCRIMINATING BETWEEN TOLERANT AND ALLERGIC PATIENTS

Extract	Positivity threshold		Allergic	Tolerant	Sensitivity (95% CI)		Specificity (95% CI)		PPV (95% CI)		NPV (95% CI)	
sIgE	**0.9**	≥	125	16	95.4	90.3-98.3	36.0	18-57.5	88.7	82.2-93.4	60.0	32.3-83.7
		<	6	9								
	13	≥	70	2	53.4	44.5-62.2	92.0	74.0-99.0	97.2	90.3-99.7	27.4	18.2-38.2
		<	61	23								
IgG:IgE	**882**	≤	125	17	95.4	90.3-98.3	32.0	14.9-53.5	88.0	81.5-92.9	57.1	28.9-82.3
		>	6	8								
	69	≤	73	2	55.7	46.8-64.4	92.0	74.0-99.0	97.3	90.7-99.7	28.4	18.9-39.5
		>	58	23								
IgG4:IgE	**110**	≤	125	18	95.4	90.3-98.3	28.0	12.1-49.4	87.4	80.8-92.4	53.9	25.1-80.8
		>	6	7								
	12	≤	89	2	67.9	59.2-75.8	92.0	74.0-99.0	97.8	92.3-99.7	35.4	23.9-48.2
		>	42	23								
IgA:IgE	**198**	≤	125	19	95.4	90.3-98.3	24.0	9.4-45.1	86.8	80.2-91.9	50.0	21.1-78.9
		>	6	6								
	20	≤	85	2	64.9	56.1-73.0	92.0	74.0-99.0	97.7	91.9-99.7	33.3	22.4-45.7
		>	46	23								

TABLE S6. SENSITIVITY, SPECIFICITY, PPV AND NPV IN DISCRIMINATING BETWEEN TOLERANT AND ALLERGIC PATIENTS (CONTINUED)

Ara h 2	Positivity threshold		Allergic	Tolerant	Sensitivity (95% CI)		Specificity (95% CI)		PPV (95% CI)		NPV (95% CI)	
sIgE	**0.2**	≥	129	12	94.2	88.8-97.5	52.0	31.3-72.2	91.5	85.6-95.5	61.9	38.4-81.9
		<	8	13								
	1.3	≥	104	2	75.9	67.9-82.8	92.0	74.0-99.0	98.1	93.3-99.8	41.1	28.1-55.0
		<	33	23								
IgG:IgE	**3214**	≤	130	16	94.9	89.8-97.9	36.0	18.0-57.5	89.0	82.8-93.6	56.2	29.9-80.2
		>	7	9								
	183	≤	95	2	69.3	60.9-76.9	92.0	74.0-99.0	97.9	92.8-99.8	35.4	23.9-48.2
		>	42	23								
IgG4:IgE	**86.2**	≤	130	13	94.9	89.8-97.9	48.0	27.8-68.7	90.9	85.0-95.1	63.2	38.4-83.7
		>	7	12								
	5.7	≤	83	2	60.6	51.9-68.8	92.0	74.0-99.0	97.7	91.8-99.7	29.9	20.0-41.4
		>	54	23								
IgA:IgE	**7.99**	≤	130	13	94.9	89.8-97.9	48.0	27.8-68.7	90.9	85-95.1	63.2	38.4-83.7
		>	7	12								
	130	≤	106	2	77.4	69.5-84.1	92.0	74.0-99.0	98.2	93.5-99.8	42.6	29.2-56.8
		>	31	23								

Sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) were calculated at threshold value corresponding to a high sensitivity (~95%) and a high specificity (~ 95%). Thresholds for the ratios are in the opposite direction, ratios are higher in tolerant than in allergic patients.

TABLE S7: SENSITIVITY, SPECIFICITY, PPV AND NPV IN DISCRIMINATING BETWEEN MILD AND SEVERE PATIENTS

Extract	Positivity threshold		Severe	Mild	Sensitivity (95% CI)		Specificity (95% CI)		PPV (95% CI)		NPV (95% CI)	
sIgE	**1.1**	≥	84	50	95.5	88.8-98.8	26.5	16.5-38.6	62.7	53.9-70.9	81.8	59.7-94.8
		<	4	18								
	83	≥	31	4	35.2	25.3-46.1	94.1	85.6-98.4	88.6	73.3-96.8	52.9	43.6-62
		<	57	64								
IgG:IgE	**568**	≤	84	54	95.5	88.8-98.8	20.6	11.7-32.1	60.9	52.2-69.1	77.8	52.4-93.6
		>	4	14								
	16	≤	32	4	36.4	26.4-47.3	94.1	85.6-98.4	88.9	73.9-96.9	53.3	44.0-62.5
		>	56	64								
IgG4:IgE	**46**	≤	84	39	95.5	88.8-98.8	42.6	30.7-55.2	68.3	59.3-76.4	87.9	71.8-96.6
		>	4	29								
	1.1	≤	25	4	28.4	19.3-39.0	94.1	85.6-98.4	86.2	68.3-96.1	50.4	41.4-59.4
		>	63	64								
IgA:IgE	**195**	≤	84	58	95.5	88.8-98.8	14.7	7.3-25.4	59.1	50.6-67.3	71.4	41.9-91.6
		>	4	10								
	2.6	≤	27	4	30.7	21.3-41.4	94.1	85.6-98.4	87.1	70.2-96.4	51.2	42.1-60.2
		>	61	64								

TABLE S7: SENSITIVITY, SPECIFICITY, PPV AND NPV IN DISCRIMINATING BETWEEN MILD AND SEVERE PATIENTS (CONTINUED)

Ara h 2	Positivity threshold		Severe	Mild	Sensitivity (95% CI)		Specificity (95% CI)		PPV (95% CI)		NPV (95% CI)	
sIgE	**0.6**	≥	84	42	94.4	87.4-98.2	42.5	31.0-54.6	66.7	57.7-74.8	86.1	70.5-95.3
		<	5	31								
	47	≥	34	4	38.2	28.1-49.1	94.5	86.6-98.5	89.5	75.2-97.1	55.6	46.5-64.6
		<	55	69								
IgG:IgE	**658**	≤	84	45	94.4	87.4-98.2	38.4	27.2-50.5	65.1	56.2-73.3	84.8	68.1-94.9
		>	5	28								
	21	≤	33	4	37.1	27.1-48.0	94.5	86.6-98.5	89.2	74.6-97.0	55.2	46.0-64.1
		>	56	69								
IgG4:IgE	**40**	≤	84	49	94.4	87.4-98.2	32.9	22.3-44.9	63.2	54.4-71.3	82.8	64.2-94.2
		>	5	24								
	0.9	≤	21	4	23.6	15.2-33.8	94.5	86.6-98.5	84.0	63.9-95.5	50.4	41.7-59.0
		>	68	69								
IgA:IgE	**348**	≤	84	49	94.4	87.4-98.2	32.9	22.3-44.9	63.2	54.4-71.3	82.8	64.2-94.2
		>	5	24								
	2.5	≤	16	4	18.0	10.6-27.6	94.5	86.6-98.5	80.0	56.3-94.3	48.6	40.1-57.1
		>	73	69								

Sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) were calculated at threshold value corresponding to a high sensitivity (~95%) and a high specificity (~ 95%). Thresholds for the ratios are in the opposite direction, ratios are higher in tolerant/ mild than in severe patients.

7

General discussion

With the studies in this thesis, the aim was to discover sensitization patterns and clinical and demographic parameters that are associated with severe phenotypes of hazelnut, peanut and walnut allergy amongst food allergic patients across Europe, whereby improvement of model-based prediction of the risk of severe reactions to these foods was sought.

In this chapter, the results of these studies will be discussed, keeping these original aims in mind, thereby focusing on the following aspects:

PART I: providing the context of the main findings in this thesis
I. The role of birch pollen exposure on hazelnut, walnut and peanut sensitization
II. Independent factors related to severity of allergic reactions
III. Predicting severity of allergic reactions

PART II: how to translate the results to the clinical practice?
IV. Factors influencing the evaluation of IgE testing methods and prediction models
V. Using CRD and prediction models in clinical practice
VI. Recommendations for future research

PART I: PROVIDING THE CONTEXT OF THE MAIN FINDINGS IN THIS THESIS

I The role of birch pollen exposure on hazelnut and walnut sensitization

The first aim of this thesis was to uncover differences in sensitization between patient populations from different geographical areas. As shown in **Chapter 2 and 4** using Component Resolved Diagnosis (CRD), birch pollen sensitization plays a dominant role in occurrence of food allergen sensitization across Europe. Northern and Central European are known to have high birch pollen exposure levels as compared to Southern European regions.[29,108]

In our study, the variation in sensitization to hazelnut and walnut Bet v 1-homologous components followed a similar pattern as birch pollen sensitization. [29,62] Patients from cities in Northern and Central European countries mainly showed IgE against food allergens that are known to be cross-reactive with the major birch pollen allergen Bet v 1, namely hazelnut Cor a 1, walnut Jug r 5 and to a lesser extent peanut Ara h 8.[6,62,63] The lower frequency of cross-reactivity to Ara h 8 can easily be explained by the closer taxonomic relation to birch of hazelnut and walnut trees than of the legume peanut. In line with this, in particular IgE levels to Cor a 1 and Jug r 5 correlated strongly with IgE levels against Bet v 1. These observations are in contrast to those made for patients from the cities in the Mediterranean area (in our studies, from Athens and Madrid). Few patients from these regions responded to Cor a 1, Jug r 5 or Ara h 8. Instead,

sensitization to the LTPs hazelnut Cor a 8, walnut Jug r 3 and peanut Ara h 9 dominated here. The broadly accepted opinion is that peach is the primary sensitizer for food LTPs in these regions. Also, it has been proposed that the absence of birch pollen exposure may contribute to the sensitization of LTP allergens in the Mediterranean, although it is unclear what immune mechanism would underlie this.[6]

Contrasting this, only slightly higher birch pollen counts in Reykjavik compared to Athens and Madrid,[29] did not lead to increased LTP sensitization: none of the participants in Reykjavik was sensitized to LTP allergens. Despite the relatively low birch pollen exposure, patients were still mainly sensitized to hazelnut Cor a 1, suggesting that birch pollen exposure is still high enough to lead to Bet v 1 sensitization. Although peach is considered the primary source of sensitization to LTP and its consumption is likely significantly higher around the Mediterranean than in Iceland, it cannot be excluded that exposure to other pollen also contributes to LTP sensitization in Spain and Greece. Where exposure to weed pollen such as mugwort and to plane tree pollen is absent in Iceland, the pollen are common in the Mediterranean cities. Both mugwort and plan tree contain LTPs that have been shown to cross-react with fruits like peach.[8,33,78,116] Whereas around the Mediterranean, primary sensitization to LTPs from these pollen is thought to play a minor role in cross-reactivity to peach, this has been reported for Northern China, an area with very high exposure to mugwort pollen.[8] This Chinese study shows convincingly that primary mugwort LTP (Art v 3) sensitization can lead to cross-reactive reactions to peach Pru p 3, suggesting that mugwort pollen sensitization could be the starting point for associated (cross-reactive) peach allergy.

While the dominant role of birch pollen has previously been described by others, less has been reported on sensitization to seed storage proteins of tree nuts and legumes across Europe. Our studies revealed that sensitization to storage proteins does not have a distinct geographical pattern for both hazelnut and walnut. Additional analysis of the EuroPrevall study (data not published) and previously published data[35] showed that this is also the case for sensitization to peanut storage proteins. Overall, storage protein sensitization was more frequently observed in patients with probable peanut allergy that in those with both tree nut allergies (24% vs 10%).

Storage protein sensitization is not associated with exposure to pollen, but is thought to be primary sensitization, usually already occurring in childhood. Birch pollen sensitization leading to cross-sensitization and food allergy commonly occurs later in life, but surpasses the frequency of storage protein sensitization in adults by far. In the EuroPrevall outpatient clinic surveys, the majority of patients that reported allergic reactions to hazelnut, walnut or peanut were adults. Only 5-14% of the adult patients were sensitized to storage proteins compared to >35-40% in children (<18 years).

In conclusion, CRD has helped revealing geographical differences in sensitization patterns that proved to be useful to understand the origins and prevalence of specific food allergies such as to hazelnut, walnut and peanut. Additionally, CRD can help to more accurately identify patients that are sensitized and possibly allergic to these foods. In diagnostic food extracts, classically used to measure sensitization, important allergens can be underrepresented. This is particularly true for Bet v 1 homologues which are labile and present in low quantities. In **Chapter 4**, this is clearly demonstrated for walnut extract on ImmunoCAP. Most patients that were sensitized to Jug r 5 did not respond to the walnut extract indicating that testing for IgE against Jug r 5 will significantly improve sensitivity to detect walnut sensitization in birch endemic areas. Additionally, around 30% of the patient with IgE against Ara h 8 tested negative to peanut extract by ImmunoCap. The reason that this problem does not occur in case of hazelnut is that the hazelnut ImmunoCAP was spiked by the manufacturer with recombinant Cor a 1 to increase sensitivity. The performance of single allergens in allergy diagnosis is further discussed in *Paragraph III* of this chapter.

II Independent factors related to severity of hazelnut, walnut and peanut allergy

What determines the severity of allergic reactions to foods? Several factors were found to be independently associated to the severity of reactions to hazelnut, walnut and peanut. The most notable associations were observed for storage proteins and skin-related atopic diseases reported in **Chapters 3, 4 and 5**.

Storage proteins

Positive testing to storage proteins in hazelnut and peanut, but not walnut, consistently showed a positive association with the severity of symptoms induced by that food. However, the levels of specific IgE against storage proteins were not always clearly associated with symptom severity. In contrast to storage proteins, sensitization to pollen-related allergens, hazelnut Cor a 1 and Cor a 2, peanut Ara h 8 and walnut Jug r 5 and Jug r 7 was associated with a mild allergy phenotype.

Sensitization to the peanut storage protein Ara h 2 (2S albumin) has frequently been reported to be positively associated with the severity of symptoms, [23,35–37] although a lack of this association has been reported in some studies that included only allergic children.[24,41–43] We found a positive relation between peanut Ara h 2 and symptom severity in adult patients (**Chapter 5**), and also in children (**Chapter 6**). Children with suspected peanut allergy from **Chapter 6** all underwent a challenge to confirm peanut allergy. Differences in stop criteria used during challenge in our study and the other reported studies might have affected the outcome with respect to severity of symptoms. Additionally, differences in patient selection can alter the outcomes. *Paragraph III* in this chapter will discuss the influence of patient selection and study methodology in more detail.

For hazelnut Cor a 9 and Cor a 14, associations with symptom severity were quite strong in our study, especially for severity scored during food-challenge (ORs of around 10). We could not

confirm the relation between walnut storage proteins and severity of reactions as reported by others. The relation between walnut storage protein sensitization and severity of reactions has been described in children.[40] That does not seem to hold true in our study, possibly because our population consisted of mostly adults. Nevertheless, another study[102] did find a relation between severity and Jug r 1 sensitization in adults, but their classification of severity differed from ours. Additionally, the number of patients positive to walnut storage in our study was quite low (< 20), thereby lacking statistical power with a larger risk of false negative results. The impact of differences in classification methods and frequency of positive tests are further discussed in *Paragraph III* in this chapter.

Skin-related atopic diseases

Our data showed that the (past) presence of skin related atopic diseases was the strongest independent predictor for severity of hazelnut, walnut and peanut allergy. This included reporting having (ever had) atopic dermatitis (AD), having probable latex allergy and reporting skin contact with the food as trigger for adverse reactions. The latter was most striking after skin contact with peanuts, with an OR of 8.0 (95% CI 4.3-16.4). For walnut allergy, a similar but weaker association was found with an OR of 2.23 (95% CI 0.77-6.19). For hazelnut allergy however, the association of skin contact and severity of symptoms was not observed. It is possible that this was less often reported because hazelnut is usually consumed as component in processed composite foods and direct skin contact is less common than with peanuts or walnuts, which are more often eaten as a snack.

The dual-allergen-exposure hypothesis proposes that exposure to foods not only occurs orally but also via the cutaneous route.[10] Food allergens may penetrate an impaired skin barrier, resulting in a Th2 response and IgE production by B cells. AD leads to an increased skin permeability, thereby increasing the risk of food allergen sensitization through the skin.[12] Evidence shows that AD usually starts during early childhood with consequently a risk of food sensitization and allergy.[12] Exposure to food allergens does not only occur upon handling of the food when eating e.g. peanut as a snack, but can also be by contact with peanut oil containing ointments.[13] Moreover, food allergens have also been shown to be present in the environment as they have been found in house dust samples.[155,156] Our results suggest that skin related atopic diseases not only increase the risk of developing food allergy, but also into a more severe phenotype. What the explanation behind this relation is remains to be explored.

III Predicting severity of allergic reactions

In **Chapters 3-6**, comparisons were made between traditional extract-based tests and CRD (single allergen molecules) in how well they are able to distinguish between patients with mild to moderate and those with severe symptoms. Results from extract-based tests cannot provide information about the allergen molecules recognized within the extract. Since it is now well-established that recognition of individual allergens can make the difference between causing

almost exclusively mild and local symptoms (PR-10 proteins like Cor a 1, Jug r 5 and Ara h 8) and having the potential to cause severe symptoms (storage proteins like Cor a 9 and 14 and Ara h 2), CRD clearly meets a need in the diagnosis of food allergy. Here we not only investigated whether CRD could assist in better estimating risks that patients run to encounter severe symptoms, but we went a step further and developed prediction models that also incorporated demographical and clinical factors together with serological tests.

In **Chapter 6**, we also compared the potential of other antibody isotypes than IgE, namely IgG, and it subclass $IgG_{4,}$ and IgA to contribute to discrimination between peanut allergic and tolerant patients, as well as between those with mild to moderate and with severe allergic symptoms to peanut.

Component resolved diagnosis (CRD): does it have an advantage to extract-based testing in predicting severity of food allergy?

The results for hazelnut in **Chapter 3** showed that single allergens perform slightly better than the whole hazelnut extract in predicting severity of allergic reactions. Hazelnut extract discriminated quite badly between patients with mild-to-moderate and patients with severe allergic symptoms to hazelnut with AUCs of 0.54 based on reported and 0.61 based on symptoms during a challenge. The best performance in discriminatory value was seen for Cor a 1, with an AUC of 0.73 (challenged based symptoms). IgE against Cor a 9 and Cor a 14, allergens associated with the severe reactions, had only slightly higher AUCs than hazelnut extract (AUC of respectively 0.57 and 0.60 based on reported symptoms and 0.70 and 0.71 (based on symptoms during the challenge). An AUC of 0.70 is acceptable for many diagnostic and prognostic situations but leaves much room for further improvement, and higher AUCs are probably required when using these markers in clinical practice.

In our study, walnut CRD did not show any significant improvement in discrimination of severity compared to whole walnut extract. For adults, this has also been reported by others previously,[102] but for children an improved discriminatory potency of Jug r 1 was found.[157] An underrepresented allergen in walnut extract is the Bet v 1 homologue Jug r 5. **Chapter 4** showed that sensitization to Jug r 5 was the most frequently one in our study population. The low sensitivity of the walnut extract-based testing for Jug r 5 implied that sensitization picked up by the extract ImmunoCAP is likely dominated by recognition of storage proteins. It is however questionable whether a spiked Jug r 5 extract improves walnut diagnosis since the AUC of the single Jug r 5 test was 0.58 in our study. In clinical practice, multiple factors such as demographics and clinical history are usually taken into account. Combining these factors walnut allergens with demographics and clinical history our results suggested an added value of Jug r 5 in predicting symptom severity.

Chapters 5 and 6 evaluate the discriminatory performance of peanut allergens. The best results were found for Ara h 2, although the AUC was found the be much higher in children (0.91) than in adults (0.70). This is in contrast with multiple studies that could not find an association between Ara h 2 and severity in children.[24,41-43] As mentioned earlier, this might be explained by differences in challenge stop-criteria, patient selection criteria and study designs.

In **Chapter 6,** IgG, IgG_4 and IgA antibody levels were tested against peanut allergens and compared to IgE responses. Unfortunately, the levels of allergen-specific antibodies of these isotypes did not help to make a better distinction between being tolerant or allergic to peanut or between a mild to moderate and severe peanut allergy. As has been convincingly shown for allergen immunotherapy,[46] IgG and IgG_4 antibodies can function as blocking antibodies that counteract the effects of IgE. Based on that, we expect a similar protective role of these isotypes in food allergy, that could as a result possibly contribute to improving the prediction of symptom severity. In addition to their protective role in immunotherapy, this expectation was also based on the observation that in children where peanut is introduced at a young age (4-6 months), an increase of the ratio of IgG_4 over IgE was observed and that, at the same time, they developed less often peanut allergy than children that did not start early with eating peanuts. During early intervention and immunotherapy, patients are continuously being exposed to high dose peanut allergen resulting in induction of high IgG levels. In contrast, patients that are diagnosed in a clinical setting to have peanut allergy, usually have been avoiding peanut, and consequently IgG levels will be much lower.

We nevertheless explored whether ratios of IgG, IgG_4 and/or IgA over IgE levels could help better explain differences in clinical responses to peanut. We observed that ratios of IgG_4 and IgG over IgE were indeed inversely correlated with the grade of symptoms severity. However, the ability to accurately distinguish between tolerant and allergic patients or between mild to moderate and severe symptoms using these ratios did not improve compared to using IgE levels exclusively. This observation can likely be explained by the greater dynamic range of IgE than of IgG_4 levels (as illustrated in figure 5.3). Calculated ratios are strongly affected by their denominator (in this case IgE). The difference between the lowest and highest levels of IgE was much greater than in case of IgG_4 levels. Let's illustrate this by taking data from a patient that experienced mild symptoms to peanuts (*patient 1*) and a patient that was severely allergic (*patient 2*). Patient 1 had low IgE levels of 1.05 ng/ml (0.44 kU_A/L) and IgG_4 levels were 90 ng/ml. This results in a IgG_4/IgE ratio of 86. Patient 2 had IgE levels of 547 ng/ml (228 kU_A/L), which is ~547 times higher than patient 1. IgG_4 levels were also higher compared to patient 1, but with a fold change of 11 (993 ng/ml). The ratio the IgG_4/IgE was 1.69. Both allergens were strongly associated with severity, but the magnitude of the effect of IgE was larger than that for IgG_4. Therefore, IgE levels strongly affect the ratio IgG/IgE. The relation with severity that we observed was no more than a difference in IgE.

To summarize; in the patient populations that we studied, measuring IgE against single allergens had advantage over food extracts (except for walnut) to predict whether a patient is at risk for severe allergic symptoms

Prediction models: what is the advantage as compared to single IgE tests?

The goal of a prediction model is to estimate the outcome as accurately as possible. The model should also be easy to use, in other words with as little predictors as possible. In **Chapters 3-5,** a selection of demographic and clinical factors was combined with food extract tests and single allergen molecules (see also the statistical analysis part in chapters 3-5) to predict severe food allergy. All prediction models improved in discriminative accuracy with AUCs up to 0.75 (peanut), 0.81 (walnut) and 0.91 (hazelnut) as compared to the best single IgE test, 0.64 (Ara h 2/6), 0.58 (Jug r 5) and 0.75 (Cor a 1). Models for all three foods included atopic dermatitis (ever in life) and pollen allergy (reported plus matching serology) and IgE against their respective food allergen components. Skin symptoms upon contact with the food was a strong predictor for severity of allergic symptoms to both peanut and walnut. Birch pollen allergy was relevant for both hazelnut and peanut allergy which contributed to a milder phenotype, while mugwort pollen allergy strongly increased the odds of severe walnut allergy. As mentioned earlier, mugwort LTP (Art v 3) sensitization has been linked to peach LTP (Pru p 3) sensitization, and sensitization to LTPs has been shown to be a risk factor for severe reactions.[6] It is however unlikely that this explains the association of mugwort pollen allergy with severity of reactions to walnut because an association with LTP sensitization was not found. Further research will therefore be needed to clarify the observed link between mugwort pollen allergy and severity of walnut allergy. The final models also included specific allergens known to be related to a more severe allergic phenotype, Cor a 14 and Ara h 2 or the contrary, being associated with a mild phenotype, Ara h 8, Jug r 5 and 7.

However, when looking more closely at the models, peanut serology tests (including whole extract and Ara h 2), did not improve the discriminative accuracy much when compared to a prediction model with clinical and demographical factors alone (AUCs of 0.74 vs 0.75, respectively). In fact, Ara h 2 was not even selected in the models that were based on symptoms during challenge. This suggests that clinical factors, and especially symptoms upon skin contact with peanuts and the absence of a birch pollen allergy, are already strong independent predictors.

PART II: HOW TO TRANSLATE THE RESULTS TO THE CLINICAL PRACTICE?

IV Factors influencing the evaluation of IgE testing methods and prediction models

Since study methods and patient selections affect the evaluation of the diagnostic value of a test, it is important to take into account in which setting the tests were evaluated. A number of factors such as the geographical background or the age of the patient played a role in the performance of specific IgE testing in this thesis. Also, the technique that was used for the index test and how the outcome of the golden standard test was determined (e.g. open challenge or double blind) affected the outcomes of the test evaluation. The Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis (TRIPOD) states that such key items should be reported in order to assess the model its generalizability and risk of bias.[158]

The patient population

The outpatient clinic surveys of the EuroPrevall project (**Chapters 2-5**) started out with enrolling patients reporting any food allergy, not specifically targeted to just one food. Unfortunately, we did not have a really detailed view of the selection procedure within the 12 different participating centers. Although any patient reporting symptoms to any food was eligible, selection bias cannot be excluded. It was not reported how many patients declined to participate and with which food allergy they came into the clinic. In a prospective study such as EuroPrevall, it proved to be very challenging to get a large number of DBPCFCs for all the different types of foods. It is doubtful whether the group of patients that consented is a fully representative sample for the total patient group reporting food allergy. Fortunately, we did not observe substantial differences within EuroPrevall in demographics between the groups with and without a hazelnut or peanut DBPCFC.

The EuroPrevall study had the advantage of comparing patient groups from different geographical areas using the same standardized procedures. The study resulted in a large number of patients which greatly enhanced the generalizability of results. However, there was inevitably substantial heterogeneity between centers and this may have confounded our analyses and it proved to be difficult to check and adjust for this. There was also wide variation in the expertise of the centers: some centers focused on allergy in general, other were more directed towards pulmonology, pediatrics, rheumatology and clinical immunology or dermatology. This variation may limit extrapolation of our multicenter results to individual clinics with a different combination of patient care focus. Additionally, some sites had more experience in conducting research projects including DBPCFCs than others which might have further introduced heterogeneity in clinical practice and decision making.

Heterogeneity between centers was handled in our analyses by random-effect models and we compared results with and without adjustment for heterogeneity; we did not find large differences

between the outcomes of the two approaches. Ideally, sources of variability would have been tested by sub-analysis for each patient group from the twelve centers, but this was not possible for the EuroPrevall study. For example, the number of patients from some centers (Athens, Madrid and Reykjavik) was too small and therefore stratified analysis lacked power to test for specific associations. Despite the multi-center confounding effects, our observations confirmed previous reports: pollen sensitization was strongly related to the occurrence of hazelnut, walnut and to a lesser extent peanut allergy in Europe.[32,40,108]

It is important to note that diagnostic-accuracy measures, such as sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) often vary across study settings. The prevalence of the outcome determinants in our studies, *food allergy* and *severity of food allergy*, strongly affects these accuracy measures. All of our study patients were selected in tertiary centers (both in the EuroPrevall study and in the Danish study). In tertiary centers, the prevalence of the suspected disease, in this case food allergy, is usually higher than in primary or secondary care as is the expected severity of the disease. That means that in this setting, the pre-test probability (the probability that a test will be positive) is higher compared to the non-tertiary care and as a result, the positive post-test probability is also higher.

The study on peanut allergy from the study in Denmark described in **Chapter 6** had a retrospective design. Patients selected from routine care basis are usually patients that are selectively referred to additional test. As compared to the prospective design in EuroPrevall (used in **Chapters 2-5**), it can better reflect on the clinical practice but may not identify all patients that match the inclusion criteria for the study. Although this is desirable in clinical practice, for evaluating diagnostic tests this leads to referral bias; not all eligible patients undergo both the index (IgE/IgG testing) and the reference test (DBPCFC). Excluding patients from a challenge with negative IgE testing inflates the sensitivity while excluding patients with very high IgE levels or those at risk for severe reactions will underestimate specificity.[159] Stop criteria for DBPCFCs would affect this as well. The result is an overestimation of the results for the evaluated test.

The patient population also influences the number of positive tests and their relation with the outcome (severity of symptoms). LTP allergens (hazelnut Cor a 8, walnut Jug r 3 and peanut Ara h 9) are not important allergens in the birch endemic European regions, but they might still be important allergens in Athens and Madrid. The importance of LTP has been shown for the peach allergen Pru p 3 and this LTP has been positively associated with severe allergic symptoms.[45] Unfortunately, patient numbers in Athens and Madrid were relatively low and larger number of patients from this area are needed to see whether LTPs in hazelnut (Cor a 8), walnut (Jug r 3) and peanut (Ara h 9) are truly related to severe symptoms. Low numbers of positive tests against storage proteins were also observed. This is likely explained by the fact that these allergies are more common in children than in adults.[26,38,79] In the heavily adult dominated EuroPrevall hazelnut population, Cor a 9 and 14 sensitizations were not frequent which has

affected the accuracy of the tests. This probably explains why the AUCs were acceptable (0.70) for discriminating between mild and severe cases of hazelnut allergy but not excellent (> 0.80). Results from a Dutch study in hazelnut allergic patients[26] also showed that the total number of adult positive to Cor a 9 was only 19/80 (24%). This means that 76% of the adult patients would be missed. In conclusion, Cor a 9 and Cor a 14 are related to severe symptoms but should be used with caution in adults because it could lead to many false negative cases.

Reported symptoms vs objective measurements

Food allergy prevalence based on reported history of symptoms is much higher than when diagnosed by double-blind placebo-controlled food challenge (DBPCFC).[15] Allergy might be confused to other adverse responses to food (e.g. food poisoning or lactose intolerance). Sometimes it is difficult to recognize the causative food when the food is one of the many ingredients in a meal. The presence of matching specific IgE (sensitization) increases the likelihood of reported food allergies to actually present as clinical food allergy. However, proof of sensitization still is no proof for clinical food allergy: specific IgE antibodies against a food can be present in people that do not adversely react to that food.

A relatively high frequency of positive challenges confirmed reported hazelnut allergy (70%) although this varied between the centers. For example, Strasbourg had a high proportion of positive challenges (89%) but only a very small number of patients was challenged (9/70, 13%). Compared to Reykjavik, 46% of all patients included were challenged but only 67% were positive to that challenge. DPCFCs were offered to all patients reporting hazelnut allergy (except to those with a history of anaphylaxis), but a majority of the patients declined to undergo a challenge.

Specific IgE testing technique

The test used to determine IgE levels also influences the final prediction model. In the **Chapters 3-5** two different test were used: ImmunoCAP (a single-plex assay) and a multiplex microarray chip. ImmunoCAP was used to test IgE levels to 24 foods, 12 inhalants and latex, 7 hazelnut allergens, 8 walnut allergens and 5 peanut allergens as well as the IgG, IgG_4 and IgA levels to peanut and its major allergenic components. The allergen molecules from these and other foods studied in EuroPrevall, were also tested on a custom-made multiplex microarray chip. The advantage of this multiplex method over ImmunoCAP is that less serum volume is needed and multiple allergens can be measured at one time. A disadvantage that has been reported is that such microarray chips are usually less sensitive than the singleplex ImmunoCAP tests.[160] In EuroPrevall, differences in positive responses between ImmunoCAP and microarray indeed varied per allergen. Only 10% of the patients with a suggestive hazelnut allergy were positive d for Cor a 9 as measured by ImmunoCAP, but by microarray this was significantly lower with 3% positive tests. When comparing patients tested for peanut allergens by both ImmunoCAP and microarray, numbers were more comparable, except for Ara h 3, where positive sensitization on the microrarray was more frequently seen (30%) than by ImmunoCAP (20%). Ara h 3 on ImmunoCAP is a recombinant allergen while on the microarray Ara h 3 is isolated from

the natural allergens source. This natural Ara h 3 protein is glycosylated while the recombinant form is produced without the carbohydrate moiety that is attached to the protein, also called cross-reactive carbohydrate determinant (CCD). IgE can bind to CCD giving a positive, but clinically irrelevant, test results while this would not happen with the recombinant allergen.

Another problem is that there were not enough ImmunoCAPs for the components to test all patients. For walnut allergy, samples were tested if they were available at a certain point of time during the study. In order to get comparable numbers for hazelnut allergy, most patients from centers that included less than 50 patients were tested. For other centers, random samples were taken. However, the first criterion to test a patient's serum was whether the patient had undergone a DBPCFC; therefore, the subsample with available chip data is probably not representative for the total group. Patients were not randomly assigned to undergo a challenge, but the option was given by choice to the patient. You can however argue that data from these patients were more reliable than from those with only reported symptoms.

Food challenges

Food allergy diagnosis is challenging. The double-blind placebo-controlled food challenge (DBPCFC) is the reference standard for food allergy diagnosis.[21,161] DBPCFCs are however time consuming and should take place in hospital settings. In EuroPrevall, the challenge took place on two different days to which the placebo and verum doses were randomly assigned, as recommended in the PRACTALL consensus report.[161] According to the criteria of this report, a challenge is positive when objective symptoms occur. However, the PRACTALL criteria do also suggest that in some cases subjective symptoms can be sufficient for a positive outcome. Subjective symptoms were included in the EuroPrevall criteria to consider a challenge positive if subjective symptoms occurred at three consecutive doses or when a severe subjective symptom lasted for more than 45 minutes. Interpretation of subjective symptoms is however difficult and a high degree (94-100%) of interobserver variability for subjective symptoms has been reported.[162] This is probably because placebo reactions are more frequently seen in patients with subjective reactions and differences on how symptoms to placebo doses are interpreted.[21] However, not including subjective symptoms in a food challenge can lead to underdiagnosis of food allergy. The definitive diagnosis in EuroPrevall was made by the physician and all cases of placebo reactions have been re-evaluated by a team of experts within the project. In our analyses, all placebo reactors have been excluded, hence the frequency of positive cases can be overestimated from the number with actual food allergy.

Stop criteria used for a food challenge also influence the classification of the severity of the symptoms. It is likely that more severe symptoms would occur when continuing the challenges after the first objective symptoms. The outcome of our DBPCFCs probably did not completely reflect real-life symptoms and could have underestimated the specificity of the markers that were evaluated. Data from the peanut challenges in **Chapter 5**, included only one patient that experienced symptoms that were classified as severe. Most of the patients with a severe

phenotype included in the analysis of the subgroup with challenge-confirmed peanut allergy had a convincing history of anaphylaxis, and were in fact not challenged. Their diagnosis of anaphylaxis was confirmed by a committee of three independent experienced clinicians; hence their food allergy was considered confirmed. The classification of severity based on history and on DBPCFC/confirmed anaphylaxis gave comparable results in associations, and resulting models. This supports the validity of our findings based on history.

Classifying severity

One of the main themes of this thesis was to identify patients with an increased risk of severe allergic reactions to hazelnut, walnut and peanut. Different severity classifications were used in different chapters of this thesis. This reflects current clinical practice, where severity is often based on patient-reported allergic symptoms or symptoms observed during a challenge and several different severity classification systems are applied. The classification systems of Sampson[105] and Mueller[163] are often used and are based on symptoms during a food challenge in which symptoms are divided into 5 grades. Other studies have classified according to the European Academy of Allergology and Clinical Immunology (EAACI) taskforce[164] or simply classified according to having local or systemic reactions. In **Chapters 3, 4 and 5**, we used a severity classification that was developed within the iFAAM project (described in the introduction of this thesis). Some studies have also included the eliciting dose in their severity classification[147] but for developing a model that can predict severity and substitute a time consuming challenge, this was not possible.

It raises the question of which classification to use and if the choice of classification would affect the outcome of the analysis. Taking Ara h 2 as an example, conflicting results are reported for the relation between severity of peanut allergy and IgE levels to Ara h 2.[24,36,37,41–43,145] These studies also use different severity classifications systems. It is possible that the population and differences in study methods play a role in the conflicting results that are reported but whether that is true is difficult to say. The data from the studies in this thesis all used patients from tertiary clinics, although with different age groups. Martinet *et al.*[36] found a positive association between Ara h 2 and severity of peanut allergy using the EAACI taskforce classification in peanut allergic children and adolescents, while Klemans *et at.*[24] and van Veen *et al.*[42] did not observe this in Dutch children using the Sampson severity classification. Song *et al.*[37] also used the Sampson classification and did find a positive, albeit relatively weak correlation between the 5 grades and IgE to Ara h 2 in a patient group of mainly teenagers and adolescents. In **Chapter 6** we used the Sampson classification and found a strong positive correlation between severity and Ara h 2 ($p < 0.001$). The age range of the patients was comparable to the group of Klemans et. al. Using the iFAAM classification in **Chapters 3, 4 and 5**, although simplified by combining grades I-III and IV-V, a positive association was found between severity and Ara h 2 in a patient group of which the majority were adults. IgE levels were measured in most studies[24,36,37](including the studies in this thesis) by ImmunoCAP and also by a multiplex chip-based assay (including the EuroPrevall studies in this thesis).

Some have argued that 5 classes are too complex and difficult to translate for patients; what are moderate symptoms? Simplification would be a better option, for example, by noting whether adrenaline treatment was needed (yes or no)? That can be a clear outcome for both the patient and the clinician. Another option is to develop a continuous score for severity. The advantaged of such a score may be that it is more sensitive to detect changes and to decide if a particular change is clinically relevant. It could be valuable for monitoring efficacy of immunotherapy treatment, but would probably be less relevant in diagnosing patients.

It is difficult to say which classification would be best. However, in order to compare results some consensus should be reached. In clinical practice, a simplified classification would probably be most useful and outcomes easier to interpret.

V Using component resolved diagnosis and prediction models in clinical practice

There remains a need to decrease the dependency on the use of time-consuming and costly double-blind placebo-controlled food challenges. It takes up almost two whole days, and is always taking place in specialized hospital settings, because of the risk of severe reactions remains. Prediction models give an estimated risk on an outcome, i.e., on severe food allergy. By entering a number of parameters into digital software, for example an app, it will return a probability score. This outcome can assist clinicians in their treatment plan and lifestyle advise without performing a food challenge. It saves time, decreases risks and is less invasive for a patient.

Before a new test or probability score can be useful in the clinic, cut-off values need to be determined. It is well known that the major problem in IgE testing is overlap in the levels of IgE measured in those that are tolerant and those that are truly allergic. Trying to distinguish within the group of allergic patients between those that are mild responders or severe responders, is even more difficult. So which cut-off value to use? This depends naturally on the desired decision making.

To *rule out* allergy: a high sensitivity is useful because it results in a low *false negative rate*. If the sensitivity of a test is 95%, it means that 95% of the patients that have food allergy are correctly indicated as having an allergy. In this situation, there can still be a relatively high proportion of *false positives*, those that are tested positive but that are in fact tolerant to the implicated food. This also means that if a patient has a positive test, the certainty that it is truly positive is not clear. However, where the sensitivity is high, *the false negative rate* or *negative predictive value* (NPV) is usually also high, which means that a negative test is quite certain and food allergy can be ruled out.

To *rule in* allergy*:*** a high specificity is useful because it results in a high *true positive rate*. The positive predictive value (PPV) is usually high; when the test is positive, it is highly likely that the patient is truly allergic.

The NPV and PPV highly depend on the prevalence of the disease. If the pre-test probability is low, then the positive post-test probability will also be low. If you have few severe patients then the likelihood that a test will be positive beforehand is low.

Although improvements are found for single allergens compared to extract-based tests, finding a cut-off value for clinical purposes remains challenging. There is often a large overlap between IgE levels and shifting the cut-off value affects false positive and negative testing. This problem is visualized in Figure 1 taking Ara h 2 IgE level data from the Danish study as an example. The bars show the range of the IgE levels against Ara h 2 for the group with mild-to-moderate symptoms and the group with severe symptoms. Both bars are divided in 4 quartiles that contain 25% of all patients in the respective bar. The upper part (**A**) shows that at a cut-off value (dashed

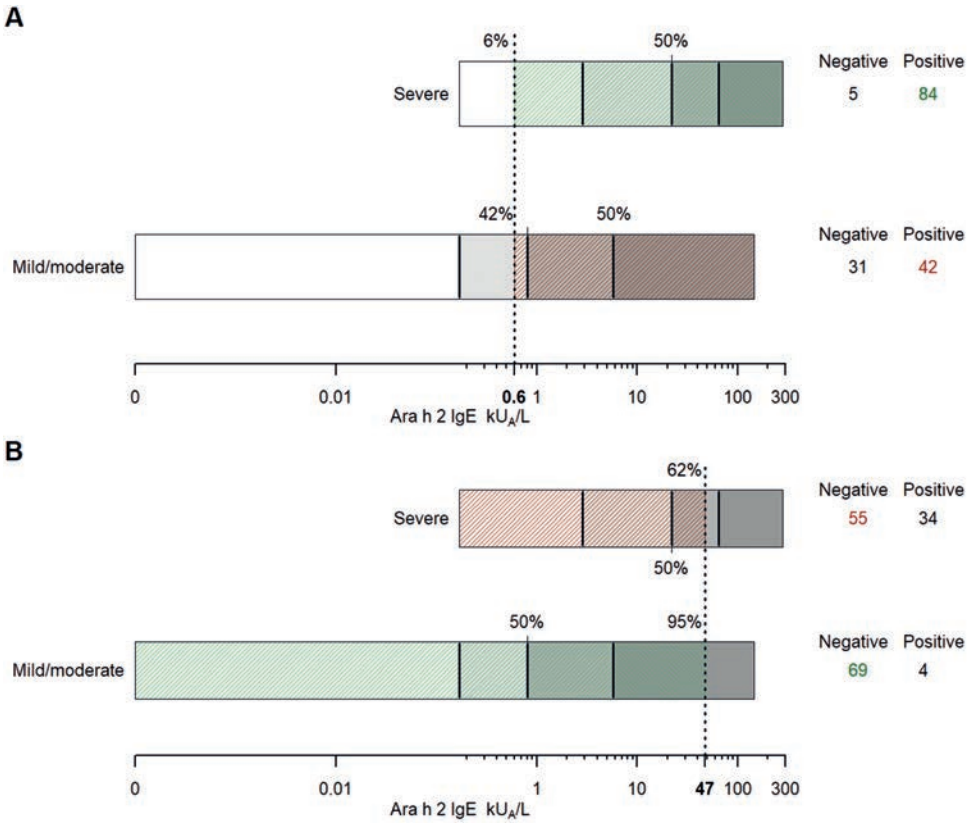

FIGURE I. Distribution of IgE levels between patients with a mild- to moderate peanut allergic phenotype and patients that experience severe symptoms after eating peanuts. The bars show the levels of IgE within that group and are dived into 4 quartiles (black vertical lines). Each quartile contains 25% of all the patients within the specific group. The dashed line indicates the level of IgE at which the test is considers that a patient has a severe phenotype (positive). The green area in the bars is the proportion of patients that were correctly classified using the cut-off level. The red area are patients that are misclassified (e.g., severe patients that are classified as mild and vice versa).

line) of 0.6 kU_A/L IgE, 94% of the severe patients are being classified as severe (green area). The second bar presents the group with mild/moderate symptoms which shows that 68% of these patients have a false positive test because they are also classified as being severe (red area). In the lower part (**B**) the cut-off value is shifted to the right, 47 kU_A/L IgE. In this situation, 95% of the patients with milder symptoms are correctly classified as a 'mild phenotype' (green area). However, this also results in missing 62% of the patients with severe symptoms (red area). As for those that are positive, it is fairly certain that they are indeed severely allergic to peanuts (PPV of 89%). The negative patients should be followed up to investigate whether they are truly negative by for example a DBPCFC.

Instead of using a single IgE test, other information can help to better predict if a patient is at risk for severe allergic symptoms to a food. An example on how to predict severe allergy when looking at multiple factors is given below. The outcomes are based on the results from **Chapter 3.**

Table below (Table 1) shows the clinical factors and serology data that were selected by building a model to predict severity of allergic symptoms to hazelnut. The final model is the result of a logistic regression analysis and for each independent variable beta values are calculated. The odds ratio of severe symptoms can be extracted from the beta values by taking its exponent (exp(β)).

The β values are used to calculate the probability on severe hazelnut allergy using the following equation:

$$1/(1+(e^{\wedge}\text{-}(\text{-}0.338 + (AD * 2.574) + (PA* \text{-}3.005) + (Cor\ a\ 14 * 0\text{-}.074) + (Walnut *0.400))))$$

For each individual patient, the *absence* (0) or *presence* (1) of a certain factor or the continuous levels are multiplied with their corresponding betas, then summed and finally transformed according to the equation at the bottom of the table. For example, a patient with *atopic dermatitis* (1 * 2.574), that is not *allergic to pollen* (0 * -3.005) and with IgE levels of 9 kU_A/L against *Cor*

TABLE I: OUTCOME OF MODEL 4 IN PREDICTING SEVERE SYMPTOMS TO HAZELNUT

			95% CI around exp(beta)	
Predictor variables	**β**	**exp(β)**	**Lower**	**Upper**
Atopic dermatitis (AD)	2.574	13.11	3.04	56.54
Pollen allergy (PA)	-3.005	0.05	0.01	0.28
Cor a 14 IgE levels	-0.074	0.93	0.80	1.08
Walnut IgE levels	0.400	1.49	1.10	2.01
Intercept	*0.338*			

Clinical and specific IgE predictor variables selected for the prediction of severe hazelnut allergy in **Chapter 4**.

a 9 (-0.074*9) and 2 kU_A/L to *walnut* (0.4*2) will have a probability of 95% on severe allergic symptoms to hazelnut. For a patient that does not have *atopic dermatitis* (0 * 2.574) but is *allergic to pollen* (0 * -3.005), who has IgE levels of 5 to *Cor a 9* (-0.074*5) and 3 kU_A/L to *walnut* (0.4*3), the probability will be 14%.

The decision at which probability-threshold a patient is considered to be negative or positive, depends on what would be the accepted number of false negative or false positive tests. Figure 2 shows the probability scores on severe hazelnut allergy and thresholds at 95% sensitivity and specificity. This illustrates that probability scores for severity of hazelnut allergy largely overlapped between the groups with severe symptoms to those with mild-to-moderate symptoms. This was similar for walnut and peanut allergy. At the hatched line in figure 3A, 95% of the patients with severe allergy are identified as such, but 94% of the patients in the other group

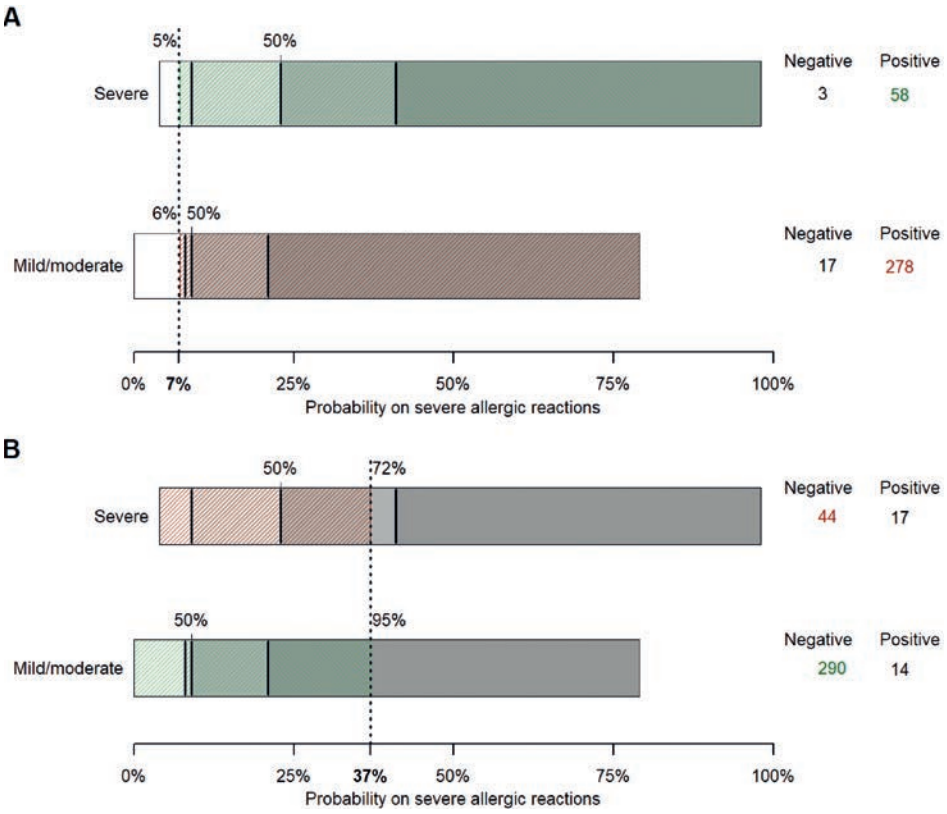

FIGURE II. Distribution of the probability scores on a severe allergic phenotype. The bars are dived into 4 quartiles (black vertical lines). Each quartile contains 25% of all the patients within the specific group. The dashed line indicates the level of probability at which the test is considers that a patient has a severe phenotype (positive). The green area in the bars is the proportion of patients that were correctly classified using the cut-off level. The red area are patients that are misclassified (e.g., severe patients that are classified as mild and vice versa).

as well. The other way around, when 95% of the patients with mild-to-moderate symptoms are identified as 'not severe', 72% of the severe patients are being missed.

In conclusion, our models do perform better than single allergens but the consequence in practice that many patients still need to be followed up after the test results. It is debatable whether these high rates of false positive or false negative (depending on the chosen cut-off value) are acceptable in the clinic.

Future perspectives

Validation studies

A key step in de the development of prediction model is validation. It helps to ensure proper classification or to point weaknesses. Ideally, our studies should have included some resampling techniques, also called internal validation of the model.[158] The next step is assessing the performance of our prediction models in a different data set. Using this dataset, the model is validated and can additionally be updated or adjusted. Reporting according to the TRIPOD statement checklist is highly advisable.

For implementing the prediction models, a decision regarding further testing depending on the number of false positives or false negatives that would be acceptable in daily clinic should also be further evaluated. Moreover, future studies should be designed for the purpose of model development and validation. In addition, although we could not confirm this, our findings and that of others suggest that the accuracy of IgE tests and perhaps our models is influenced by the age of a patient.[26,35,40] Children were underrepresented in our prediction studies. Geography might affect the outcomes as well, especially since its well-known that patients from different areas respond to different allergens in foods. Important to note is that results cannot be directly translated to other clinical settings or populations. Therefore, validation of the prediction models is needed in other tertiary settings, and in primary and secondary settings as well to identify severe food allergy in an earlier stage. Additionally, stratification of different age groups as well as in patients from different geographical areas should also be made.

Novel biomarkers

Despite the relatively good performance of some specific food allergens and the additional value of the prediction models, there remains a grey area when it comes to correlating test results with clinical outcomes. As illustrated in this chapter, there is a large overlap between patients with mild and patient with severe allergic reactions to a food. Therefore, it would still be preferable to have a surrogate test. Basophil activation tests (BAT) have shown some promising results although further research is needed to assess useful diagnostic thresholds.[165] Beside the traditional approaches, more modern techniques could also help better understand food allergen phenotypes. Omics sciences use advanced high-throughput approaches to investigate

a patient's entire collection of cells and molecules. It can create a detailed network map that can help understanding different allergy phenotypes and finding new biomarkers.[166] Although promising, it does face some challenges regarding data output including technologies to create data, heterogeneity of biological data and, not unimportantly, the volume of the data.

Clinical application

If severity of allergic reactions to foods can be better predicted, less patients have to undergo DBPFCs, which are time-consuming, burdensome for the patients and carry risks. Clinicians can make quicker decisions on what is needed for daily management of the patient's food allergy. Since clinical history remains the most important step in food allergy diagnosis, all clinical factors that we evaluated in our model are already collected in daily care. When this information is added into an equation, it will help to inform clinicians whether a patient is at risk for severe allergic reactions. In an ideal situation, a supporting tool or app is developed. By simply adding a set of key parameters it calculates the likelihood of having a severe allergy.

In summary, there is a further need to improve the prediction of severe reactions to hazelnut, walnut and peanut. Our findings should be validated but are a good starting point and give insight in which parameters are helpful in capturing patients that are at risk of severe allergic symptoms to foods.

8

Summary

English summary

Symptoms of food allergy can vary from mild to life-threatening, typically with a rapid onset upon exposure to the food. The symptoms are caused by an abnormal hypersensitivity reaction where the immune system produces unwanted antibodies of the IgE isotype, against proteins of a food. The process of induction of IgE antibodies is called *sensitization*, and the proteins capable of inducing IgE sensitization are referred to as *allergens*.

When clinical symptoms are reported to be associated with a specific food, confirmation of their causative role us often supported by IgE sensitization tests. However, the presence of IgE antibodies does not always imply food allergy. Additionally, due to large overlap in the distribution of IgE levels, distinguishing between patients with mild oral symptoms or those at risk for severe reactions has proven to be difficult.

This thesis focused on sensitization patterns of hazelnut, peanut and walnut allergy and factors that are related to severe allergic reactions to these foods. Most of the studies in this thesis used data of the large EuroPrevall study that gathered data on sensitization patterns of many persons with food allergy on a large standardized multilevel scale. Sensitization patterns were studied using component-resolved diagnosis (CRD) with which a distinction between different types of allergens can be made. This is an important advantage of CRD over extract based tests that contain both allergens and proteins that are not clinically relevant.

The first aim of this thesis was to describe sensitization patterns of food allergens across Europe. **Chapters 2 and 4** confirm the dominant role of birch pollen exposure for allergy to hazelnut and to walnut. The results from EuroPrevall clearly demonstrated that molecular geographical recognition patterns of hazelnut allergen Cor a 1 and walnut allergen Jug r 5 were similar to sensitization patterns of Bet v 1 ,the major birch pollen allergen. Bet v 1 is known as a primary sensitizer that can cross react with allergens from several plant food allergens, such as Cor a 1 and Jug r 5. Birch pollen-related food sensitization to those cross-reactive allergens was mainly observed in Northern and Central European countries. To some extent, we also observed this for peanut Ara h 8 sensitization, the Bet v 1-homologue in peanut, but association were less strong than to those of hazelnut and walnut. In Southern Europe, where birch pollen exposure is virtually absent, patients showed IgE sensitization against lipid transfer proteins (LTP) from hazelnut, Cor a 8, walnut, Jug r 3, and peanut, Ara h 9.

Storage protein sensitization (hazelnut Cor a 9, Cora 11, Cora 14, walnut Jug r 1, Jug r 2, Jug r 4, Jug r 6, and peanut Ara h 1, Ara h 2, Ara h 3 and Ara h 6) showed a less clear geographical pattern. We observed that sensitization to these allergens occur more often in children than in adults and that sensitization to these allergens was more common in patients with peanut allergy (Ara h 1, Ara h 2 and Ara h 3) than in patients with hazelnut or walnut allergy.

The second aim of this thesis was to evaluate the relation between clinical and demographic patient characteristics and IgE recognition patterns of food allergen molecules with severity of allergic symptoms. We further investigated whether single food allergens performed as a good diagnostic marker of food allergy. **Chapter 3** focused on sensitization to the relation between IgE recognition of specific hazelnut allergens and the severity of the symptoms that were reported by the patients or that were recorded during a Double-blind placebo-controlled food challenge (DBPCFC). A positive association was found between sensitization (having IgE ≥ 0.35 kU_A/L) to hazelnut storage proteins Cor a 9 and Cor a 14 and severe symptoms to hazelnuts, both reported by patients and recorded during DBPCFC. An opposite association was found for Cor a 1: sensitization was less frequently seen in patients with severe symptoms but very common in patients with mild symptoms. As for their diagnostic performance, the results for Cor a 9 and Cor a 14 were poor to moderate, with areas under the curves (AUCs) of 0.57 and 0.60 for reported symptoms and 0.70 and 0.71 for symptoms during the DBPCFC. **Chapters 4 and 5,** report on similar studies performed for walnut and peanut, respectively. IgE levels to the walnut allergen Jug r 5 showed the strongest (negative) association with severity of symptoms. High Jug r 5 IgE levels were mostly seen in patients with mild symptoms. Also, IgE against peanut Ara h 8, was significantly higher in patients with mild symptoms compared to patients with a severe peanut allergic phenotype. Similar to what was observed for hazelnut, peanut storage proteins Ara h 2/6, Ara h 1 and Ara h 3 IgE levels were found to be higher in patients with severe symptoms. For the walnut storage proteins, no association with symptom severity was found, but this may be explained by the small number of patients with storage protein allergens. Additionally, CRD alone did not accurately discriminate between patients with mild symptoms to walnut or peanut and those with severe symptoms.

Our next step was to combine the IgE levels to the individual allergens with other possible predictive factors to see if a combination of all these factors could improve the prediction of severity. We developed prediction models including demographic and clinical characteristics from the patients in combination with CRD but also extract-based sensitization data.

For severity of hazelnut allergy (**Chapter 3**), the first model that we built included information available from patients' histories, such as the age of the patient or whether he or she had co-morbidities such as atopic dermatitis, asthma, or other respiratory allergies. Then we added sensitization data against hazelnut extract and against extracts of other food sources. In our third model sensitization data against single allergen molecules were added. All models showed significantly higher AUCs as compared to single allergens. The final model based on symptoms during DBPCFC included having atopic dermatitis (yes/no), having a pollen allergy (yes/no), the levels of IgE against Cor a 14, and levels of IgE against walnut. This resulted in an AUC of 0.91 (95%CI: 0.84-0.97).

A similar modeling exercise was carried out for severity of walnut allergy in **Chapter 4** and severity of peanut allergy in **Chapter 5.** The inclusion of clinical history and demographic

background for both walnut and peanut allergy improved predicting severity compared to models using CRD serology data alone. The predictors for severe peanut and walnut allergy included having a family member with allergy, having atopic dermatitis, and reactions after skin contact with the culprit food. Additionally, mugwort pollen allergy was also predictive for severe symptoms to walnuts and house dust mite allergy for a severe peanut allergy.

For predicting severe peanut allergy serology data did not show any value when added to clinical patient characteristics. This implies that clinical characteristics, collected from a patient history in a standardized matter, are very valuable for estimating the risk for severe reactions to peanuts.

Noteworthy is that for all three foods, skin related atopic diseases where strong predictors for severity of symptoms, individually and in the prediction models; atopic dermatitis ever in life (hazelnut, walnut, peanut), latex allergy (hazelnut, peanut) and symptoms that occurred upon skin contact with the culprit food (hazelnut, walnut, peanut). There is strong evidence that exposure to foods via the skin is involved in the process of sensitization, often facilitated by an impaired barrier such as in case of atopic dermatitis single nucleotide polymorphisms (SNP) linked to barrier dysfunction are associated with an increased risk of developing food allergy. Our results also suggest that skin-related factors are associated with more severe phenotypes.

Mild allergic symptoms to hazelnut, walnut and peanut were as expected mostly associated with birch pollen sensitization, focused around cross-reactive PR-10 allergens. Other predictive inhalant sensitizers such as mugwort pollen (walnut) and house dust mite (peanut) were related to severe symptoms.

Finally, we explored the possible added value of measuring IgG, IgG_4 and IgA antibodies to predict severity of peanut allergy in **Chapter 6**. These isotypes can block pro-allergenic activity of IgE antibodies and we hypothesized that they would perhaps improve prediction of severity. In a Danish cohort of patients with challenge-proven peanut allergy (and tolerant controls with peanut sensitization), we demonstrated that ratios of IgG, IgG_4 and IgA, especially the IgG_4/IgE ratio, were significantly higher in tolerant than peanut allergic patients. Also, for severity of symptoms such an association was found, i.e., IgG_4/IgE decreased along with increasing symptom severity. These ratios could however not improve further diagnostic performance of IgE levels alone. We further confirmed the dominant role of Ara h 2 in peanut allergy; Ara h 2 was the best discriminator for both peanut allergy and tolerance and for estimating severity of symptoms.

The general discussion of the thesis (**Chapter 7**) addresses the reliance of the analyses in the present thesis on patient-reported symptoms, in the light of the consensus that challenge-proven food allergy is the gold-standard for food allergy diagnosis. In fact, this is well-established for distinguishing food allergy from tolerance, but not really for predicting severity. This is not so unexpected if one realizes that challenge procedures usually stop before severe reactions occur. Although self-reported data have the weakness of being retrospective and subjective,

they do probably reflect severity better than controlled challenges in the clinic. Nevertheless, analyses in this thesis using challenged sub-populations gave very similar results to using self-reported data. The models that were developed to predict severity may find their way to the clinic in the future, although there still is quite some overlap between mild/moderate and severe populations, resulting in high numbers of false classifications depending on the chosen thresholds. Moreover, the models will need to be validated in other patient cohorts. Application of (improved and validated) models may in the future decrease the need for food challenges.

What do we need for the future?

Ideally, novel biomarkers that color the grey area that remains in translating study results into clinical practice. In order to replace the time-consuming and burdensome food challenges, we need biomarkers and models that accurately identify patients at risk of severe allergic symptoms to foods. Crucial information on risk factors is necessary and our results are a good starting point for future studies. Ideally, study populations should be well-defined based on the culprit food, age groups, clinical settings and geographical area, collecting data from both clinical history and food challenges. Prediction models should be validated and adjusted when needed. Hopefully, supporting tools can be developed from novel findings and help clinicians in decision making, with less patients undergoing a DBPCFC.

Nederlandse samenvatting

Voedselallergische symptomen kunnen uiteenlopen van een milde lokale reacties tot ernstige levensbedreigend reacties. Ze uiten zich typisch korte tijd na blootstelling aan het voedingsmiddel. De symptomen worden veroorzaakt door een abnormale overgevoeligheidsreactie van het immuunsysteem waarbij ongewenste antistoffen van het IgE isotype tegen het voedingsmiddel worden aangemaakt. Dit proces van aanmaak van IgE antistoffen wordt *sensibilisatie* genoemd. Eiwitten die de capaciteit hebben om IgE sensibilisatie te induceren worden *allergenen* genoemd.

Wanneer allergische klachten gerapporteerd worden tegen een specifiek voedingsmiddel, dan wordt de betrokkenheid van dit voedingsmiddel in de praktijk vaak ondersteund door IgE sensibilisatie testen. Tegelijkertijd betekent de aanwezigheid van IgE antistoffen niet automatisch dat er sprake is van een voedselallergie. Daarnaast maakt dat een grote overlap in de verdeling van de hoeveelheid gemeten IgE het onderscheid tussen patiënten met milde en met ernstige klachten erg moeilijk maakt.

Dit proefschrift richt zich op IgE sensibilisatiepatronen bij hazelnoot-, pinda- en walnootallergie en op demografische en klinische factoren die gerelateerd zijn aan de ernst van de reactie tegen deze voedingsmiddelen. Daarbij is gebruikt gemaakt van gegevens die verzameld zijn in het kader van het Europese onderzoeksproject "EuroPrevall". Dit project heeft het mogelijk gemaakt om op een gestandaardiseerde manier en op grote schaal vergelijkingen te maken tussen verschillende sensibilisatiepatronen. Sensibilisatiepatronen werden vooral bestudeerd door gebruik te maken van zogenaamde 'component-resolved diagnosis' (CRD). Hiermee kan onderscheid worden gemaakt tussen IgE tegen individuele allergeenmoleculen met potentieel verschillende klinische relevantie, van mild tot potentieel levensbedreigend. Dit is een duidelijk voordeel ten opzichte van de klassieke extract-gebaseerde testen waarin allergenen van verschillende klinische relevantie gemengd getest worden.

Het eerste doel van dit proefschrift was het onderzoeken van voedselsensibilisatie patronen in Europa. **Hoofdstukken 2 en 4** bevestigen de dominante rol van blootstelling aan berkenpollen bij hazelnoot- en walnootallergie. De EuroPrevall resultaten laten een duidelijke moleculair geografische patroon zien: de spreidingspatronen van IgE tegen het hazelnoot allergeen Cora 1 en walnoot allergeen Jug r 5 komen overeen met het spreidingspatroon van IgE tegen het berkenpollen allergeen Bet v 1. Bet v 1 is de primaire bron van sensibilisatie, en IgE antistoffen hiertegen zijn zeer kruisreactief met verwante allergenen in verschillende plantaardige voedingsmiddelen, zoals Cor a 1 in hazelnoot, Jug r 5 in walnoot en Ara h 8 in pinda. Berkenpollen gerelateerde voedselsensibilisatie werd voornamelijk gezien in noord en centraal Europa, zoals hier waargenomen voor Cor a 1 en Jug r 5 in respectievelijk hazelnoot en walnoot. Tot op zekere hoogte was dit ook het geval voor het pinda-allergeen Ara h 8, maar deze relatie was minder sterk dan die voor hazelnoot en walnoot. Blootstelling aan berkenpollen komt vrijwel niet voor

in zuid Europa. Hier waren patiënten voornamelijk gesensibiliseerd tegen de zogenaamde 'lipid transfer proteins' (LTP), in hazelnoot Cor a 8, in walnoot Jug r 3, en in pinda Ara h 9.

Sensibilisaties tegen opslageiwitten (hazelnoot Cor a 9, Cora 11, Cora 14,walnoot Jug r 1, Jug r 2, Jug r 4, Jug r 6, en pinda Ara h 1, Ara h 2, Ara h 3 en Ara h 6) lieten minder duidelijke geografische patronen zien. Verder zagen we dat sensibilisatie tegen deze allergenen vaker voorkwam bij kinderen dan bij volwassenen. Tot slot werd sensibilisatie tegen opslageiwitten vaker waargenomen bij patiënten met een pinda allergie (Ara h 1, Ara h 2, Ara h 3 en Ara h 6) dan bij patiënten met een hazelnoot of walnoot allergie.

Het tweede doel van dit proefschrift was het evalueren van de relatie tussen demografische en klinische patiënt karakteristieken en IgE sensibilisatiepatronen, en de ernst van gerapporteerde allergische klachten. **Hoofdstuk 3** richtte zich op sensibilisatie tegen specifieke hazelnoot allergenen en de ernst van gerapporteerde klachten en van klachten vastgelegd gedurende een dubbelblinde placebo-gecontroleerde voedselprovocatie (DBPCVP). Deze analyses lieten een positieve associatie zien tussen sensibilisatie (het hebben van IgE ≥ 0.35 kU_A/L) tegen de hazelnoot opslageiwitten Cor a 9 en Cor a 14 en het hebben van ernstige klachten tegen hazelnoten, zowel gerapporteerd als tijden de provocatie.

Een omgekeerde associatie werd gevonden voor Cor a 1, sensibilisatie werd minder frequent gezien in patiënten met ernstige klachten dan in patiënten met milde klachten. De diagnostische prestaties van Cor a 9 en Cora 14 waren matig tot slecht, met 'areas under the curve' (AUC)s van respectievelijk 0.57 en 0.60 voor gerapporteerde klachten, en 0.70 en 0.71 voor klachten tijdens de DBPCVP. **Hoofstukken 4 en 5** rapporteren vergelijkbare studies voor respectievelijk walnoot en pinda. IgE waardes tegen het walnoot allergeen Jug r 5 liet de sterkste associatie (negatief) zien met ernstige klachten. Sensibilisatie voor dit allergeen werd voornamelijk gezien in patiënten met milde klachten. Ook IgE tegen pinda Ara h 8 was significant hoger in patiënten met milde klachten dan in patiënten met een ernstig pinda-allergie fenotype. Vergelijkbaar met wat er werd geobserveerd voor hazelnoot, waren IgE waardes tegen opslageiwitten, Ara h 2/6, Ara h 1 en Ara h 3, hoger in patiënten met ernstige klachten. Er werd geen associatie gevonden tussen walnoot opslageiwitten en de ernst van gerapporteerde klachten, maar dit kan mogelijk worden verklaard door de lage aantallen van patiënten met dergelijke sensibilisatie in de studie. Associaties betekenen niet automatische dat er sprake is van goede discriminatie. Zoals er ook werd aangetoond in **Hoofdstuk 3**, kon CRD op zichzelf niet accuraat discrimineren tussen patiënten met milde klachten tegen walnoot of pinda en degenen met ernstige klachten.

Onze volgende stap was om te onderzoeken of het combineren van IgE waardes tegen de individuele allergenen en andere mogelijk voorspellende factoren uit demografische en klinische karakteristieken van de patiënten, het voorspellen van de ernst van reacties zou kunnen verbeteren. Ons doel was hierbij om voorspelmodellen te bouwen waarin dus demografische en

klinische factoren werden gecombineerd met sensibilisatiepatronen, zowel verkregen uit CRD maar ook met extract-gebaseerde testen.

Het eerste model voor het voor spellen van de ernst van hazelnoot allergie bevatte de beschikbare informatie over de achtergrond van de patiënt zoals leeftijd, of hij/zij co-mobiliteiten had zoals atopische dermatitis, astma of andere respiratoire allergieën. We vervolgden met variabelen geselecteerd in model 1 en voegden daar sensibilisatie data tegen hazelnoot extract en extracten van andere voedingsbronnen aan (model 2). In het derde en laatste model werden sensibilisatie data tegen individuele allergeen moleculen toegevoegd. Alle drie de modellen lieten significant hogere AUC's zien in vergelijking met de individuele allergenen. Het uiteindelijk best voorspellende model was gebaseerd was op klachten waargenomen tijdens de DBPCVP, en bestond uit het hebben van eczeem (ja/nee), het hebben van een pollen allergie (ja/nee) en de IgE waardes tegen Cor a 14 en tegen walnoot. Dit model resulteerde in een AUC van 0.91 (95%BI: 0.84-0.97).

Een vergelijkbare benadering om voorspelmodellen te ontwikkelen werd ook uitgevoerd voor walnoot allergie in **Hoofdstuk 4** en voor pinda allergie in **Hoofdstuk 5**. De inclusie van klinische historie en demografische achtergrond voor zowel walnoot en pinda allergie lieten een verbetering zien in het voorspellen van de ernst van allergische reacties dan het gebruik van enkel CRD serologie data. De voorspellers voor ernstige pinda en walnoot allergie bevatten de volgende factoren: het hebben van een familielid met allergie, het ooit atopische dermatitis hebben gehad, en het rapporteren van klachten die ontstaan na huidcontact met het voedingsmiddel. Daarnaast was het hebben van een allergie tegen bijvoet pollen ook voorspellend voor de ernstige klachten tegen walnoot en van het hebben van een huisstofmijtallergie voor een ernstige pinda allergie.

Serologie data had echter, naast klinische karakteristieken van de patiënt, geen toegevoegde waarde in het voorspellen van een ernstige pinda allergie. Dit impliceert dat klinische karakteristieken verzameld uit de achtergrond van de patiënt het meest waardevol zijn in het voorspellen van ernstige reacties tegen pinda.

Het is noemenswaardig dat voor alle drie de voedingsmiddelen, huid-gerelateerde atopische aandoeningen sterke voorspellers waren voor de ernst van klachten, zowel individueel als in de predictiemodellen: eczeem ooit (hazelnoot, walnoot en pinda), latex allergie (hazelnoot en pinda), en klachten die ontstonden na huidcontact met het voedingsmiddel (walnoot en pinda). Er zijn sterke aanwijzingen dat blootstelling aan een voedingsmiddel via de huid betrokken en wellicht doorslaggevend is bij het sensibilisatie proces. Blootstelling wordt vaak gefaciliteerd door een aangetaste huidbarrière wat het geval is bij eczeem; 'single nucleotide polymorphisms'(SNPs) die zijn gerelateerd met een disfunctionerende huidbarrière worden geassocieerd met een verhoogd risico op het ontwikkelen van een voedselallergie. Onze resultaten suggereren daarnaast nu dat huid-gerelateerde factoren ook geassocieerd zijn met het risico op een meer ernstige vormen van voedselallergie.

Milde allergische symptomen tegen hazelnoot, walnoot en pinda waren zoals verwacht voornamelijk geassocieerd met berkenpollen sensibilisatie, met daarbij de focus op kruisreactieve PR-10 allergenen. Andere voorspellende vormen van sensibilisatie tegen inhalatieallergenen waren die tegen bijvoet pollen (in het geval van walnoot) en tegen huisstofmijt (in het geval van pinda). Beide waren gerelateerd aan ernstige klachten.

Tot slot hebben we de mogelijke meerwaarde van het meten van allergeen-specifieke IgG, IgG_4 en IgA antistoffen bij het voorspellen van ernstige pinda allergie onderzocht in **Hoofdstuk 6.** Deze isotypes staan er bekend om dat ze pro-allergische rol van IgE antistoffen kunnen blokkeren. Onze hypothese was deze isotypes daarom mogelijk zouden kunnen bijdragen aan een betere voorspelling van de ernst van symptomen. In een Deens cohort van patiënten met een met een provocatie-bevestigde pinda allergie (en tolerante controles met een pinda sensibilisatie), lieten we zien dat de ratios van IgG en IgE en IgA en IgE, en vooral de IgG_4/IgE ratio, significant hoger waren in tolerante dan pinda allergische patiënten. Ook voor ernst van klachten werd deze associatie gevonden: de IgG_4/IgE ratio nam af met toenemende ernst van klachten. Deze ratio's konden echter de diagnostische prestatie niet verbeteren ten opzichte van alleen de voorspellende waarde van specifiek IgE. Bij deze studie konden we de dominante rol van Ara h 2 bevestigen voor pinda allergie: Ara h 2 is de beste discriminator voor pinda allergie en tolerantie en voor het inschatten van ernstige klachten.

In de algemene discussie (**Hoofdstuk 7**) werd tot slot de betrouwbaarheid van de analyses op patiënt-gerapporteerd klachten besproken met daarbij als uitgangspunt dat de algemeen aanvaarde gedachte dat een voedselallergie bewezen door een voedselprovocatie de gouden standaard is. Dit is inderdaad de meest betrouwbare manier om voedselallergie van tolerantie te onderscheiden, maar waarschijnlijk minder succesvol voor het voorspellen van de ernst van klachten. Dit is niet geheel onverwacht omdat de provocatie meestal wordt gestopt voordat zich er ernstige klachten voordoen. Hoewel gerapporteerde klachten retrospectief en subjectief, en dus waarschijnlijk vaak minder betrouwbaar zijn, reflecteren ze, door de stopcriteria tijdens provocaties, de ernst van reacties toch beter dan klachten die zich uiten tijdens een gecontroleerde provocatie. Desondanks gaven de analyses in de subpopulatie met een provocatie vergelijkbare resultaten. De modellen die zijn ontwikkeld om de ernst van reacties te voorspellen vinden mogelijk in de toekomst hun weg naar de kliniek, al is er momenteel nog een grote overlap tussen milde/matige en ernstige populaties. Dit resulteert in een hoog aantallen vals positieve classificaties, afhankelijk van de gekozen afkapwaardes. Daarnaast is het essentieel dat de modellen geverifieerd en gevalideerd worden in andere populaties patiënten. De toepassing van (verbeterde en gevalideerde) modellen kunnen in de toekomst de noodzaak van voedselprovocaties doen afnemen.

Wat is er nodig voor de toekomst?

Ideaal gezien hebben we nieuw biomarkers nodig die het grijze gebied dat er bestaat tussen het vertalen van de studieresultaten naar de kliniek kunnen inkleuren. Om de voedselprovocaties, die veel tijd kosten en belastend zijn, te kunnen vervangen hebben we biomarkers en modellen nodig die accuraat patiënten kunnen identificeren die het risico lopen op een ernstige voedselallergische reactie. Daarbij is cruciale informatie over risicofactoren een vereiste. Onze resultaten zijn daarin een goed starpunt voor toekomstige studies. Idealiter is er een goed gedefinieerde studiepopulatie met een focus op leeftijdsgroepen, klinische settings en geografische gebieden. Hierbij wordt data verzameld van zowel de klinische achtergrond van de patiënt en van voedselprovocaties. Voorspelmodellen moeten worden gevalideerd en aangepast waar nodig. Hopelijk kunnen ondersteunende middelen worden ontwikkeld op basis van nieuwe bevindingen en kan daarmee besluitvorming in de kliniek worden ondersteund, zodat het aantal voedselprovocaties kan worden teruggebracht.

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Abbreviations

Author affiliations and contribution

Portfolio

List of Publications

Dankwoord

About the author

Bibliography

Abbreviations

AD	Atopic dermatitis
AUC	Area under the curve
BAT	Basophil activation test
CCD	Cross-reactive carbohydrate determinants
CI	Confidence interval
CRD	Component-resolved diagnosis
DBPCFC	Double-blind placebo-controlled food challenge
FA	Food allergy
FN	False negative
FP	False positive
HDM	House dust mite
(s)IgA	(specific) Immunoglobulin A
(s)IgE	(specific) Immunoglobulin E
(s)IgG	(specific) Immunoglobulin G
LASSO	Least Absolute Shrinkage and Selection Operator
IQR	Interquartile range
LMW	Low-molecular weight
LTP	Lipid transfer protein
NPV	Negative predictive value
OAS	Oral allergy syndrome
OFC	Open food challenge
OR	Odds ratio
PA	Peanut allergy
PR-10	Pathogenesis-related protein family 10
PPV	Positive Predictive value
ROC	Receiver operating characteristic curve
SD	Standard deviation
SNP	Single nucleotide polymorphisms
SPT	Skin prick test

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General courses	
AMC world of Science	2012
Scientific Writing for English Publication	2013
Oral presentation in English	2013
Reference manager	2013
End note	2014
Epidemiology & biostatistics	
Advanced topics in biostatistics	2013
Advanced topics in clinical epidemiology	2013
Genetic epidemiology	2014
Immunology	
Advanced immunology	2014
Computer skills	
Computing in R	2012
R programming (Online course John Hopkins University)	2014
Seminars, workshops and master classes	
General	
Weekly department seminars	2012-2016
2-weekly journal clubs	2012-2016
Author and Reviewer work shop	2012
Masterclass “How to deal with biomedical journals?”	2016
Food allergy	
Food allergy summer school in El Escorial (Spain)	2015
Food allergy workshop by Jonathan Hourihane	2016
Teaching	
General courses	
Critical appraisal of published medical literature	2016-2017

PORTFOLIO (CONTINUED)

Peer-reviewing	**Year**
International Archives of Allergy and Immunology	
Critically evaluating submitted manuscripts for publication on allergic sensitization and molecular diagnosis in pollen and food allergy	2015-2016
Other activities	
Organization of the Triple I Retreat	
Organizing a 2-day event to bring PhD students, who work in the field of infection and immunology, together to present and discuss their research.	2016-2017
Presentations & Conferences	
Presentations	
Presentation	
Can we improve the prediction of severe reactions to hazelnuts?	2016
The Food Allergy and Anaphylaxis Meeting (FAAM), October 2016**,** Rome. e-Poster.	
Sensitization patterns of hazelnut allergens across Europe.	2016
Nederlandse vereniging voor allergologie (NVvA), Breukelen. Invited speaker.	
Non-IgEs in the diagnosis of peanut allergy: is it useful?	2016
Integrated approaches to food allergen and allergy risk management (iFAAM) meeting, Amsterdam, Poster.	
Identification of risk factors and biomarkers for severity of food allergy.	2016
Integrated approaches to food allergen and allergy risk management (iFAAM) meeting, Amsterdam, Poster.	
The role of non-IgE component resolved diagnosis (CRD) in peanut allergic phenotypes.	2015
International Symposium on Molecular Allergology (ISMA), Lisbon. Invited speaker.	
Food sensitization profiles in school-aged children from China and Russia.	2014
The Food Allergy and Anaphylaxis Meeting (FAAM), October 2014**,** Dublin. e-Poster.	
Hazelnut allergy in outpatient clinics across twelve European countries.	2013
The annual European Academy of Allergy and Clinical Immunology (EAACI) congress, Milan. Oral presentation.	
(Inter)national conferences and symposia	
The European Academy of Allergy and Clinical Immunology (EAACI)	
Annual Congress EAACI, 11-15 June, Vienna (Austria)	2016
Annual Congress EAACI, 6-10 June, Barcelona (Spain)	2015
Food Allergy and Anaphylaxis Meeting (FAAM), 9-11 Oct, Dublin (Ireland)	2014
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Food Allergy and Anaphylaxis Meeting (FAAM), 7-9 Feb, Nice (France)	2014
Annual Congress EAACI, 16-20 June, Geneva (Switzerland)	2012
International Symposium on Molecular Allergology (ISMA)	***Year***
ISMA, 19-21 Nov, Lisbon (Portugal).	2015
ISMA, 5-7 Dec, Vienna (Austria).	2015

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PhD Retreats	**Year**
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22-23 May, Vinkeveen	2014
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List of Publications

Datema MR, Zuidmeer-Jongejan L, Asero R, et al. Hazelnut allergy across Europe dissected molecularly: A EuroPrevall outpatient clinic survey. *Journal of Allergy and Clinical Immunology*. (2015), 136(2):382-91. DOI: 10.1016/j.jaci.2014.12.1949

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Dankwoord

Het boekje is er, eindelijk! Een thesis afronden en twee kinderen op de wereld zetten is een zeer uitdagende combinatie. Toch zou ik het niet anders hebben willen doen, maar zonder alle ondersteuning van anderen zou ik mijn proefschrift nooit hebben kunnen afronden. Daarom wil ik deze mensen ook graag bedanken.

Ronald, van jou heb ik enorm veel geleerd op het gebied van (moleculaire) allergologie. Je dacht groots en zag overal mogelijkheden om met de EuroPrevall data aan de slag gegaan. Sommige periodes was je veel op reis, maar daardoor niet minder bereikbaar. Wanneer je in het AMC was, stond jouw deur altijd open en zat je tijdens de lunchpauzes met het hele Allergieteam aan tafel. Ik ben dankbaar voor het vertrouwen dat je in mij had als PhD'er. Bedankt voor alle reizen die ik heb mogen maken en het vertrouwen om mij zelfs als genodigd spreker op het programma van een conferentie te zetten.

Koos, ook bij jou stond de deur altijd open. De laagdrempeligheid om bij je naar binnen te lopen, maakte het erg plezierig om met je te werken en alle vragen te stellen die ik had. Wat heb ik veel geleerd van jouw statistische kennis! Je dacht altijd mee, wist ingewikkelde analyses begrijpelijk uit te leggen en daarbij elke formule zo op het whiteboard te toveren. Ik was ontzettend blij met het plekje wat je mij aanbood op jouw afdeling. Alle contacten op de KEBB waren een zeer welkome aanvulling in mijn netwerk. Jouw kennis, expertise en netwerk zijn onmisbaar geweest voor het tot stand komen van dit boekje.

Laurian, dank voor alle steun die je als co-promotor hebt geboden. Dank voor al je pragmatische input en feedback bij het schrijven van de artikelen en voorbereiden van presentaties. Fijn dat je me wegwijs gemaakt hebt in het vakgebied en tijdens de congressen. We hebben er heel wat gezamenlijke treinritjes en buitenlandse reisjes opzitten met veel gesprekken over werk, maar ook het leven daarbuiten. Dank voor al je interesse.

En de rest van de allergie groep (van toen), **Leonie, Serge, Adrian, Jaap, Derya, Lara** en **Hans**. We waren een bijzonder gemêleerd gezelschap. Dank voor de fijne tijd op K0 en gezellige lunch momenten. Hans, zet 'm op met jouw proefschrift, het gaat je lukken!

Veel dank aan de overige leden van de beoordelings- en promotiecommissie **prof. dr. P.M.M. Bossuyt, prof. dr. A.J. Bredenoord, prof. dr. P.I. Spuls, prof. dr. L.K. Poulsen, Prof dr. M Yazdanbakhsh, prof. dr. W.J. Fokkens** voor de bereidheid dit proefschrift te willen lezen en beoordelen. Speciaal dank aan **Maria** en **Lars**. **Maria**, wat een eer dat jij nu in mijn commissie zit. Jouw aanmoediging en enthousiasme voor onderzoek heeft mij ertoe gezet de stap te maken om te gaan promoveren. **Lars,** thank you for your scientific collaboration, talks and conference drinks & dinners. I am very grateful for your time to assess this thesis and coming to the Netherlands for my defence.

I would like to acknowledge the entire **EuroPrevall and iFAAM team.** Thanks to all co-authors, for your valuable contributions and suggestions to the manuscripts. Special thanks to **Monsterrat Fernandez-Rivas**, for sharing your medical experience and insights of the EuroPrevall project and your warm welcome when I visited Madrid. Also, many thanks to **Carsten Bindslev-Jensen** and **Esben Eller** for your collaboration and your willingness to share your data, which resulted in Chapter 6 of this thesis. It's too bad I did not get to see more of your beautiful home city, Odense.

Sarah, dank voor je fijne samenwerking. Jouw bijdrage (en natuurlijk dat van jouw team) aan hoofdstuk 5 was zeer waardevol. En natuurlijk ook erg veel dank dat ik betrokken mocht zijn bij het totstandkomen van het walnoot manuscript.

Uiteraard was dit proefschrift was nooit tot stand gekomen zonder de onmisbare bijdrage van alle **patiënten** die wilden deelnemen aan dit onderzoek.

Mijn oud-kamergenoten van de KEBB, **Daniël**, **Maurice**, **Nina** en (ook al was je officieel geen kamergenoot) **Annefloor**. Het lijkt alweer zo ver weg, maar wat heb ik het fijne tijd gehad met jullie op een kamer. Ik heb goede herinneringen aan onze gezamenlijk borrels en feestjes (mede tot stand gekomen door ons puntensysteem), het volgen van de dagelijkse meeuwen-soap op het dak van collegezaal 1, Daniël die in het heetst van de strijd tijdens wielerwedstrijden iedereen bij z'n scherm bijeen riep, onze tripjes naar Parijs, maar uiteraard ook jullie waardevolle kennis en onze wetenschappelijke discussies. Hopelijk komen we elkaar zo nu en dan nog weer eens tegen.

Mijn huidige **AMC collega's,** dank voor jullie interesse en aanmoediging om mijn proefschrift af te ronden.

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Dan wil ik tot slot ook al mijn lieve vrienden en familie bedanken voor al jullie steun en interesse de afgelopen jaren. Dank dat jullie wilden luisteren naar al mijn verhalen over allergenen, antilichamen, complexe analyses of gewoon het lief en leed van een promotietraject. Een aantal van jullie wil ik graag speciaal noemen.

Irma, Wytske, Leonie en Ingrid. Jullie mag ik niet vergeten te bedanken. Al zijn we inmiddels allemaal onze eigen weg gegaan, jullie hebben een groot deel uitgemaakt van de periode tijdens onze studie gezondheidswetenschappen en de stap die ik daarna maakte om een PhD traject te gaan volgen. We hebben heel wat lief en leed gedeeld over promoveren. Dank jullie wel.

Susanne, wat fijn dat wij elkaar toevallig tegenkwamen op een van de eerste APH-bijeenkomsten. Ik vond het prettig dat ik nu en dan even met je kon sparren over mijn proefschrift. En als ik het even niet meer zag zitten gaf je me bemoedigende woorden om toch door te zetten. Dank voor het meedenken en je luisterend oor. Ik hoop dat we nog veel gezellige koffiemomenten samen mogen hebben, zowel binnen als buiten het AMC.

Lieneke - lieve Lieneke, bij jou kan ik altijd steun vinden. Je luistert, zegt lieve woorden en kan me weer vrolijk maken als het even tegen zit. Straks in Amsterdam zit je dan misschien niet direct naast me, maar hopelijk wel heel dichtbij in de zaal. Ik kijk uit naar de tijd dat we weer een festivalletje mee kunnen pikken en broodjes frikandel kunnen nuttigen.

Tessa en Michiel, dank voor al jullie ondersteuning aan ons gezin het afgelopen jaar!

Emily, samen succesvol een PhD-retraite organiseren en er een mooie vriendschap aan overhouden! Jij weet als geen ander hoe het voelt een PhD traject af te ronden. Alle tegenslagen heb jij al overwonnen en met succes je heb je jouw proefschrift mogen verdedigen. Ik vind het superfijn dat jij mijn paranimf wil zijn.

Dan zijn er tot slot nog een paar vrienden die niet wil vergeten te bedanken, **Nadine, Johannes**, **Elise,** dank voor al jullie interesse en steun

Evert en Lenie, dank voor al jullie interesse in mijn werk en ondersteuning bij ons thuis de afgelopen jaren. Evert, speciaal dank voor het maken van de coverfoto voor mijn boekje! Ik ben erg blij met het resultaat.

Sia – lieve Sia, gesprekken met jou zijn altijd fijn. Je hebt een speciaal plekje in mijn hart.

Lisette & Alex - lieve **Lisette**, dankjewel dat je straks naast me staat als paranimf. Niet helemaal hetzelfde als getuige bij een bruiloft, maar het komt toch wel aardig in de buurt ;) Het is altijd fijn om iemand die je hele leven al zo dicht bij je staat, nu ook naast je te hebben bij een belangrijke gebeurtenis als deze. Lieve **Alex**, ik heb je gemist het afgelopen jaar. Nu eindelijk weer eens een feestje. Hopelijk volgen er nog vele.

Papa, mama - lieve allebei, dank voor jullie onvoorwaardelijke steun in al mijn keuzes die ik de afgelopen jaren heb gemaakt. Ik ben vele richtingen opgegaan. Dit was een van de moeilijkste maar ook meest uitdagende. Nu is deze dan ook eindelijk afgerond. Dankjewel voor alles!

Ruben - lieve Ruben zonder jou had ik dit nooit kunnen afronden. Je hebt ongekend veel vertrouwen in mij. Het afgelopen jaar was ontzettend pittig. Toch kon je mij helpen om elke keer weer opnieuw moed te verzamelen en mij de ruimte te geven om verder te werken aan mijn proefschrift. En dit had nogal wat voeten in de aarde. Wat ben ik blij met al jouw steun en enorm trots dat je straks tijdens mijn verdediging zo dicht bij me zit... Ik kijk uit naar onze toekomst samen met daarin veel fietsen, reizen, cappuccino's en onze kinderen zien groeien en bloeien.

Aurora en Elian, jullie hebben ons gezin compleet gemaakt. Ik geniet intens van jullie enthousiasme en nieuwsgierigheid. Jullie helpen mij om de wereld met andere ogen te bekijken.

About the author

Mareen was born on July 24th 1984 in Groningen, the Netherlands. Her education and career path was not standard but filled with insights and discoveries which each time took her a step further. She started her secondary school at her the local village and obtained her VMBO diploma in 2000. After completing an education to become a social worker, she was dedicated to take the next step and started to study physiotherapy. She discovered her fascination of the human body and her experience from her social worker training gave her a huge advantage in working with patients. During her study, she did several internships including one in Paramaribo, Suriname. After successfully obtaining her Bachelor's degree Physiotherapy in 2008, she started working in a rehabilitation center treating patients with neurological conditions. While working as a physiotherapist, she was dedicated to continue studying and started orientating for different master programs. To be better prepared for University, she took higher secondary education mathematical lessons and took the official Dutch National exam successfully. In 2009, after a full year of working and studying math, Mareen enrolled the Health Sciences (pre) Master program in Amsterdam. Never did she imagine that she could be so fascinated by infectious diseases, and choosing the specialization Infectious Disease & Public Health was a very easy choice. During the Masters years, she did an internship at the Parasitology Department at the Leiden University Medical Center, under supervision of Prof. dr. Maria Yazdanbakhsh. Her research was focused on Immune responses in *Schistosoma* infections and their local spatial patterns. After obtaining her Master's degree, she was introduced by Maria to Ronald van Ree, Professor at the Department of Experimental Immunology at the Academic Medical Center and he gave her the opportunity to obtain a PhD degree. This included the analyses of epidemiological and immunological data aiming to understand differences in sensitization profiles and severity of allergic to foods.

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While finishing her PhD, she was offered a job in het AMC as a data steward. During the last years, she developed herself as an expert of all activities that involve supporting researchers to make their data findable, accessible, interoperable and reusable.

Mareen lives together with Ruben and their two children, Aurora and Elian.

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